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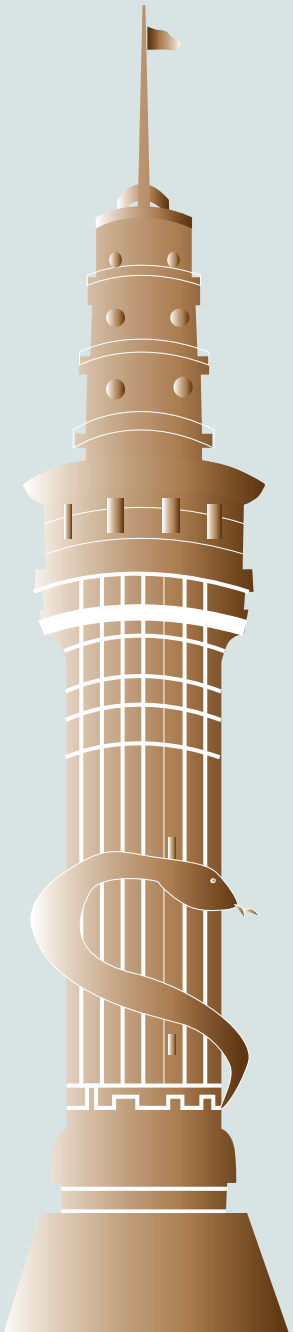

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Original Article

Dynamic pricing as an innovative approach in drug sales and its effect on consumers' perception of pre-purchase and post-purchase behavior

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ABSTRACT

Background and Aims: Drug companies worldwide engage in intense global competition, focusing on consumer satisfaction, profitability, and promotion to gain an edge. This study aims to explore the effects of dynamic pricing on pre-purchase and post-purchase behaviors among drug consumers in Turkey.

Methods: The study employed a quantitative research design, utilizing a structured questionnaire administered to 414 employed individuals in Turkey. Data were analyzed using a structural equation modeling (SEM) approach to assess the relationships between consumers' perception of dynamic pricing and their behaviors accordingly.

Results: Statistical analyses revealed that consumers' perception of dynamic pricing significantly influences pre- and post-purchase behavior, satisfaction levels, loyalty, and positive word-of-mouth communication. Moreover, the study highlights the interconnectedness of consumers' post-purchase behavior, especially regarding experiences with dynamically priced drugs.

Conclusion: Dynamic pricing significantly influences pre-purchase decisions and post-purchase loyalty, highlighting the importance of customer-centric strategies for pharmaceutical companies. Post-purchase satisfaction and loyalty enhance word-of-mouth communication, fostering long-term customer relationships. However, broader studies with diverse participants and timelines are recommended to strengthen the statistical insights and applicability of findings.

Keywords: Drug Sales, Dynamic Pricing, Pre-Purchase Behavior, Post-Purchase Behavior, Financial Effect.

INTRODUCTION

Due to advancements in technology and the Information Technology (IT) sector, businesses now operate in an increasingly competitive environment, making customer retention and acquisition critical (Porter, 2008). Modern consumers, who act more rationally than ever, drive businesses to adopt customer-centric approaches (Simonson & Winer, 2014). The activities companies undertake regarding their products and services significantly influence consumer purchasing behaviors across three stages: before, during, and after purchase (Engel, Blackwell, & Miniard, 1995). To succeed in this dynamic landscape, businesses must gather comprehensive market information and develop strategies aligned with consumer decision-making processes. After filtering the thoughts of elements such as quality, price, promotion and presentation, they usually make choices that they perceive will maximize their benefits for themselves (Nagle & Hogan, 2006). Ultimately, it is an uncertain fact that ethical physiological characteristics and activities are important in the variety of sustainable and long-term business relationships (Laczniak & Murphy, 1993).

This dynamic care mentioned is an example of the pharmaceutical industry, where fierce competition is experienced and innovation is at the forefront (Chen & Zhang, 2014). Brands and products in this area, product features, advertising and installation strategies are the link to minimize perceived financial risks and ensure trust. Dynamic structural elements that can be adjusted according to price and time and provided play an important role in shaping the perception of adjustment. Due to this importance, distributed solutions are provided with analysis views related to the behaviors of the applications of dynamic processes before and after the purchase of consumption.

Before defining dynamic pricing, it is useful to explain the concept of price. Price is one of the marketing mix components that businesses manipulate to influence demand for goods or services. Tek (1999) describes price as a differentiation tool that balances supply and demand while ensuring measurability.

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Consumer purchasing decisions can change randomly or systematically due to factors like seasonal effects, fashion trends, or shifts in purchasing power. Philips (2005) emphasizes that understanding such changes is critical for predicting customer price expectations and maintaining organizational continuity. Organizations tailor pricing processes based on consumer demand, market conditions, and financial circumstances. For instance, airline companies adjust ticket prices as the flight departure date approaches, reflecting fluctuating demand.

Rohani & Nazari (2012) highlight that dynamic pricing is driven by consumer demand, with prices changing based on feedback. Kleindle (2003) defines it as selling goods or services at prices that vary to balance supply and demand. While originally used as a bargaining strategy, dynamic pricing has evolved with technological advancements and is now widely applied, especially in sectors like aviation. Ancarani (2002) notes that analyzing dynamic pricing data enables organizations to adapt to market conditions and achieve profit maximization. The rise of e-commerce has further integrated dynamic pricing into everyday practices, allowing consumers to compare prices and enabling businesses to forecast demand and create personalized strategies (Önder & Oktay, 2011).

Dynamic pricing adjusts prices flexibly based on consumer interest, demand patterns, and competitor pricing. Monahan, Petruzzi, & Zhao (2004) explain that the goal is to maximize income, while Korkmaz, Öztürk, Eser, & Işın (2009) emphasize its role in reducing stock levels and increasing profitability through targeted price policies. However, Megep (2011) identifies disadvantages, such as reduced customer loyalty, dissatisfaction with frequent price changes, and challenges in maintaining quality perceptions. Implementing dynamic pricing also requires significant investments in technology and expertise.

Recent literature shows growing interest in dynamic pricing. Studies highlight its impact on stock management, retail, and products with short sales lifespans (Chatwin, 2000; Zhao & Zheng, 2000). Research by Bilisik & Gurgen (2012), Rahimi (2014), and Mammadli (2017) demonstrates how dynamic pricing affects repurchase intentions by shaping price perceptions. Kuzay (2018) found that dynamic pricing on websites positively influenced consumer value when coupled with additional benefits. Similarly, Machmud & Minghat (2020) analyzed the impact of dynamic pricing on hand sanitizer during COVID-19, finding that prices stabilized post-pandemic. Despite customer concerns, businesses defend dynamic pricing as a response to global changes, using it across various sectors to adapt strategically.

The fields of pharmacology and medicine have always collaborated to develop essential drugs, making them indispensable to human life. Globally and in Turkey, the pharmaceutical industry holds a significant share in the economy, driven by the growing market and competition with derivative drugs. Since 1970, Turkey's pharmaceutical industry has seen substantial growth, with exports to approximately 160 countries, including EU nations. Özçelikay & Bilginer (2002) note that advancements in technology have brought significant developments to the sector, with Turkey aligning its practices with international standards. Fırat & Asil (2006) highlight that meeting global quality standards is now a necessity rather than a luxury, supported by legal regulations and modernization.

The pharmaceutical industry is distinct from other sectors due to its economic and health impacts. Increasing diseases and patient numbers boost medicine demand, driving investments in research and development (Kayserili & Kiyak, 2019; Konca, Özer, & Uğurluoğlu, 2015). While global pharmaceutical activity is concentrated in developed countries, Turkey has emerged as a key player, attracting international investment and addressing challenges like raw material imports, exchange rate fluctuations, and licensing issues (Fırat & Asil, 2006; Gumus, 2014).

Purchasing behavior, as defined by Pride & Ferrell (2000), involves consumers acquiring goods to meet personal or family needs. Pharmacies, combining commercial and public service roles, navigate industrial and situational factors influencing their purchasing decisions (Bilginer & Unal, 2019). Marley, Collier, & Meyer Goldstein, (2004) describe satisfaction derived from pharmacy services as "patient satisfaction" or "customer satisfaction." Satisfied customers tend to increase demand and generate repeat business, highlighting the importance of meeting expectations throughout the supply chain, from pharmaceutical companies to patients.

According to the World Health Organization, medicines are formulated combinations of active substances used for diagnosis, prevention, or treatment (Bayrac, 2011). Advances in the pharmaceutical sector reflect changes in disease patterns, demographics, and healthcare services. PMAT (2020) reports attribute the industry's expansion to globalization, increased healthcare access, and longer life expectancies. Globally, international companies dominate 95% of the market, with the USA, EU countries, and Japan leading in production and imports (KPMG, 2018). In 2019, global pharmaceutical imports grew by 4.6%, reaching $706.7 billion.

Turkey's pharmaceutical industry demonstrates strong production capacity and advanced technology, with significant contributions to trade in medical supplies (PMAT, 2020). High production costs and technological requirements have led to reliance on imports for certain biotechnological products, vaccines, and cancer drugs (KPMG, 2018). PMAT (2020) highlights 77 pharmaceutical manufacturing facilities in Turkey, with a notable presence of multinational companies. However, challenges such as raw material shortages and high costs persist, impacting foreign trade. Despite this, Turkey continues to manufacture cutting-edge biotechnological and medical products in specialized centers

MATERIALS AND METHODS

The aim of this study is to statistically determine the impact of dynamic pricing, one of the innovative pricing elements, on consumers' pre- and post-purchase behavior when purchasing medicines. In this study, a quantitative research method was preferred, and 14,925,783 paid employees affiliated with the social insurance institution in Turkey were selected as the research population in June 2023 (TSI, 2023). The reason for choosing this research population is that certain health expenses are deducted from the premium payments of the employees. Considering the purpose of the research, it was thought that the individuals who are financially affected the most by drug prices are these paid employees. A survey was chosen as the data collection technique in the research, and in this survey, in addition to questions to determine the demographic information of the participants, questions were asked to understand the perception of dynamic pricing and the pre-purchase and post-purchase behaviors of consumers (satisfaction, loyalty, repurchase intention, word of mouth communication). It was planned to use the scales (pre-purchase, satisfaction, loyalty, repurchase intention, word of mouth communication) included in the questionnaire. The first three questions were used in order to determine attitudes about dynamic pricing. These were related to price consciousness and were taken from the scale developed by Donthu & Gilliland (1996). The other six questions related to dynamic pricing were about perceived price fairness and procedural price fairness scales and were based on the Martin, Ponder, & Lueg, (2009) study. Statements aimed at determining the pre-purchase behavior of consumers who planned to take part in the survey were taken from İşlek's (2012) study. The four statements to measure participants' post-purchase satisfaction with dynamic pricing were from Casalo et al. (2008). The seven-item scale, which includes statements to measure participants' loyalty to the same product or company after purchasing regarding dynamic pricing, was taken from the scale developed by Anderson & Srinivasan (2003). The survey includes a three-item word-of-mouth scale developed by Babin, Lee, Kim, & Griffin, (2005), which includes statements about participants recommending the seller of the product they purchased to others after their purchasing experience. Ethics committee approval for this study was obtained from Istanbul Aydin University (Report No: 2024/12), confirming compliance with ethical standards. The survey was digitized and conducted online from 01.09.2023 to 14.09.2023. Of 457 responses, 43 were excluded due to inconsistencies, leaving 414 usable datasets. Structural equation model is used in the analysis of the data obtained according to the research purpose. The research model created according to the research purpose regarding the scales planned to be used in the research is given in Figure 1 below.

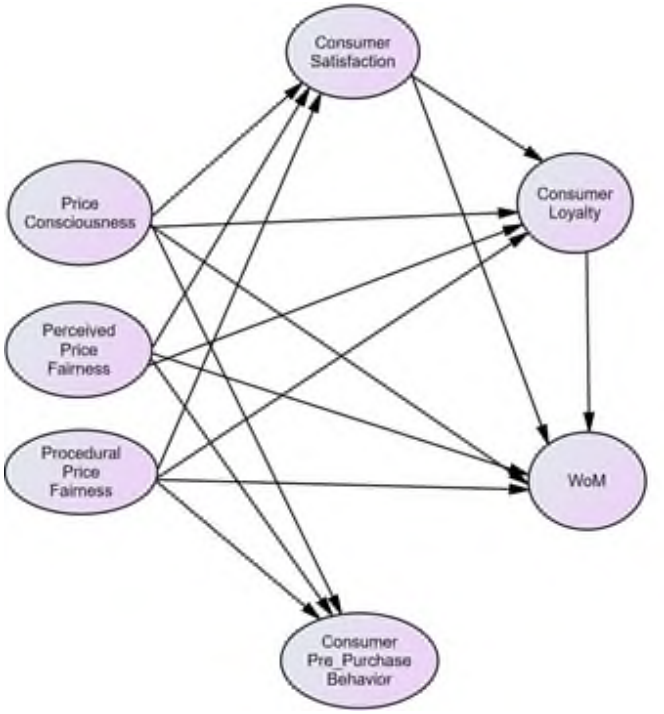

Figure 1. Research Model

RESULTS

A pilot questionnaire comprising scale expressions and demographic questions was administered to 50 participants to evaluate scale suitability. Confirmatory factor analysis (CFA) was applied to the dynamic price application scales, revealing acceptable fit values (X2/df: 3.913, GFI: .946, AGFI: .894, CFI: .957, RMSEA: .079, P=0.000<0.05) (Shermelleh-Engel et al., 2003). Reliability tests showed Cronbach's Alpha values exceeding 0.70 for all scales: dynamic price (α = .872), pre-purchase behavior (α = .906), satisfaction (α = .791), loyalty (α = .893), and word of mouth (α = .786), indicating reliability. Demographic characteristics of the participants showed in the Table 1 below.

Taking the research model of this study into account, the structural equation model (SEM) was used to analyze the relationship between variables. SEM is a statistical technique that allows examining the relationship between continuous or discrete independent variable(s) and continuous or discrete dependent variable(s) (Collier, 2020). SEM was created in accordance with the research model and is shown in the path diagram in Figure 2 below.

According to Figure 2, which shows the path diagram drawn with the research variables, the effects of price consciousness, perceived price fairness, and procedural price fairness subdimensions of the dynamic price scale on satisfaction, loyalty, word-of-mouth communication, and pre-purchase behavior were examined. Additionally, the effect of satisfaction on

Table 1. Demographic Distribution of Participants

		Frequency	Percent			Frequency	Percent
Gender	**Male**	213	51.4	**Education Level**	**High school**	21	5.0
	Female	201	48.6		**Undergraduate**	100	24.2
	Total	**414**	**100%**		**Graduate**	217	52.4
Marital Status	**Married**	252	60.9		**Postgraduate**	76	18.4
	Single	162	39.1		**Total**	**414**	**100%**
	Total	**414**	**100%**				
		Frequency	**Percent**			**Frequency**	**Percent**
Age	**18-23**	32	7.7	**Household Income**	**14500 TL and less**	86	20.8
	24-29	61	14.8		**14501-18000 TL**	71	17.1
	30-35	185	44.7		**18001-21500 TL**	97	23.4
	36-41	70	16.9		**21501-25000 TL**	101	24.4
	42 and more	66	15.9		**25001 TL and more**	59	14.3
	Total	**414**	**100%**		**Total**	**414**	**100%**
		Frequency	**Percent**			**Frequency**	**Percent**
Drug Use (Month)	**Never**	77	18.6	**Chronic Disease**	**Yes**	127	30.7
	1 – 5	261	63.1		**No**	287	69.3
	6 – 10	63	15.2		**Total**	**414**	**100%**
	11 and more	13	3.1				
	Total	**414**	**100%**				

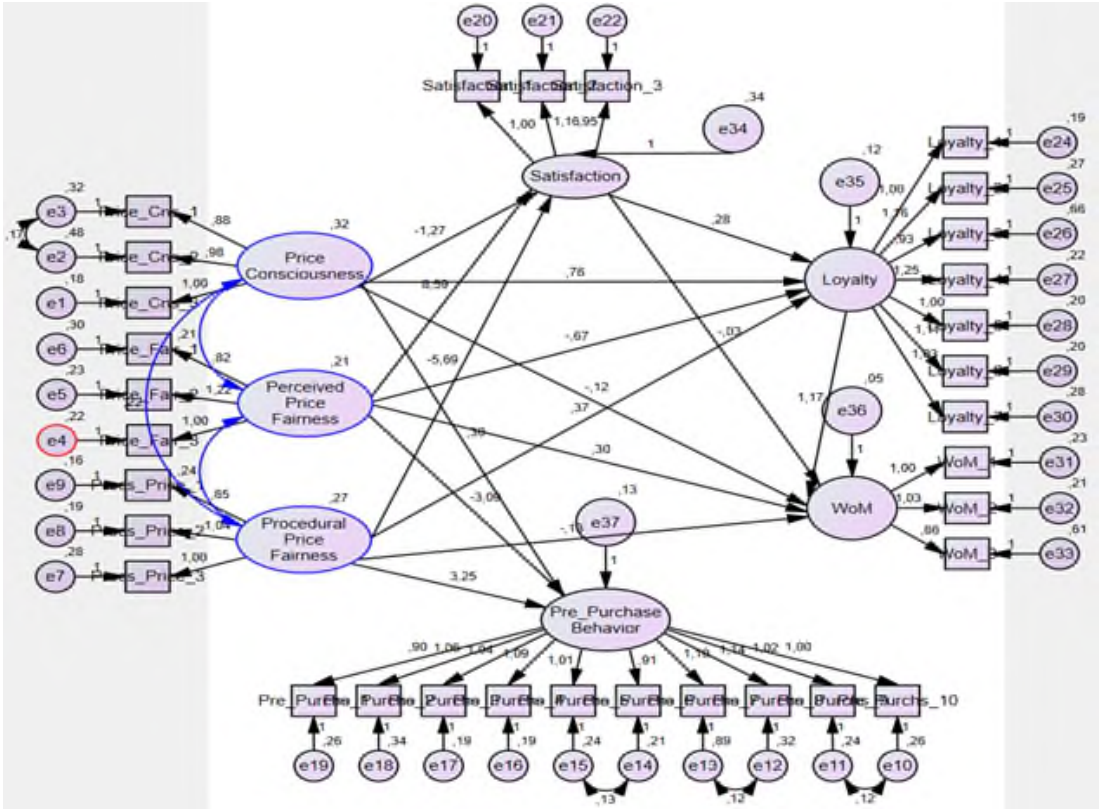

Figure 2. Path Diagram

loyalty and word-of-mouth communication and finally the effect of loyalty on word-of-mouth communication were examined. When the goodness of fit values of the established structural model are examined (X2/df= 2.970, CFI= .903, GFI= .9, AGFI= .852, RMSEA= .069), it is clear that it falls within the acceptable goodness of fit values. The values of the relationship between the variables in the established model are given in Table 2 below.

An examination of Table 2, where the regression weights of the path diagram created with the research variables are given, is examined, shows that there are four situations with significance levels below .05. For this reason, it is understood that four situations in which the effect exists will be mentioned in

Table 2. Regression Weights

			Estimate	S.E.	C.R.	P
Satisfaction	<---	Price Consciousness	-1.268	.985	-1.288	.198
Satisfaction	<---	Perceived Price Fairness	8.588	6.942	1.237	.216
Satisfaction	<---	Procedural Price Fairness	-5.695	5.517	-1.032	.302
Loyalty	<---	Price Consciousness	.757	.128	5.917	***
Loyalty	<---	Perceived Price Fairness	-.673	.468	-1.439	.150
Loyalty	<---	Procedural Price Fairness	.373	.448	.833	.405
Loyalty	<---	Satisfaction	.276	.100	2.754	**.006**
WoM	<---	Price Consciousness	-.119	.126	-.947	.343
Pre_Purchase	<---	Price Consciousness	.305	.378	.806	.420
WoM	<---	Perceived Price Fairness	.297	.383	.777	.437
Pre_Purchase	<---	Perceived Price Fairness	-3.087	1.700	-1.816	.069
WoM		Procedural Price Fairness	-.132	.354	-.373	.709
Pre_Purchase		Procedural Price Fairness	3.252	1.250	2.601	**.009**
WoM		Satisfaction	-.027	.092	-.298	.766
WoM		Loyalty	1.167	.101	11.501	***

- ***** : p<0.05**
- Estimate: Regression weight

which an effect exists, according to the statistical result of the study. The first of these is the positive effect of price consciousness, one of the sub-dimensions of the dynamic price scale, on customer loyalty (.757). The second effect is the positive effect of the procedural price justice sub-dimension of the dynamic price scale on pre-purchase behavior (3.252). The effect of satisfaction, one of the research variables, on loyalty is another effect obtained from the structural model (.276). Finally, the positive effect of loyalty, one of the research variables, on word-of-mouth communication is another effect obtained from the model (1.167). When the other relationships in the table are examined, it cannot be said that there is a statistical effect because their significance level is greater than the margin of error (p>.05).

DISCUSSION

Today, people adopt a rational approach when purchasing consumer goods, prioritizing quality at affordable prices. However, this rationality often diminishes in health-related expenditures. Public authorities enforce regulations and inspections to protect consumers, particularly regarding drug expenses. Pharmaceutical companies may apply dynamic pricing strategies within the limits of legal regulations, but they must prioritize customer satisfaction and long-term sustainability. Dynamic pricing is critical to minimize sociological and psychological impacts on consumers who allocate limited resources to health expenses, making this an essential area of study.

This research focused on paid working individuals in Turkey, aiming to assess their perceptions of dynamic pricing in drug purchases and the effects on pre- and post-purchase behavior. A questionnaire was used to collect data from 414 participants, and the structural equation model was applied to analyze the relationships between dependent and independent variables. Findings revealed that procedural price fairness influences pre-purchase behavior, indicating that consumers' perception of fairness in dynamic pricing impacts their purchasing decisions.

Post-purchase behavior analysis showed that price consciousness affects customer loyalty. This suggests that consumers' awareness of dynamic pricing strategies influences their loyalty in drug purchases. Additionally, satisfaction was found to impact loyalty, which, in turn, affects word-of-mouth communication. Loyal customers contribute to positive word-of-mouth, enhancing brand reputation.

Literature on dynamic pricing supports these findings, showing no negative impact on satisfaction (Haws & Bearden, 2006; Kuzay, 2018; Kolsuz & Erenkol, 2021) and a positive relationship with word-of-mouth communication (Martin et al., 2009; Weisstein, Monroe, & Kukar-Kinney, 2013; Ajorlou, Jadbabaie, & Kakhbod, 2018). These studies emphasize that dynamic pricing strategies can foster loyalty and improve customer relationships.

CONCLUSION

When consumers' pre- and post-purchase behaviors regarding dynamic pricing are examined structurally with the statistical findings obtained as a whole, it is seen that dynamic pricing effects on pre-purchase customer decisions. It is also understood that post-purchase decisions have an impact on loyalty.

Based on the results, it is clear that pharmaceutical companies planning to carry out long-term operations should be customer-centered in dynamic pricing decisions. Considering the impacts of post-purchase behaviors observed in research, satisfaction and loyalty variables are noted to positively influence word-of- mouth communication, which in turn typically effects long term behaviors.

Although the research is informative for companies operating in this sector with its statistical findings, it has limitations as it is only applied to employed people in one month. For this reason, in future studies in this field, it is recommended that the research survey be administered at different times and to other individuals with purchasing power in order to provide more comprehensive statistical information.

Ethics Committee Approval: Ethics committee approval for this study was obtained from Istanbul Aydin University (Report No: 2024/12)

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Original Article

Knowledge, attitude and practice of pharmacoepidemiology in paediatric pharmacists: A nationwide questionnaire-based study

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ABSTRACT

Background and Aims: Pharmacoepidemiology evidence are crucial in the development and evaluation of drugs in paediatrics. Data on the knowledge, attitude, and practice (KAP) of paediatric pharmacists regarding pharmacoepidemiology are limited. This study aimed to evaluate the KAP of pharmacoepidemiology in Malaysian paediatric pharmacists.

Methods: A total of 149 paediatric pharmacists from Malaysian public hospitals were invited to participate in this cross-sectional study. Data were collected between April and June 2023 using a self-administered online questionnaire on KAP regarding pharmacoepidemiology. Bloom's cut-off value of ≥ 81% denoted high knowledge, positive attitude, and good practice, respectively. Descriptive and inferential data analyses were performed using SPSS v20.

Results: Ninety-nine paediatric pharmacists (response rate 66.44%; mean age 34.3±3.99 years) participated in this study. The majority (61.62%, n=61) worked at major specialist hospitals, with an overall mean working experience of 5.2±4.29 years. About 22.22% of pharmacists had a high level of knowledge, 15.15% had a positive attitude, but none had a good level of pharmacoepidemiology practice. On-the-job training (89.9%) and networking on paediatric pharmacy research (86.87%) were strongly recommended as key facilitators of pharmacoepidemiology. Knowledge [OR = 1.067, 95% CI (1.023–1.112), p=0.02] and attitude [OR = 1.118, 95% CI (1.044–1.198), p=0.02] scores significantly correlated with pharmacoepidemiology practice.

Conclusion: Paediatric pharmacists demonstrated moderate knowledge, a neutral attitude, but poor practice towards pharmacoepidemiology. Future initiatives should emphasise collaborative efforts among academic institutions, professional bodies and practitioners to address knowledge and attitude through the provision of on-the-job training and networking to enhance pharmacoepidemiology application among paediatric pharmacists in Malaysia.

Keywords: Pharmacoepidemiology, Paediatric, Knowledge, Attitude, Pharmacists

INTRODUCTION

Pharmacoepidemiology is the study of the use and effects of drugs in large numbers of people (Strom, Kimmel, & Hennessy, 2013). In comparison with experimental studies or clinical trials, pharmacoepidemiologic studies have the potential to descriptively evaluate drug use and effects in patients experiencing real-life conditions using data collected retrospectively, prospectively, or cross-sectionally (Montastruc et al., 2019). The ability to evaluate drug use and effects in real-world settings makes pharmacoepidemiologic studies a more suitable approach for generating evidence in populations underrepresented in clinical trials, such as children.

The numerous drugs used in children lack age-specific studies, rendering them unapproved for paediatric use by regulatory authorities. Recruiting children for clinical trials has proven challenging (Lagler, Hirschfeld, & Kindblom, 2021). As a result, pharmacoepidemiologic studies have been employed to assess prescription drug safety (Luo, Doherty, Cappelleri, & Frush, 2007), develop tools for detecting irrational drug use (Prot-Labarthe et al., 2014), evaluate prescription drug-related adverse events (Luo, Cappelleri, & Frush, 2007), and describe dosing practices (Thompson et al., 2020) in children. However, only a minority of healthcare professionals caring for children expressed interest in the epidemiology associated with prescribing and medication use (MacLeod, 2018).

Globally, paediatric pharmacy services operate under different operational models, with the majority being integrated into larger hospitals, while some are freestanding for children (Webster et al., 2019). In Malaysia, paediatric pharmacy services were introduced under the Ministry of Health (MOH) Malaysia's pharmacy programme in 2006. Subsequently, the

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first paediatric pharmacists working group committee was formed in 2009 (Pharmaceutical Services Programme, 2015). The establishment and growth of paediatric pharmacy services are heavily reliant on data-driven attributes, including benchmarking, the anticipated impact on the quality of care and patient safety, interdisciplinary support, national organisation guidelines and costing (Webster et al., 2019). Given the specific indications and limitations of various pharmacoepidemiological study designs, understanding the different methods involved is crucial to ensure optimal use and accurate interpretation of the data (Lasky et al., 2016).

Based on current information, no studies have been conducted to evaluate the knowledge, attitude, and practice (KAP) of paediatric pharmacists in Malaysia regarding the pharmacoepidemiology approach and its application. Consequently, this study was undertaken to evaluate the KAP of pharmacoepidemiology among paediatric pharmacists in Malaysia. The findings of this study will be of paramount importance in identifying the need for improvement of in-service training and areas where future research or intervention programmes should be focused on the use and application of pharmacoepidemiology data in providing safe and effective medicine for children.

MATERIALS AND METHODS

Operational definitions

According to the classification by the European Medicines Agency (EMEA) and the International Council for Harmonisation (ICH), paediatric patients were categorised by age, including neonates (below 28 days), infants (28 days to 23 months), children (2 to 11 years) and adolescents (12 to 18 years) (Ceci et al., 2002).

Paediatric pharmacists are clinical pharmacists working in various ward settings, including general paediatrics, paediatric intensive care, neonatal intensive care, and paediatric subspecialty wards, such as nephrology, neurology, dermatology, cardiology, and oncology. Their role is to optimise and influence the safe and effective use of medications for paediatric patients (Pharmaceutical Services Programme, 2015).

The definitions of KAP used in a previous study (Balan, 2021) were applied in this study.

Knowledge: Theoretical or practical understanding of the concept and application of pharmacoepidemiology.

Attitude: Predisposition to respond positively or negatively to pharmacoepidemiology approach and application.

Practice: Application of knowledge or practical approaches related to pharmacoepidemiology.

Ethical consideration

Ethical approval was obtained from the ethics committee for the Ministry of Health (MOH) facilities in Malaysia, i.e., the Medical Research and Ethics Committee (MREC) (NMRR ID-23-00214-2RW). The personal identifiers of respondents were not collected. Electronic informed consent was obtained from the respondents before the commencement of the study. The reporting of the study followed the consensus-based Checklist for Reporting Results of Internet E-Surveys (CHERRIES) (Eysenbach, 2004) (Supplementary material: Appendix A).

Study design

Cross-sectional, questionnaire-based study.

Study setting

Ministry of Health hospitals in Malaysia are generally classified as major, minor, or non-specialist hospitals and special medical institutions (regarded collectively as 'hospitals' hereinafter). Major and minor specialist hospitals differ by scope of specialty services and workload, whereas non-specialist hospitals provide visiting specialist services, and special medical institutions have specific resident specialties. In total, 146 MOH hospitals are in Malaysia (Health Informatics Center, 2022). As recommended by the Pharmaceutical Services Programme, the ratio of paediatric ward pharmacist to patient is 1:20 for general or sub-specialty wards and 1:10 for critical care wards (Pharmaceutical Services Programme, 2023). All hospitals with paediatric pharmacists were involved in this study.

Eligibility criteria

Paediatric pharmacists practicing in MOH hospitals with at least 12 months of clinical experience were included in the study. Paediatric pharmacists on leave (study leave, maternity leave etc.) during the study period and paediatric pharmacy trainees were excluded from the study.

Sampling technique and sample size

The convenience sampling technique was used in this study. The sample size was calculated using Raosoft online software available at "http://www.raosoft.com/samplesize.html". At the time of study conception, there were 149 paediatric pharmacists practising in MOH facilities in Malaysia. The sample size was calculated based on the assumption of a precision of 0.05 with a 95% confidence interval and response distribution of 50%. Based on this, the required number of respondents was 108.

Data collection

The data were collected using a validated, piloted, and self-administered online questionnaire. A closed survey approach was used whereby the email addresses of all paediatric pharmacists were obtained from the Pharmaceutical Services Programme, MOH. Invitations to participate, including the study brochure (Supplementary material: Appendix B), information sheet, and questionnaire link, were sent via email to all eligible paediatric pharmacists. Data were collected between April and June 2023. To increase participation and response rates, reminder emails were sent at one-month intervals. Respondents received no incentives because participation was voluntary. Consent to participate was obtained through the online questionnaire link. Paediatric pharmacists who agreed were directed to respond to the questionnaire, whereas those who declined were automatically redirected to exit the online questionnaire platform. The estimated time to complete the questionnaire was approximately 20 minutes. Respondents could review their responses through a 'Back' button, before submission.

Questionnaire development

A specific questionnaire was developed to assess KAP on pharmacoepidemiology and its application. The initial questions were formulated by the researcher based on the information gathered from a similar questionnaire (Norhayati & Nawi, 2021) and a thorough review of the literature (Barzkar, Baradaran, & Koohpayehzadeh, 2018; Lasky et al., 2016; S. Li, Cao, & Zhu, 2019; MacLeod, 2018; Osokogu, Verhamme, Sturkenboom, & Kaguelidou, 2018; Reali et al., 2021; Strom et al., 2013; Verhamme & Sturkenboom, 2011). The final wording of the questions was expressed as consensus in consultation with two academicians with expertise in paediatrics. The task undertaken in several sessions led to the first version of the questionnaire, which was subjected to face and content validation by a panel of experts, including former paediatric pharmacists, academicians, and pharmacoepidemiology specialists.

Former paediatric pharmacists identified through a snowballing method pilot-tested the final questionnaire draft. The usability and technical functionality of the electronic questionnaire were also included in the pilot testing. The final version of the questionnaire was refined based on the comments from the expert panel and feedback received during the pilot testing. The comments received during the pilot testing suggested adding definitions for the categories of duty (i.e., full-time and part-time) and including a time frame for questions related to conducting pharmacoepidemiology research and attending training.

The questionnaire was designed using Google Forms, and all questions were mandatory to respond. Key sections were presented in a logical order, but within sections, questions were randomised to reduce bias. The questionnaire consisted of five sections: Section 1 collected the sociodemographic data of the respondents, including age, gender, ethnicity, academic qualification, place of practice, years of experience, and information on training and research experience.

Section 2 assessed knowledge on pharmacoepidemiology using 16 items that encompassed an understanding of the concept (8 items) and application of pharmacoepidemiology (8 items). Responses were scored as 'Correct=1', 'Wrong=0,' and 'Unsure=0'.

Section 3 included 15 attitude items that were divided into three categories i.e. applicability (7 items), effect on practice (4 items), and learning (4 items); in keeping with previous studies (Barzkar et al., 2018; S. Li et al. (2019). Responses for attitude items were evaluated on a five-point Likert scale: Strongly Agree = 5, Agree = 4, Neutral = 3, Disagree = 2, and Strongly Disagree = 1.

Sections 4 contained 10 practice items which were assessed using a five-point Likert scale as follows: Always = 5, Often = 4, Sometimes = 3, Seldom = 2, Never = 1. The items within the practice section were categorised as either 'knowledge application' (3 items) or 'practical approach' (7 items) (Balan, 2021).

Section 5 contained nine facilitators of the pharmacoepidemiology approach and its application, which were identified from previous studies on practice-based research participation among hospital pharmacists (Reali et al., 2021) and challenges in paediatric pharmacoepidemiology (Osokogu et al., 2018). Responses to the items were evaluated on a five-point Likert scale: Strongly Agree = 5, Agree = 4, Neutral = 3, Disagree = 2, and Strongly Disagree = 1.

Data analysis

Response rate was calculated as the percentage of participants who submitted responses out of the total number of individuals invited to participate in the study. The total scores were calculated for each knowledge, attitude, and practice domain. Each total raw score was transformed into a "percent score" and categorised based on Bloom's cut-off point. In accordance with a previous KAP study (Zanaridah, Norhayati, & Rosnani, 2021), scores less than 59% denoted low, negative, and poor levels of knowledge, attitude, and practice. Scores within 60%–80% were equated with a moderate, neutral, and fair level of knowledge, attitude and practice. Scores exceeding 80% denote high, positive, and good levels of knowledge, attitude, and practice, respectively. Responses to facilitators of pharmacoepidemiology application are presented as raw scores. The pharmacoepidemiology attitudes and facilitators domains were categorised by grouping "strongly agree" and "agree" as positive responses and "disagree" and "strongly disagree" as negative responses. For the practice domain, 'always' and 'often' were categorised

as positive responses while 'seldom' and 'never' were categorised negative responses.

The data was entered and analysed using SPSS version 20. (IBM SPSS Statistics, IBM, New York, US). Descriptive analyses were conducted to define high levels of knowledge, positive attitudes, and a good practice of pharmacoepidemiology among paediatric pharmacist in Malaysia. Simple and multiple logistic regression analyses were performed to identify factors associated with pharmacoepidemiology practice.

A simple logistic regression analysis was performed to determine the potential associated factors for pharmacoepidemiology practice category. Independent variables that were statistically (p-value <0.25) and clinically significant were chosen for multivariate analysis using multiple logistic regression. The final variable selection was conducted using an automatic backward and forward stepwise procedure. Interactions and multicollinearity were checked. A model fit assessment was performed to obtain the final model. Crude and adjusted regression coefficients with 95% confidence intervals and p-values are presented. A $p < 0.05$ level was considered statistically significant.

RESULTS

Socio-demographic characteristics

A total of 149 paediatric pharmacists were invited to participate in the study, and 99 responded to the questionnaire (response rate of 66.44%). Most respondents (n=82, 82.83%) were practising in a single sub-discipline, while others practiced in multiple sub-disciplines (Table 1).

Knowledge of pharmacoepidemiology

High and moderate levels of knowledge were found in 22.22% and 53.54% of respondents, respectively. The average number of respondents with correct answers for items related to the application of pharmacoepidemiology (n=72) was higher than those related to the concept of pharmacoepidemiology (n=56). The knowledge items and corresponding responses are presented in Table 2.

Attitude towards pharmacoepidemiology

The responses to each attitude item are shown in Table 3. Overall, the majority (82.83%) of the respondents had a neutral attitude towards pharmacoepidemiology approach and its application. Items categorised as "effect" and "learning" received more positive responses compared to those categorised as "applicability". Specifically, up to 89.89% and 86.86% positive responses were recorded for items in the "effect" and "learning" categories, respectively. For items in the "applicability" category, positive responses reached up to 78.78%.

Practice of pharmacoepidemiology

None of the respondents had a good level of pharmacoepidemiology practice, with 58.59% and 41.41% reported to have poor and fair levels of pharmacoepidemiology practice, respectively. Positive responses for practice items related to the application of practical approaches (up to 43.43%) were slightly higher than those related to the application of knowledge (up to 26.26%) (Table 4). The responses for each practice items are shown in Table 4.

Facilitators of the pharmacoepidemiology approach and its application

On-the-job training (89.9%) and networking on paediatric pharmacy research (86.87%) were strongly recommended as key facilitators of pharmacoepidemiology. On the other hand, the least preferred facilitator (81.82%) was providing scheduled protected time for paediatric pharmacists to conduct pharmacoepidemiology research (Table 5).

Factors associated with pharmacoepidemiology practice

In the univariate analyses, ethnicity, workplace, category of duty, and knowledge and attitude scores were statistically significant and were subsequently included in the multivariate analysis. The overall fit of the model was checked and reported with Hosmer-Lemeshow test (p=0.768) and Pearson Chi-Square Test (p=4.904). The model explained 32.4% (Nagelkerke R2) of the variance in pharmacoepidemiology practice and correctly classified 73.7% of the cases. Multivariate logistic regression analyses demonstrated that knowledge and attitude scores were significantly associated with pharmacoepidemiology practice level among paediatric pharmacists (Table 6).

Table 1. Socio-demographic Characteristics of the Respondents (n = 99)

Variables	n (%)
Age (years ± SD)	34.3±3.99
Gender	
Female	86 (86.87)
Male	13 (13.13)
Ethnicity	
Malay	51 (51.52)
Chinese	40 (40.40)
Indian	7 (7.07)
Others	1 (1.01)
Highest academic qualification	
Degree	76 (76.77)
Masters	21 (21.21)
PhD	2 (2.02)
Current workplace	
Major Specialist Hospital	61 (61.62)
Minor Specialist Hospital	26 (26.26)
Non-specialist Hospital	11 (11.11)
Special Medical Institution	1 (1.01)
Working experience as paediatric pharmacist (years ± SD)	5.2±4.29
Category of duty	
Full-time	89 (89.9)
Part-time	10 (10.10)
Current sub-discipline	
General paediatrics	47 (47.47)
General paediatrics + NICU	3 (3.03)
General paediatrics + NICU + PICU + SCN	2 (2.02)
General paediatrics + NICU + SCN	6 (6.06)
General paediatrics + PICU	2 (2.02)
General paediatrics + SCN	1 (1.01)
NICU	20 (20.20)
NICU + PICU + SCN	1 (1.01)
NICU + SCN	2 (2.02)
PICU	10 (10.1)
SCN	1 (1.01)
Haemato-oncology	3 (3.03)
Paediatric surgery	1 (1.01)
Conducted pharmacoepidemiology research in the past 12 months	
Yes	1 (1.01)
No	95 (95.96)
Unsure	3 (3.03)
Attended training related to pharmacoepidemiology research methods	
Yes	0 (0)
No	97 (97.98)
Unsure	2 (2.02)

NICU=Neonatal Intensive Care Unit, PhD=Doctor of Philosophy, PICU=Paediatric Intensive Care Unit, SCN=Special care Nursery, SD=Standard Deviation.

Table 2. Knowledge Items with Percentage of Responses

Item (Category)	Description	Correct n (%)	Unsure n (%)	Wrong n (%)
K1 (Concept)	Pharmacoepidemiology is a bridge of science connecting both pharmacology and epidemiology.	96 (96.97)	3 (3.03)	-
K2 (Concept)	Pharmacoepidemiology research investigates the use of drug in the post marketing phase.	73 (73.74)	20 (20.2)	6 (6.06)
K3 (Concept)	Cohort, case-control and cross-sectional studies are examples of study designs used in pharmacoepidemiology.	70 (70.71)	24 (24.24)	5 (5.05)
K4 (Application)	Real-life clinical impact of a medication can be clearly demonstrated using pharmacoepidemiology approach.	87 (87.88)	10 (10.1)	2 (2.02)
K5 (Application)	Pharmacoepidemiology approach can be used to identify and evaluate causes or risk factors of diseases.	78 (78.79)	11 (11.11)	10 (10.1)
K6 (Concept)	Prospective studies are less prone to bias and can more easily demonstrate causation.	71 (71.72)	21 (21.21)	7 (7.07)
K7 (Concept)	Meta-analysis is superior to case-control studies in evidence-based medicine.	76 (76.77)	16 (16.16)	7 (7.07)
K8 (Application)	Pharmacoepidemiology approach can be used in tool development to evaluate rational drug use.	88 (88.89)	9 (9.09)	2 (2.02)
K9 (Concept)	Pharmacoepidemiology studies utilise both observational and experimental methods.	9 (9.09)	23 (23.23)	66 (67.68)
K10 (Application)	Pharmacoepidemiology study findings is suitable for making decisions about patient care rather than for policy making.	20 (20.2)	24 (24.24)	55 (55.56)
K11 (Application)	Pharmacoepidemiology approach can be used in supporting the rational and cost-effective use of drugs in the population.	93 (93.94)	3 (3.03)	3 (3.03)
K12 (Application)	In clinical settings, pharmacoepidemiology studies can be used for hypothesis generating and testing.	71 (71.72)	21 (21.21)	7 (7.07)
K13 (Application)	Drug utilisation studies in children may be used to identify the major therapeutic problems in this population.	84 (84.85)	13 (13.13)	2 (2.02)
K14 (Application)	The study of adverse drug reactions (ADRs) in a pharmacovigilance database is a type of pharmacoepidemiology study.	79 (79.8)	18 (18.18)	2 (2.02)
K15 (Concept)	The STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) checklist is an instrument to evaluate the quality of observational research.	3 (3.03)	50 (50.51)	46 (46.46)
K16 (Concept)	The measure of risk that is calculated in case-control studies is the odds ratio, which are the odds of having the exposure if an individual has the disease.	56 (56.57)	37 (37.37)	6 (6.06)

DISCUSSION

Approximately 20% of the respondents reported having good knowledge about pharmacoepidemiology. Considering the educational background and working experience, we inferred that the participants' knowledge of pharmacoepidemiology was acquired during their undergraduate years. The observed low level of knowledge underscores the previously identified disparities between the curriculum (Herrera Comoglio, 2020) and the impact (M. Li, Schulz, Wang, & Lu, 2019) of pharmacoepidemiology in both undergraduate and postgraduate programmes at universities. Although refinement of university curricula is important, these findings also highlighted the necessity of discovering methods to enhance the preparedness of paediatric pharmacists with the requisite knowledge and skills in pharmacoepidemiology. A collaborative approach involving educational institutions and practicing professionals to ensure a comprehensive understanding of pharmacoepidemiology will foster a more proficient and well-equipped workforce in the field.

The overall attitude of paediatric pharmacists towards pharmacoepidemiology was neutral. Although majority of the respondents expressed that pharmacoepidemiology can yield favourable effects on their practice, lower scores were given regarding its applicability. Similar findings were reported by another local study, in which pharmacists gave lower scores for the attitude domain, namely, implementing research into practice' compared to other domains (Tan & Hatah, 2017). While performing their duties, pharmacists typically rely more on formularies and drug information sources rather than evidence from research articles (Iheanacho, Odili, & Oluigbo, 2021). Furthermore, prescribers tend to have greater discretion in medical decision-making, although a multidisciplinary approach is advocated (Coughlin, 2018). These circumstances may have led pharmacists to perceive the implementation of pharmacoepidemiological evidence as challenging.

Table 3. Attitude Items with Percentage of Responses

Item (Category)	Description	Strongly agree n (%)	Agree n (%)	Neutral n (%)	Disagree n (%)	Strongly disagree n (%)
A1 (Effect)	I believe practicing pharmacoepidemiology approach improves patient health outcome	57 (57.58)	32 (32.32)	10 (10.1)	-	-
A2 (Learning)	I am willing to learn about pharmacoepidemiology approach and application if given the opportunity	51 (51.52)	35 (35.35)	10 (10.1)	3 (3.03)	-
A3 (Effect)	I believe that pharmacoepidemiology approach and application is a threat to good clinical practice	31 (31.31)	19 (19.19)	12 (12.12)	26 (26.26)	11 (11.11)
A4 (Applicability)	I am ready to practice pharmacoepidemiology approach and application in my work	34 (34.34)	41 (41.41)	21 (21.21)	3 (3.03)	-
A5 (Applicability)	I feel that pharmacoepidemiology research findings are very important in my day-to-day management of patients	38 (38.38)	40 (40.4)	20 (20.2)	1 (1.01)	-
A6 (Applicability)	I feel that pharmacoepidemiology approach and application is of limited value in paediatric medicine	20 (20.2)	23 (23.23)	20 (20.2)	26 (26.26)	10 (10.1)
A7 (Applicability)	I believe that years of clinical experience is more valuable than evidence derived from pharmacoepidemiology studies	19 (19.19)	15 (15.15)	32 (32.32)	27 (27.27)	6 (6.06)
A8 (Effect)	I am convinced that pharmacoepidemiology approach and application in clinical practice increases the effectiveness of my work	36 (36.36)	45 (45.45)	17 (17.17)	1 (1.01)	-
A9 (Applicability)	I feel confident managing patients with evidence derived from pharmacoepidemiology studies	36 (36.36)	41 (41.41)	20 (20.2)	1 (1.01)	1 (1.01)
A10 (Applicability)	I believe that understanding the basic drug effect and outcome is sufficient for good clinical practice	27 (27.27)	24 (24.24)	23 (23.23)	20 (20.2)	5 (5.05)
A11 (Effect)	I feel that practicing pharmacoepidemiology approach and application would produce better health practitioners	42 (42.42)	43 (43.43)	12 (12.12)	2 (2.02)	-
A12 (Applicability)	I often feel burdened whenever needing to use pharmacoepidemiology approach in practice	8 (8.08)	20 (20.2)	47 (47.47)	20 (20.2)	4 (4.04)
A13 (Learning)	I am happy if it is mandatory for paediatric pharmacists to learn about pharmacoepidemiology	18 (18.18)	32 (32.32)	41 (41.41)	6 (6.06)	2 (2.02)
A14 (Learning)	I think that continuous education and incorporating formal teaching of pharmacoepidemiology approach and application is very important	38 (38.38)	45 (45.45)	13 (13.13)	3 (3.03)	-
A15 (Learning)	I am willing to attend training programmes specifically dedicated to paediatric pharmacoepidemiology	44 (44.44)	39 (39.39)	14 (14.14)	2 (2.02)	-

Table 4. Practice Items with Percentage of Responses

Item (Category)	Description	Always n (%)	Often n (%)	Sometimes n (%)	Seldom n (%)	Never n (%)
P1 (Knowledge application)	I use pharmacoepidemiology approach and application in my daily practice	4 (4.04)	20 (20.2)	41 (41.41)	19 (19.19)	15 (15.15)
P2 (Practical approach)	I use multiple search engines to look for pharmacoepidemiology study articles	10 (10.1)	24 (24.24)	33 (33.33)	19 (19.19)	13 (13.13)
P3 (Practical approach)	I search for pharmacoepidemiology articles from published journal only	3 (3.03)	28 (28.28)	37 (37.37)	18 (18.18)	13 (13.13)
P4 (Practical approach)	I do not have enough time to study on pharmacoepidemiology approach and application	18 (18.18)	35 (35.35)	33 (33.33)	9 (9.09)	4 (4.04)
P5 (Knowledge application)	I do not apply pharmacoepidemiology approach in my professional duties due to limitations of the management that I can offer to paediatric patients	10 (10.1)	23 (23.23)	41 (41.41)	18 (18.18)	7 (7.07)
P6 (Knowledge application)	I use pharmacoepidemiology approach and application for answering the questions in clinical setting	7 (7.07)	19 (19.19)	48 (48.48)	14 (14.14)	11 (11.11)
P7 (Practical approach)	I join continuous medical education for updates regarding pharmacoepidemiology approach and application	4 (4.04)	18 (18.18)	24 (24.24)	30 (30.3)	23 (23.23)
P8 (Practical approach)	I share knowledge on pharmacoepidemiology approach and application with my colleagues	1 (1.01)	13 (13.13)	27 (27.27)	32 (32.32)	26 (26.26)
P9 (Practical approach)	I promote pharmacoepidemiology approach and application to my colleagues at workplace	2 (2.02)	13 (13.13)	26 (26.26)	26 (26.26)	32 (32.32)
P10 (Practical approach)	I do not need to conduct pharmacoepidemiology research as evidence is available about many interventions I make in my clinical practice	6 (6.06)	13 (13.13)	37 (37.37)	26 (26.26)	17 (17.17)

Table 5. Facilitators of Pharmacoepidemiology with Percentage of Responses

Item	Description	Strongly agree n (%)	Agree n (%)	Neutral n (%)	Disagree n (%)	Strongly disagree n (%)
S1	Inclusion of pharmacoepidemiology approach and application in decision making process	25 (25.25)	58 (58.58)	15 (15.15)	1 (1.01)	-
S2	Provide opportunities and assistance to increase publication of pharmacoepidemiology study(s) in peer-reviewed journals	28 (28.28)	55 (55.55)	16 (16.16)	-	-
S3	Provide opportunities and assistance to present pharmacoepidemiology study(s) in local and international conferences.	28 (28.28)	55 (55.55)	15 (15.15)	1 (1.01)	-
S4	Easy accessibility to pharmacoepidemiology outcome data and research articles/reports	42 (42.42)	42 (42.42)	13 (13.13)	1 (1.01)	1 (1.01)
S5	Establishing a network of paediatric pharmacists' research group to discuss pharmacoepidemiology outcome data and research articles/reports	44 (44.44)	42 (42.42)	13 (13.13)	-	-
S6	Regular e-mail update on recent pharmacoepidemiology outcome data and research articles/reports involving paediatric patients	38 (38.38)	44 (44.44)	14 (14.14)	3 (3.03)	-
S7	Providing adequate on-the-job training in conducting pharmacoepidemiology studies	41 (41.41)	48 (48.48)	9 (9.09)	1 (1.01)	-
S8	Providing incentive for paediatric pharmacists who conduct and publish/present pharmacoepidemiology studies	46 (46.46)	38 (38.38)	15 (15.15)	-	-
S9	Providing scheduled protected time for paediatric pharmacists to conduct pharmacoepidemiology study(s)	40 (40.4)	41 (41.41)	16 (16.16)	2 (2.02)	-

Table 6. Factors associated with pharmacoepidemiology practice

Factors	Practice category		b	Adjusted OR (95% CI)	p-value
	Fair, n=41	Poor, n=58			
Knowledge score, % (SD)	73.2 (11.6)	61.9 (17.4)	0.064	1.067 (1.023, 1.112)	0.02
Attitude score, % (SD)	77.5 (8.7)	71.3 (6.9)	0.112	1.118 (1.044, 1.198)	0.02

Poor pharmacoepidemiology practice was observed among paediatric pharmacists, reinforcing the notion that translating research into actionable outcomes is a common challenge within the profession. Additionally, knowledge and attitude scores were associated with pharmacoepidemiology practice among paediatric pharmacists. Emphasising theoretical knowledge is crucial in the approach and application of pharmacoepidemiology, considering the numerous challenges unique to the field (Beyene, Chan, & Man, 2023), particularly those specific to paediatric pharmacoepidemiology (Osokogu et al., 2018). By addressing knowledge and attitude factors, there is a potential to enhance the overall competence and practice of paediatric pharmacists in pharmacoepidemiology, ultimately leading to improved patient care and outcomes in the field.

Paediatric pharmacists strongly recommended on-the-job training and the establishment of a research group network as facilitators of pharmacoepidemiology. These findings align with the mission statement and objectives of the Paediatric Special Interest Group (SIG) of the International Society for Pharmacoepidemiology (ISPE) (Pharmacoepidemiology, n. d.). The feasibility of designing and implementing a pharmacy-tailored research training programme has been shown to positively impact pharmacists' knowledge and attitudes (Awaisu et al., 2015). Locally, a research technical committee for pharmacoepidemiology and data analysis, along with a paediatric pharmacists' working group committee, exists within the Pharmaceutical Services Programme. Close collaboration between these entities could foster the development and implementation of a training programme to equip paediatric pharmacists with the necessary skills and knowledge for effective pharmacoepidemiology practice.

The proposed training module development process can be divided into three phases. In Phase I, the educational and training needs of paediatric pharmacists can be evaluated. The questionnaire used in this study is easily adaptable for this purpose. Phase II includes the design and delivery of training. The active-learning method i.e. learning-centred paradigm, can be considered as it has been proven successful in providing on-the-job training for practicing pharmacists (Peletidi & Kayyali, 2022). As observed in the current study, knowledge of the concept of pharmacoepidemiology could be emphasised in the training module. As poor practice of pharmacoepidemiology was observed, the training module should also consist of hands-on research discussions and group assignments to conduct pharmacoepidemiology studies. The research topics can be predetermined based on professional or national research priorities. Finally, in Phase III, the evaluation of the training programme can be conducted to assess its effectiveness and identify aspects that can be improved.

To the best of our knowledge, this is the first study, both in local and global contexts, to assess the KAP of paediatric pharmacists in the pharmacoepidemiology field. The items included in the main domains of the questionnaire incorporated the element of applicability, emphasising the practical relevance of the gathered information. Nevertheless, this study has some limitations. The study used a self-reporting questionnaire, which may have been subject to response bias. However, efforts were made to minimise response bias by ensuring participant anonymity and confidentiality. The study items excluded open-ended questions that were deemed appropriate for focusing on the breadth rather than the depth of the information. The generalisability of the findings beyond the Malaysian context may be limited. However, considering the richness of the information presented, the study is easily replicable in other settings. This can potentially lead to the customisability of the proposed training module development process to specific contexts. Future studies could use a qualitative study design to explore in-depth information regarding pharmacoepidemiology facilitators, barriers, and application strategies among paediatric pharmacists. Findings of the qualitative study can be considered in Phase I of the proposed training module development process.

CONCLUSION

Paediatric pharmacists demonstrated moderate knowledge and a neutral attitude, but poor practice towards pharmacoepidemiology. Future initiatives should emphasise collaborative efforts between academic institutions, professional bodies and practitioners to address the knowledge and attitude of paediatric pharmacists. This can be achieved through the development of a training module and the provision of on-the-job training to enhance the pharmacoepidemiology approach and application among paediatric pharmacists in Malaysia.

Ethics Committee Approval: Ethical approval was obtained from the ethics committee for the Ministry of Health (MOH) facilities in Malaysia, i.e., the Medical Research and Ethics Committee (MREC) (NMRR ID-23-00214-2RW)

Informed Consent: Written consent was obtained from the participants.

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Original Article

Turkish version of the patient safety culture survey for community pharmacies: Evaluation of patient safety culture perceptions of pharmacy employees*

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*This study was produced from the master's thesis titled "Turkish Version of the Patient Safety Culture Survey for Community Pharmacies: Evaluation of Patient Safety Culture Perceptions of Pharmacy Employees"

ABSTRACT

Background and Aims: This study adapted the Community Pharmacy Survey on Patient Safety Culture developed by the Agency for Healthcare Research and Quality for independent pharmacies into a Turkish survey and evaluated the perception of patient safety culture of 392 pharmacy employees operating in Çankaya district of Ankara province.

Methods: The method developed by Brislin was used in the adaptation process to Turkey. After ensuring the language validity of the questionnaire, its construct validity was examined through explanatory factor analysis and confirmatory factor analysis. In this descriptive and cross-sectional study, the participants' patient safety culture perception scores and demographic characteristics were compared.

Results: According to the results of the study, the questionnaire, as adapted to Turkish, was found to be a valid, reliable, and usable questionnaire for measuring and evaluating patient safety culture. The highest percentage of positive responses was for "Teamwork" (95.3%), "Staff Training and Skills" (91.7%) and "Physical Space and Environment" (90.6%), while the lowest percentage of positive responses was for "Staffing, Work Pressure and Pace" (56.2%). In this study, it was determined that the perception of patient safety culture was higher in female employees, those with higher education levels, those who were pharmacists, those with more years of employment, and those with less than 60 hours of weekly working hours.

Conclusion: As a result of the findings, it was determined that factors such as working environment, training of employees, work intensity, working hours, and the adequacy of the number of personnel affected the perception of the patient safety culture of independent pharmacists. Therefore, improvements should be made in these areas to improve the patient safety culture in pharmacies.

Keywords: Patient safety, Pharmacy, Reliability and validity, Surveys and questionnaires

INTRODUCTION

According to the Preventing Medication Errors report published by the Institute of Medicine (IOM) in 2006, one and a half million people are injured every year due to medication errors, which are considered among medical errors, and these errors increase medical expenditure costs and decrease productivity (Partin, 2006). 34-56% of medication errors are preventable (AHRQ). In the report, the IOM stated that medical errors are caused by faulty systems and recommended that systems be analysed and redesigned at all levels (Partin, 2006). All measures taken to ensure patient safety in pharmacies are aimed at preventing potential medication errors. Prescribing errors can be detected and prevented not only by nurses but also by pharmacy staff. In a study conducted in the United States, it was reported that prescribing errors detected in 0.3-1.9% of prescriptions were detected and prevented by pharmacists (Dean, Schachter, Vincent, & Barber,2002). In 2022, WHO presented its third challenge for patient safety as "harmless medicines", aiming to reduce serious preventable harm from medicines by 50% globally in the next 5 years (WHO, 2022).

Medical errors are less common in health systems with well-functioning patient safety. Patient safety starts when an individ-

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ual receives healthcare and continues until the need for healthcare is no longer needed (Sayek, 2011). Organisations with a culture of safety are willing to evolve, change, learn from mistakes, and create strategies to prevent accidents (Ovalı, 2010). The Agency for Healthcare Research and Quality (AHRQ) recommends developing safe approaches to ensure a culture of safety, providing employees with environments where they feel comfortable reporting errors, and collaboratively approaching patient safety issues in a multidisciplinary team (AHRQ).

Community pharmacies, which are defined as a type of health facility that provides specific medicines-related services, play an important role in the health system. Community pharmacy workers are recognised as key health professionals who make dynamic and evolving contributions to improving the health of the communities they serve (PGEU, 2019). Crucial but underappreciated in the healthcare system, community pharmacy workers play a vital role in patient safety by ensuring that medicines are used safely by patients (Aboneh, Stone, Lester, & Chui, 2020; Jassim & Jamal; 2021). Community pharmacies have become an integral part of the health system, actively contributing to ensuring patient safety through education, collaboration, accurate dispensing, monitoring, and implementation of safety protocols (Khatri *et al*., 2024; Sharma, Kumar, Sharma, & Verma 2024). Previous studies on safety culture assessments have largely focused on hospital settings.

Studies have reported that medication errors are common in community pharmacies, and many are linked to organisational culture (Knudsen, Herborg, Mortensen, Knudsen, & Hellebek, 2007; Odukoya, Stone, & Chui, 2014). Therefore, safety culture assessments may be useful for organisational improvement efforts. Pharmacists' medication dispensing and patient care services increase patients' understanding of their medications and contribute to improving patient safety (American Pharmacists Association).

As of the end of 2019, there were 26 558 independent pharmacies in Turkey, which are primary healthcare institutions where retail sales in the pharmaceutical sector are mostly carried out (Bagcı & Atasever, 2020). According to the Turkish Pharmacist Information System data, the total number of pharmacists in Turkey in 2019 was 37,442, of which 26,177 were independent pharmacists (Turkish Pharmacists Association, 2019). Independent pharmacies sell both prescription and over-the-counter medicines. Today's pharmacy curriculum focuses on patient oriented pharmacy applications and have courses on drug safety , rational drug use and pharmaceutical care which are important features in maintaing patient safey. In addition, in-service training is provided by the Pharmacies Association for community pharmacy employees (Turkish Pharmacists Association). Although there are legislative regulations for ensuring patient safety in public pharmacies, there are no independent pharmacies. However, community pharmacies are inspected at least twice a year by the Turkish Medicines and Medical Devices Agency under the Ministry of Health in terms of the characteristics and qualifications of the place to be used as a pharmacy, supply and storage of medicines, employment of personnel, registration, and disposal (Turkish Medicines and Medical Devices Agency, 2022).

In the literature, no study has evaluated the patient safety culture of community pharmacy employees in Turkey. The aim of this study is to adapt the The Community Pharmacy Survey on Patient Safety Culture (CPSPSC) into Turkish and evaluate the perception of the patient safety culture of pharmacy employees. This study is the first to investigate patient safety culture in community pharmacies in Turkey.

MATERIALS AND METHODS

Study Design and Population

This study is a descriptive and cross-sectional study in terms of determining the perception of patient safety culture of employees working in community pharmacy with CPSPSC adapted into Turkish.

This study was conducted with 392 pharmacy employees who voluntarily agreed to fill out the questionnaire from 598 pharmacies operating in Çankaya district of Ankara, Turkey. Pharmacy pharmacists and non-phaarmacist employees who worked full-time at their current pharmacy for at least 6 months were included in the study. The mean age of the participants was 32.53±7.52 years, 51.3% (n=201) were female, 52.6% (n=206) were high school graduates, and 80.4% (n=315) were working as pharmacist journeymen. Participants had been working in the pharmacy for 7.32±5.91 years and 2.64±3.43 months, with a mean weekly working time of 60.76±6.25 hours (Table 1).

Data Collection

CPSPSC published by the AHRQ in 2012 was used as a data collection tool in this study. The CPSPSC was designed to determine the attitude of community pharmacy employees towards patient safety, to measure their awareness of medication safety, to determine the current state of patient safety culture in the community pharmacy environment, and to determine the steps to be taken to improve this culture (AHRQ). The original questionnaire consisted of 11 headings (Communication About Mistakes, Communication Openness, Communication About Prescriptions Across Shifts, Organisational Learning—Continuous, Improvement, Overall Perceptions of Patient Safety, Patient Counselling, Physical Space and Environment, Response to Mistakes, Staff Training and Skills, Staffing, Work Pressure and Pace, Teamwork) and 36 items. The questions are scored as a five-point Likert scale.

Data were collected face-to-face by the researcher between February and June 2020. Before administering the question-

Table 1. Demographic characteristics of participants

		N	%
Gender	Female	201	51.3
	Male	191	48.7
Educational background	Primary school	0	0.0
	Middle school	7	1.8
	High school	206	52.6
	Associate degree	117	29.8
	Licence	52	13.3
	Postgraduate	10	2.6
Working position in a pharmacy	Pharmacist (Pharmacy manager, Lead person, Responsible pharmacist, staff pharmacist)	56	14.3
	Journeyman pharmacist (journeyman leader, journeyman employee)	315	80.4
	Pharmacy staff	21	5.4

	N	Min	Max	Mean	Ss
Age	392	19.00	66.00	32.53	7.52
Working time in the pharmacy (years)	392	0.00	41.00	7.32	5.91
Working time in the pharmacy (months)	392	0.00	11.00	2.64	3.43
Total working hours per week in the pharmacy (hours)	392	35.00	72.00	60.76	6.25

naire, information about the study was given and the questionnaire was filled out by the participants. Participants were included in the study on a voluntary basis. The average time to complete a questionnaire was 15 minutes.

Adaptation Process of the Questionnaire to Turkish

The original CPSPSC is written in English. The method developed by Brislin (1976) was used in the adaptation process. The pilot study of the adapted Turkish CPSPSC was conducted with 30 pharmacy employees. During the application, the comprehensibility of the questions was discussed, the incomprehensible items were revised, and the final version of the questionnaire was given after making the necessary corrections. The questionnaires of the employees who participated in the pilot study were not included in the analysis. According to the reliability analysis result, the Cronbach alpha value of the survey is 0.896. Accordingly, it was determined that the survey was highly reliable.

Data Analysis

Kaiser-Meyer-Olkin (KMO) and Barlett Sphericity tests were performed to determine whether the questionnaire was suitable for factor analysis. In order to determine the factor structure of the questionnaire, the scatter of the eigenvalues was examined using a Scree Plot graph. However, if the number of factors was more than one, varimax vertical rotation was used to assign items to relevant factors. In the factor analysis process, factor loading values were examined in the process of assigning the questionnaire items to the factors or removing them from the questionnaire. The construct validity of the questionnaire was first examined by explanatory factor analysis (EFA) and then by confirmatory factor analysis (CFA).

In order to determine the perceptions and levels of patient safety culture of independent pharmacy employees, correlations were calculated with the percentages of positive responses over 11 domains determined by AHRQ. Among the comparison tests, t and ANOVA tests were used. The difference scores according to categorical variables with 2 groups was analysed by t-test, and the difference of scores according to categorical variables with 3 or more groups was analysed by ANOVA. In the results found to be significant in the ANOVA test, each group was examined using the Bonferroni test from the post-hoc analysis, and pairwise comparisons were made in the ANOVA.

Ethics Approval

Ethical approval for the study was obtained from the Ankara Yildirim Beyazit University's ethics committee (16.10.2019 and 22 number). Permission was obtained from the AHRQ to adapt the questionnaire to Turkish and use it as a measurement tool in Turkey. Permission was obtained from the Ankara Chamber of Pharmacists to apply the questionnaire to independent pharmacies in Ankara. Participation in the study was voluntary, and participants were informed about the study and provided verbal consent.

RESULTS

Validity Analysis Results

In factor analysis, the KMO test was used to determine whether the sample size was sufficient, and Barlett's sphericity test was used to determine whether the data were normally distributed. The total KMO value of the questionnaire was calculated as 0.839 (KMO>0.500) and the chi-square value because of Barlett's test was calculated as 8440.776 and found statistically significant ($p<0.05$). According to the results of the KMO and Bartlett's test, it was determined that the data were suitable for factor analysis.

The maximum Cronbach alpha coefficient in the survey is 0.941 and belongs to the "Working Environment and Staff Competencies" sub-dimension. The minimum Cronbach alpha value is 0.759 and belongs to the "Teamwork and Communication" sub-dimension. The Cronbach alpha value of the "Information Sharing Between Shifts" sub-dimension is 0.886. The Cronbach alpha value of the "Attitude Towards Errors" sub-dimension is 0.859. According to the reliability analysis result, the Cronbach alpha value of the survey is 0.896. Accordingly, it was determined that the survey was highly reliable.

After ensuring the Turkish language validity of the questionnaire, its construct validity was examined using EFA and CFA. In the factor analysis process, factor load values were examined when assigning survey items to factors or removing them from the survey. Items with factor loadings less than 0.300 were excluded from the analysis. Since the factor loading was less than 0.300, articles B9, B16, C3 and C8; B1 due to overlapping. Items were not included in the analysis. After the analysis, a four-factor and 31-item structure was obtained. The factors were named as Working Environment and Staff Competencies (A1-A2-A2-A3-A3-A4-A4-A5-A5-A6-A7-A8-A9-A10), Information Sharing Between Shifts (B2-B3-B4-B4-B5-B6-B7-B8-B10-B11), Teamwork and Communication (B12-B14-B15), and Attitude Towards Errors (C1-C2-C4-C5-C6-C7- C9-C10) (Table 2).

The factor loadings of the questionnaire after CFA ranged between 0.522-0.899. Item B13 was excluded from the analysis because of its low factor loading (Table 3).

When all the fit indices calculated in the CFA analysis were examined, the questionnaire showed acceptable fit indices. After EFA and CFA, it was determined that the questionnaire consisted of four factors and 30 items, and a validity analysis was completed.

Reliability Analysis Results

The internal consistency method was used to determine questionnaire reliability. According to the results of the reliability analysis, the total internal consistency coefficient of the questionnaire was found to be highly reliable, with a Cronbach's alpha value of 0.896. According to this result, we determined that the reliability values of the questionnaire and the original questionnaire were similar (Table 4).

The average positive response percentages for the 11 topics in the questionnaire were determined (Table 6). Accordingly, "Teamwork" (95.3%), "Staff Training and Skills" (91.7%) and "Physical Space and Environment" (90.6%) had the highest percentage of positive responses. The lowest percentage of positive responses belongs to "Staffing, Work Pressure and Pace" with 56.2% (Table 5).

Correlation Results

Participants' patient safety culture perception scores were analysed in terms of demographic characteristics. Only statistically significant results were presented (Table 7). A statistically significant difference was found between men and women, between groups with different educational status, between groups with different working hours in pharmacy, between groups with different total weekly working hours, and between groups with different positions in terms of " Information Sharing Between Shifts" ($p<0.05$). Accordingly, the mean score of women was higher than that of men. The mean score of those with more than 10 years of employment was the highest, while the mean score of those with 7-10 years of employment was the lowest.

A statistically significant difference was found between groups with different educational statuses and groups with different total weekly working hours in terms of "Communication and Functioning Total" ($p<0.05$). Accordingly, the mean score of those with undergraduate/graduate degrees was the highest, while the mean score decreased as the level of education decreased (Table 6).

A statistically significant difference was found between the groups with different total weekly working hours in terms of "Teamwork and Communication" ($p<0.05$). Accordingly, although the mean score of employees who worked less than 60 h was the highest, the mean decreased as the duration increased (Table 6).

A statistically significant difference was found between the groups with different positions in terms of "Communication and Operation Dimension" "Information Sharing Between Shifts" and "Attitude Towards Errors" ($p<0.05$). In terms of, "Communication and Operation Dimension" "Information Sharing Between Shifts" the average score of pharmacists is the highest, while the average of journeyman pharmacist is the smallest. In "Recording of Errors", the average score of pharmacy staff is the highest, while the average of journeyman pharmacist is the smallest. (Table 6).

Table 2. Factor structure of the survey after EFA

Factor 1: Working Environment and Staff Staff Competencies (Cronbach α = 0.941)	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**	**Explained variance ratio**
(A4) The staff in this pharmacy clearly understood their roles and responsibilities.	.902				
(A6) The staff in this pharmacy have the skills they should do their jobs well.	.889				
(A3) Technicians in this pharmacy receive the training they should perform their jobs.	.854				
(A9) The staff will work together as an effective team	.838				67.270
(A2) The staff treat each other with respect.	.834				
(A1) This pharmacy is well-organised.	.824				
(A5) This pharmacy is free of clutter.	.808				
(A8) Staff who are new to this pharmacy receive an adequate orientation.	.803				
(A7) The physical layout of this pharmacy supports good workflow.	.780				
(A10) Employees working in the pharmacy receive adequate training.	.640				
Factor 2: Information Sharing Between Shifts (Cronbach α = 0.886)	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**	**Explained variance ratio**
(B4) We expect that important prescription information will be exchanged across shifts.		.872			
(B7) Our pharmacists spend enough time talking to patients about how they use their medications.		.792			
(B10) It is easy for staff members to speak up to their supervisors or managers about patient safety concerns. In this pharmacy.		.792			
(B2) We encourage patients to talk to pharmacists about their medications.		.759			
(B5) The staff members feel comfortable asking questions when they are unsure about something.		678			37.033
(B8) Staff at this pharmacy discuss mistakes.		.674			
(B11) Our pharmacists will inform patients about their new prescriptions.		.641			
(B6) Standard procedures are in place for communicating prescription information across shifts.		.571			
(B3) Staff take adequate breaks during shifts.		.566			

Table 2. Continued

Factor 3: Teamwork and Communication (Cronbach α = 0.759)	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**	**Explained variance ratio**
(B15) In this pharmacy, we discuss ways to prevent mistakes from happening again.			.842		
(B14) The status of problematic prescriptions is well communicated across shifts.			.842		
(B13) When patient safety issues occur at our pharmacy, staff will discuss them with us.			.758		21.995
(B12) We have enough staff to handle the workload.			.556		
Factor 4: Attitude Towards Errors (Cronbach α = 0.859)	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**	**Explained variance ratio**
(C2) When a mistake occurs, we try to identify the problems in the work process that led to the mistake.				.820	
(C1) The staff are treated fairly when they make mistakes.				.804	
(C6) This pharmacy is good at preventing mistakes.				.764	
(C4) This pharmacy helps staff learn from their mistakes rather than punishing them.				.754	
(C10) Mistakes have led to positive changes in the pharmacy.				.704	
(C5) When the same mistake continues to happen, we change the way we do things.				.651	
(C7) We examine staff actions and how we do things to understand why mistakes happen. This pharmacy.				.625	51.943
(C9) The way we do things in this pharmacy reflects a strong focus on patient safety.				.613	
Total (Cronbach α = .859)					

DISCUSSION

The four-factor structure we obtained as a result of statistical analyses in our questionnaire adaptation study was similar to the study conducted by Aboneh *et al* (Aboneh *et al*., 2020). Aboneh et al reported that the original questionnaire was inadequate for the 36-item, 11-factor structure and failed to meet the fit index criteria (Aboneh *et al*., 2020). The EFA after CFA indicated that a 27-item, 4-factor structure better reflected the dimensions of safety culture in community pharmacies. In a study conducted in China with independent pharmacists, a 7-factor structure was obtained after EFA (Jia *et al*., 2014). In their study, Rawlings *et al*. (2018) did not define any correlation as very strong, although all the correlations were positive and most of the correlations were statistically significant. Therefore, the CPSPSC should not be used as a single assessment tool to measure patient safety culture in community pharmacies. In contrast, the 11-factor CPSPSC was used in other studies evaluating the perception of the patient safety culture of community pharmacy employees (Alsaleh *et al*., 2018; Herner, Rawlings, Swartzendruber, & Delate, 2017; Owusu, Abouelhassan, & Awaisu , 2021; Sivanandy *et al*., 2016; Yismaw, Tesfaye, Hailu, Tegegn, & Gebreyohannes, 2020). Similar to our study, in a study conducted during the COVID-19 pandemic, it was determined that employees working in Community pharmacies generally had a positive patient safety culture and received significantly higher scores for the dimensions of "Teamwork", "Personnel, Work Pressure and Tempo", "Response to Errors", "Organisational Learning - Continuous Improvement" and "General Perceptions of Patient Safety" (Abu Assab, Jaber, Basheer, Abu Assab, & Al-Atram, 2022). Rapid generalisations about the applications of safety culture dimensions in health services may be misleading. In addition, there may be a different number of constructs in the dimensioning of the questionnaire as practise settings may differ significantly in terms of the norms and working procedures of the organisations, i.e. cultural aspects (Waterson, Griffiths, Stride, Murphy, & Hignett, 2010).

Table 3. Factor loadings of ıtems after CFA

Factor Name	Items	Factor load
Working Environment and Staff Staff Competencies	A4	.817
	A6	.853
	A3	.721
	A9	.899
	A2	.753
	A1	.764
	A5	.742
	A8	.617
	A7	.657
	A10	.591
Information Sharing Between Shifts	B4	.793
	B7	.724
	B10	.721
	B2	.596
	B5	.710
	B8	.669
	B11	.678
	B6	.575
	B3	.522
Teamwork and communication skills	B15	.803
	B14	.851
	B12	.677
Attitude Towards Errors	C2	.795
	C1	.542
	C6	.704
	C4	.701
	C10	.556
	C5	.621
	C7	.659
	C9	.592

Table 4. Internal consistency coefficient and descriptors of the total and subfactors of CPSPSC

Factors	**Number of Items**	**Min.-Max.**	**Cronbach Alpha**	**Description**	**Hydrangea (min.-max.)**	**Median in 0-100 Survey (Min.-Max.)**
Working Environment and Staff Competencies	10	10-50	0.941	High reliability	40(7-50)	80(0-100)
Information Sharing Between Shifts	9	9-45	0.886	High reliability	36(5-45)	80(0-100)
Teamwork and communication skills	3	3-15	0.759	Highly reliable	12(3-15)	75(25-100)
Attitude Towards Errors	8	8-40	0.859	High reliability	32(5-40)	88(0-100)
Total	30	30-150	0.896	High reliability	120(18-150)	84(0-100)

Table 5. Average positive response percentages

Dimensions	Average Positive Response Percentages (%)
Communication About Mistakes	**75.3**
Communication Openness	87.6
Communication About Prescriptions Across Shifts	82.6
Organisational Learning—Continuous Improvement	87.3
Overall Perceptions of Patient Safety	87.6
Patient Counselling	87.3
Physical Space and Environment	90.6
Response to Mistakes	88.7
Staff Training and Skills	91.7
Staffing, work pressure, and pace	56.2
Teamwork	**95.3**

Table 6. Examination of scores in relation to participants' demographic characteristics

Gender		n	Average	ss	t	P
Information Sharing Between Shifts	Female	201	4.11	0.61	2.073	.039*
	Male	191	3.98	0.62		
Educational background		n	Average	ss	F	P
Information Sharing Between Shifts	Middle/High School	213	4.00	0.62	4.471	.012*
	Associate degree	117	4.01	0.60		
	Undergraduate/graduate	62	4.26	0.62		
Working time in the pharmacy		n	Average	ss	F	P
Information Sharing Between Shifts	3 years or less	87	4.04	0.60	3.937	.009*
	4-6 years	128	4.02	0.60		
	7-10 years	72	3.87	0.64		
	More than 10 years	105	4.19	0.61		
Total working hours per week in the pharmacy		n	Average	ss	F	P
Information Sharing Between Shifts	Less than 60 h	55	4.26	0.63	4.068	.018*
	60 h	179	4.02	0.62		
	More than 60 h	158	3.99	0.60		
Teamwork and communication skills	Less than 60 h	55	4.34	0.67	5.848	.003*
	60 h	179	4.03	0.71		
	More than 60 h	158	3.94	0.82		
Communication and Functioning Total	Less than 60 h	55	4.30	0.62	4.068	.002*
	60 h	179	4.02	0.60		
	More than 60 h	158	3.96	0.62		
Working position in a pharmacy		n	Average	ss	F	P
Information Sharing Between Shifts	Pharmacist	56	4.25	0.63	4.501	.012*
	Journeyman pharmacist	315	4.00	0.60		
	Pharmacy staff	21	4.18	0.69		
Communication and Functioning Total	Pharmacist	56	4.21	0.65	3.160	.044*
	Journeyman pharmacist	315	4.00	0.60		
	Pharmacy staff	21	4.13	0.68		
Attitude Towards Errors	Pharmacist	56	2.69	1.38	3.900	.021*
	Journeyman pharmacist	315	2.36	1.46		
	Pharmacy staff	21	3.16	1.47		

***p<0,05**

In the AHRQ data, "Patient Counselling" (95%), "Communication Openness" (87%) and "Communication about Errors" (85%) had the highest positive response rates, while " Staffing, Work Pressure and Pace" had the lowest positive response rate of 45%. When the results were compared with the AHRQ data, the positive response rates in our study were higher. In both studies, the lowest positive response rate belongs to " Staffing, Work Pressure and Pace" (56.2%). In our study, "Teamwork" (95.3%), "Staff Training and Skills" (91.7%) and "Physical Space and Environment" (90.6%) had higher positive response rates. The reason for the high perception scores of employees in these dimensions is; the fact that they give more importance to physical space in community pharmacy inspections in Turkey may indicate that teamwork can be good because the employees are personally selected by the pharmacist and that regular in-service training is provided by the Pharmacists' Association. The percentages of positive response in the findings of our study are similar to those of both studies conducted in the USA, China, Malaysia, Qatar, Ethiopia, Saudi Arabia, and Kuwait (Almalki *et al*., 2021; Alsaleh *et al*., 2018; Jia *et al*., 2014; Qwusu *et al*., 2021; Sivanandy *et al*., 2016; Yismaw *et al*. 2020) and the results of the literature review conducted by Kown *et al*. Because of these studies, it was reported that areas with low positive response percentages should be developed and improved to improve the perception of patient safety culture in pharmacies. In a study conducted by Kown *et al* (2023), it was reported that pharmacy employees did not have the staff to handle the workload, were rushed, could not take breaks, and thought that the work could not be completed correctly due to distraction. However, several studies have emphasised the negative effects of workload on patient safety (Kown *et al*; 2023). Independent pharmacy workers should ensure the control of workload by allocating human resources appropriately to improve patient safety.

It was determined that the mean score of the perception of "Information Sharing Between Shifts" culture increased as the educational level of the employees increased. In a study conducted by Owusu *et al* (2021) it was determined that non-pharmacist employees (such as journeymen, interns, trainees) felt a greater sense of urgency when preparing prescriptions than pharmacist employees. This is believed to be related to the level of education. Therefore, this gap can be closed by providing training on patient safety to low-education employees. In a study conducted by Sivanandy *et al* (2016), it was suggested that pharmacists' general perceptions of patient safety could be improved by providing training on the importance and principles of patient safety. Pharmacy employees with more than 10 years of work experience and less than 60 h of weekly work hours had higher communication and functioning culture perception scores. The level of work experience and length of working hours are closely related to the level of patient safety. More work experience provides the ability to effectively manage patient safety issues and control workload. In addition, employee confidence based on experience can contribute significantly to the development of a patient safety culture (Kown *et al*; 2023). Owusu *et al*. (2021) found that pharmacy workers with six years or more experience had better teamwork and that working 40 h or more per week had a positive effect on patient safety. Therefore, experiential competence is a prerequisite for a positive response to teamwork.

In our study, the mean score for the perception of culture of "Information Sharing Between Shifts" was the highest among those working as pharmacists, while the mean score was the lowest among those working as pharmacist journeymen. Pharmacists have an important role in ensuring patent safety and efficacy in regard to medication use. In addition, the high level of education that pharmacists receive in relation to patients' health care and rational drug use has an important place in the formation of patient safety culture perception before graduation. Similarly, the mean score of the perception of the culture of "Attitude Towards Errors" was the highest among pharmacy staff and the lowest among pharmacist journeymen. In a study conducted by Herner *et al*. (2017), the level of communication regarding errors was found to be statistically higher among pharmacy technicians than among pharmacists. Jia *et al* (2014) found that the positive response rate of highly qualified pharmacists (senior pharmacists) was higher than that of those with a low level of competence in the area of "staffing, work intensity, and speed" (junior pharmacists). In this direction, training programmes on patient safety can be organised for employees with less pharmacy experience and less competence in terms of the position they work in (pharmacist journeyman, employee).

Although this study is important in terms of being the first in Turkey to measure the perception of the patient safety culture of employees working in independent pharmacies, several important limitations should be discussed. First, our study was conducted in one district of one province in Turkey due to time constraints and factors such as cost and transportation. Therefore, it may not reflect the general trends across the country. However, conducting the study in Ankara, Turkey's capital, may yield results that are close to the general trend in Turkey due to its multicultural structure. Second, the findings of this study are limited to those working in independent pharmacies. This does not reflect the perceptions of pharmacy employees operating in public health institutions.

CONCLUSION

Based on the findings obtained because of the validity and reliability study, it was determined that the CPSPSC is a valid and reliable questionnaire with linguistic equivalence and is culturally appropriate for use under Turkish conditions. This study emphasised the importance of pharmacy staff perception in ensuring patient safety at community pharmacies. This study showed that factors such as the adequacy of vocational training received, the professional competence and experience

of pharmacy employees, the ability to perform teamwork well in many pharmacies, and the adequacy and equipment of the physical environment in pharmacies are effective in ensuring patient safety. As a result, it is thought that the perception of patient safety culture is high in the study group we surveyed. However, pharmacists were found to have higher perceptions of patient safety culture dimensions of "Communication and Operation Dimension" and "Information Sharing Between Shifts" compared to non-phaarmacist employees.

Since our study was limited to self-employed pharmacy employees working in Çankaya District of Ankara, Turkey, the capital of Turkey, conducting more comprehensive studies is expected to contribute to the literature. Future studies should also focus on the differences in the perception of patient safety culture among pharmacy employees in the community and the public sector.

Ethics Committee Approval: Ethical approval for the study was obtained from the Ankara Yıldırım Beyazıt University's ethics committee (16.10.2019 and No: 22).

Informed Consent: Written consent was obtained from the participants.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study: S.H.K., N.T.; Data Acquisition: S.H.K.; Data Analysis/Interpretation: S.H.K., N.T.; Drafting Manuscript: S.H.K., N.T.; Critical Revision of Manuscript: İ.N.T..; Final Approval and Accountability: S.H.K., N.T.

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Appendix 1. Serbest Eczaneler İçin Hasta Güvenliği Kültürü Anketi

	Kesinlikle Katılmıyorum	Katılmıyorum	Ne Katılıyorum Ne De Katılmıyorum	Katılıyorum	Kesinlikle Katılıyorum	Cevap Vermek İstemiyorum Ya Da Cevabı Bilmiyorum
Bu eczanede çalışanlar rollerinin ve sorumluluklarının bilincindedirler.						
Bu eczanede çalışanlar işlerini iyi yapmak için gerekli yeteneğe sahiptirler.						
Bu eczanede çalışan eczane kalfaları işlerinde ne yapmaları gerektiğiyle alakalı gerekli eğitimleri almaktadır.						
Çalışanlar bir arada etkili birtakım olarak çalışmaktadır.						
Çalışanlar birbirine saygılı davranır.						
Bu eczane iyi şekilde organize olmuş durumdadır.						
Bu eczane dağınıklıktan uzaktır.						
Bu eczanede yeni işe başlamış çalışanların uyum sağlaması için yeterli destek sağlanmaktadır.						
Bu eczanenin fiziksel düzeni işlerin yapılmasını kolaylaştırmaktadır.						
Bu eczanede çalışanlar yeterli düzeyde eğitim almaktadırlar.						
Bu eczanede yapılan hataların tekrarlanmamasının yollarını aramızda konuşuruz.						
Reçetelerdeki problemler vardiya değişikliklerinde etkili şekilde aktarılmaktadır.						
İş yükünün paylaşılabilmesi için yeterli çalışana sahibiz.						

Appendix 1. Continued

	Asla	Nadiren	Bazen	Çoğu Zaman	Her Zaman	Cevap Vermek İstemiyorum Ya Da Cevabı
Vardiyaların değişimlerinde önemli bilgilerin diğer çalışanlara aktarılması konusunda açık beklentilerimiz bulunmaktadır						
Eczacılarımız hastalarla ilaçların nasıl kullanılması gerektiği hakkında konuşmak için gerekli zamanı harcamaktadır.						
Bu eczane çalışanları hasta güvenliği konularını üstleriyle rahatlıkla konuşabilmektedir.						
Bu eczane çalışanları hatalar üzerinde tartışmaktadır.						
Eczacılarımız hastaların reçetelerindeki yenilikler hakkındaki önemli bilgileri hastalara aktarmaktadır.						
Vardiyalar arasında önemli bilgilerin aktarılması konusunda standart prosedürlerimiz bulunmaktadır.						
Çalışanlar vardiyaları sırasında yeterli molayı alabilmektedirler.						
Hastaları, ilaçları hakkında eczacılarla konuşması için cesaretlendiririz						
Çalışanlar, emin olmadıkları herhangi bir konu hakkında soru sorarken rahat hissetmektedirler						
Bir hata oluştuğunda hangi problemlerin bu hataya yol açtığını bulmak için çaba gösteririz.						
Çalışanlara hata yapmaları durumunda adil davranılmaktadır.						
Bu eczane hataların engellenmesinde başarılıdır.						
Bu eczanede çalışanların hatalarından dolayı cezalandırılması yerine hatalarından ders çıkarmasına yardımcı olunmaktadır.						
Bu eczanede hatalar pozitif değişimlerin olmasına yol açmaktadır.						
Aynı hatalar oluşmaya devam ediyorsa işin yapılma şeklini değiştiririz.						
Bu eczanede oluşan hataların nedenini anlamak için çalışanların davranışlarına ve işleri halletme biçimimize bakarız.						
Bu eczanede işlerin yapılma şekli hasta güvenliğine etkili bir şekilde odaklanıldığını yansıtmaktadır.						

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Original Article

Evaluation of Turkish women's attitudes and perceptions regarding medication use in pregnancy: A pilot study

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ABSTRACT

Background and Aims: Medication use for pre-existing or pregnancy-induced conditions/diseases is common during pregnancy. Understanding the attitudes and perceptions of pregnant women regarding medication use is crucial for healthcare professionals in providing safe and rational drug use during pregnancy. The aim of this pilot study was to evaluate the attitudes and perceptions of Turkish women regarding medication use during pregnancy.

Methods: A cross-sectional, questionnaire-based study was conducted with 60 pregnant/lactating women who applied to a community pharmacy in Istanbul, Türkiye. The questionnaire focused on women's attitudes and perceptions regarding the use of medications and supplements during pregnancy.

Results: 65% of women stated that the use of certain medications is harmful, while 31.7% believed that all medications are harmful in pregnancy, particularly in the first trimester. The majority of participants (90%) consulted with their physicians before using any medication, whereas a few received advice from pharmacists and nurses. Most women adhered to the prescribed treatment regimen for pre-existing (83.3%) and pregnacy-induced diseases (75%), while a few stopped taking medication for not to harm their baby. None of the participants had a habit of self-medication.

Conclusion: The majority of the women used medication with high adherence and had high confidence in the advice from a physician. However, the tendency to avoid medication use due to concerns about fetal harm, and the low consultation rate with pharmacists are notable findings. More effort is needed to encourage pregnant women to obtain information regarding medication use during pregnancy from community pharmacists.

Keywords: Pregnancy, Medication use, Attitudes, Perceptions

INTRODUCTION

Medication use during pregnancy is on the rise globally. Recent studies in developed countries have shown that most women take at least one medication, whether prescribed or over the counter (OTC), during pregnancy (Lupattelli et al., 2014). Medication is often necessary for the effective management of pre-existing chronic diseases such as hypertension, diabetes, asthma, hypothyroidism or epilepsy, as well as acute illnesses like flu/cold, bacterial infections, headaches, toothaches etc., observed during pregnancy. In addition, medication is commonly used to manage pregnancy-induced conditions, including nausea-vomiting, gastroesophageal reflux, and diseases such as gestational diabetes or gestational hypertension (Mitchell et al., 2011).

The use of medication during pregnancy involves carefully weighing the risks and benefits for both the mother and the fetus. Previous studies have shown that the irrational use of medicine and/or supplements may worsen the underlying condition/disease in the mother or can lead to potential harm to the fetus (Sharma, Kapoor, & Verma, 2006; Nordeng, Ystrom & Einarson, 2010; Kassaw & Wabe, 2012).

Besides ensuring the safety of maternal medication use, it is crucial for healthcare providers to understand women's attitudes and perceptions regarding medication use during pregnancy in order to maintain high adherence to necessary treatment regimens as well as to prevent unnecessary usage (Devkota, Khan, Alam, Sapkota, & Devkota, 2017). In this relation, community pharmacists are in a prime position to answer questions

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and alleviate the concerns of pregnant women. Several studies have investigated women's attitudes and perceptions concerning the potential risks and benefits of medication use during pregnancy, emphasising the pivotal role of pharmacists' practices in promoting rational drug use. (Lupattelli et al., 2014; Petersen, McCrea, Lupattelli, & Nordeng, 2015; Lynch et al., 2017; Ceulemans, Liekens, Van Calsteren, Allegaert, & Foulon, 2020). However, research on pregnant women's attitudes and perceptions on medication use within the Turkish population is limited (Göker et al., 2012; Terzioğlu Bebitoğlu, Hıdıroğlu, Ayaz, Sarısaltık, & Koc, 2022; Albayrak, Demir, Sezik, 2024). The current study is a pilot study with a particular focus on the patterns of medication use in pregnancy for acute and chronic conditions or diseases, as well as the use of OTC medicines and supplements.

MATERIAL AND METHODS

This pilot cross-sectional, questionnaire-based study was conducted between August and September 2024 with 60 pregnant or lactating Turkish women who visited a community pharmacy in Istanbul, Türkiye. A questionnaire was designed to gather information on the demographic and clinical characteristics of the participants, as well as their attitudes and perceptions regarding the use of prescribed and OTC medications or supplements for managing pre-existing or pregnancy-induced conditions and diseases. After obtaining oral and written consent from the participants, a face-to-face questionnaire was administered. This study was conducted in accordance with the approval of the Istanbul University Faculty of Dentistry Clinical Research Ethics Committee (30/07/2024, No:747). Data analyses were performed using GraphPad Prism 10.3.1.

RESULTS

Study Population

Fifty pregnant and 10 lactating women, 60 in total, participated in this pilot study. The demographic and clinical characteristics of the women are shown in Table 1.

Among the women who participated in the survey, 70% were between the ages of 25 and 35. Women under 25 and over 35 years old comprised 30% of the survey participants, indicating that pregnancy is less common in these age groups. A significant portion of the participants (46.6%) had a university or postgraduate degree. In addition, 15% of the women were in the first trimester of their pregnancy, which is considered as the most critical period for teratogenic risks. While the majority (45%) of the participants were in the final term of their pregnancy. A few percent (16.7%) of women were in the lactation period and completed the survey according to their medication use in the recent pregnancy period. Pre-existing chronic diseases such as Type-2 Diabetes Mellitus (T2DM), hypertension, hypothyroidism, asthma, thalassaemia and kidney failure were determined in some of the participants. None of them reported alcohol consumption, and only one (1.7%) reported occasional smoking.

Women's perceptions of medication use during pregnancy

31.7% of the women believed that medication use in pregnancy is harmful, while 65% of the women stated that the use of certain medications is harmful during pregnancy (Figure 1a). Of those who were concerned about medication risks, 51.7% believed that particularly the first 3 months, while 44.8% reported that the whole pregnancy period was harmful for medication use (Figure 1b).

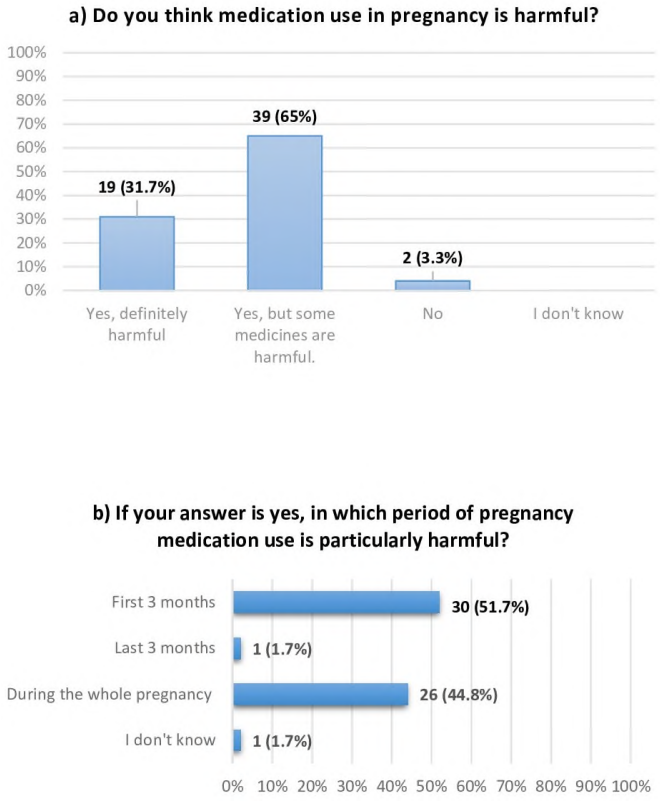

Figure 1. Women's perceptions regarding the safety of medication use in pregnancy. Data are shown as n (%) on the bar graphics

Counselling with healthcare professionals regarding medication use during pregnancy

The majority of the participants (90%) consulted with their physicians before using any medication during pregnancy, while only a few women had received advice from pharmacists (5%), nurses (3.3%) and non-professionals (friends/relatives, 1.7%) (Figure 2).

Table 1. Demographic and clinical characteristics of the women involved in the study

Specifications	Number (n)	(%)
Age		
18-25	10	(16.7)
26-30	24	(40)
30-35	18	(30)
35-50	8	(13.3)
Education Level		
Primary School	17	(28.3)
Middle School	9	(15)
High School	6	(10)
University	26	(43.3)
Master/PhD degree	2	(3.3)
Pregnancy/Lactation		
First trimester of pregnancy	9	(15)
Second trimester of pregnancy	14	(23.3)
Third trimester of pregnancy	27	(45)
Lactation	10	(16.7)
Pre-existing Chronic Diseases		
Asthma	2	(3.3)
Type 2 Diabetes Mellitus	1	(1.7)
Hypertension	1	(1.7)
Hypothyroidism	1	(1.7)
Thalassaemia	1	(1.7)
Renal Failure	1	(1.7)

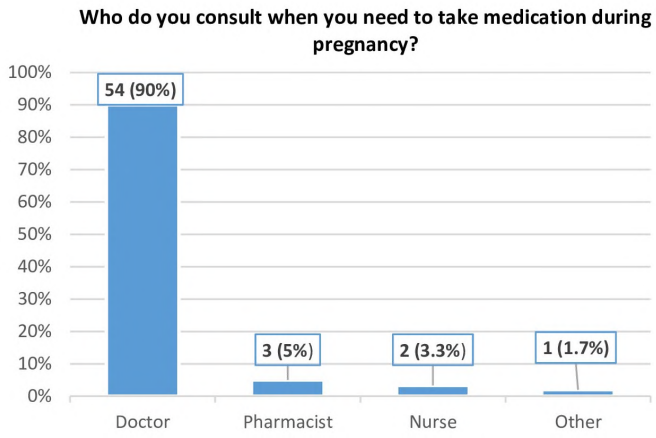

Figure 2. Counselling with healthcare professionals regarding medication use during pregnancy

Medication use for pre-existing chronic diseases

Some participants had chronic conditions such as T2DM, hypertension, hypothyroidism, asthma, thalassaemia, and kidney failure prior to pregnancy (Table 1). The medications prescribed for these pre-existing chronic diseases were insulin, methyldopa, levothyroxine, salbutamol, and iron, respectively. The women's attitudes and perceptions regarding medication use for pre-existing chronic diseases in pregnancy are shown in Table 2. Most women involved in this study adhered to the prescribed treatment regimen, by taking medicine consistently and in the recommended dosage. However, 1 of 6 women discontinued their medications due to concerns about potential harm to the fetus.

Medication use for pregnancy-induced conditions and diseases

All participants were asked whether they experienced pregnancy-induced conditions/diseases or not. 51.7% (n=31) of the women reported that they experienced nausea and vomiting, while 40% (n=24) reported gastroesophageal reflux as pregnancy-induced conditions. These women's attitudes and perceptions regarding medication use on related conditions are given in Table 3. Among these women, 48.4% and 50% used medication under a physician's supervision for nausea-vomiting and gastroesophageal reflux, respectively. The most commonly prescribed medications were metoclopramide and trimethobenzamide for nausea and vomiting and antacids for gastroesophageal reflux. Notably, approximately half of the women did not use any medication for these conditions, instead they attempted to take some supplements (Table 3).

Concerning pregnancy-induced diseases, gestational diabetes (n=3) and gestational hypertension (n=1) were observed

Table 2. Women's attitudes and perceptions regarding medication use for pre-existing chronic diseases during pregnancy

Which of the following describes how you use the medication for your pre-existing chronic diseases?	**n**	**(%)**
I adhered to the prescribed medication regimen, taking it consistently and in the dosage recommended by my doctor.	5	83.3
I only took the medication when my symptoms worsened, as I was concerned it might harm my baby.	0	0
I stopped taking the medication, as I was concerned it might harm my baby.	1	16.7
I never used medication, as I was concerned it might harm my baby.	0	0

Table 3. Women's attitudes and perceptions of medication use in pregnancy-induced conditions

	Nausea and Vomiting (n=31)		**Gastroesophageal Reflux** (n=24)	
What did you do for your nausea and vomiting or gastroesophageal reflux during pregnancy?	**n**	**(%)**	**n**	**(%)**
I consulted with my doctor and used a medication under his/her supervision.	15	48.4	12	50
I consulted with a pharmacist and used a medication based on his/her advice.	0	0	0	0
I took a medication that I used before pregnancy.	0	0	0	0
I did not use medication; instead, I attempted to take supplements.	16	51.6	12	50

in 4 (6.7%) out of 60 women. Among these women, 75% reported that they appropriately adhered to the prescribed medication regimen, namely, insulin or methyldopa. While, 1 woman (25%) with gestational diabetes avoided using medication due to concerns about potential harm to the fetus (Table 4).

Medication use for acute conditions during pregnancy

Participants of the survey were asked whether they experienced any acute conditions during their pregnancy. 46.7% (n=28) of the women reported that they experienced flu or cold, while 11.7% (n=7) reported bacterial infection and 48.3% (n=29) reported headache, toothache or joint pain. These women's attitudes and perceptions regarding medication use for such acute conditions are given in Table 5.

Women who experienced flu or cold during pregnancy used medications containing acetaminophen (paracetamol) under a physician's supervision (32,1%, n=9) or consulted a pharmacist (3.6%, n=1) to alleviate their symptoms. Most women who had bacterial infection used penicillin antibiotics (85,7%, n=6) under the supervision of their physicians. Additionally, pregnant women who experienced headache, toothache, or joint pain during pregnancy used paracetamol under the supervision of their physician (51.7%, n=15) or a pharmacist (6.9%, n=2). Interestingly, 64.3% (n=18) of the women with flu/cold and 41.4% (n=12) of the women with headache, toothache, or joint pain reported that they did not use medication, instead they attempted to take supplements.

Supplement use during pregnancy

Women's attitudes regarding supplement use during pregnancy are given in Table 6. We determined that 96.7% (n=58) of women used iron and folic acid supplements during the first trimester of pregnancy according to their physician's recommendation (n=57) or their own preference (n=1). Other supplements/herbal products including fish oil, vitamin D, calcium, multivitamins, and fennel were also determined to be used mostly under the physician's supervision (n=12) or according to the advice of friends/relatives (n=2), but not with the recommendation of a pharmacist.

Table 4. Women's attitudes and perceptions regarding medication use during pregnancy-induced diseases

	Gestational Diabetes or Hypertension (n=4)	
Which of the following describes how you use the medication for your gestational diabetes or hypertension?	**n**	**(%)**
I adhered to the prescribed medication regimen, taking it consistently and in the dosage recommended by my doctor.	3	75
I only took the medication when my symptoms worsened, as I was concerned it might harm my baby.	0	0
I stopped taking the medication, as I was concerned it might harm my baby.	0	0
I never used medication, as I was concerned it might harm my baby.	1	25

Table 5. Women's attitudes and perceptions regarding medication use for acute conditions during pregnancy

	Flu/Cold (n=28)		**Bacterial Infection** (n=7)		**Headache/Toothache/ Joint pain** (n=29)	
What did you do for your flu/cold, bacterial infection, and headache/toothache/joint pain during pregnancy?	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**
I consulted with my doctor and used the medication under his/her supervision.	9	32.1	6	85.7	15	51.7
I consulted with a pharmacist and used a medication based on his/her advice.	1	3.6	0	0	2	6.9
I took a medication that I used before pregnancy.	0	0	0	0	0	0
I did not use medication; instead, I attempted to take supplements.	18	64.3	1	14.3	12	41.4

Table 6. Women's attitudes regarding the use of supplements during pregnancy

	Yes		**No**	
	n	**(%)**	**n**	**(%)**
Did you take iron and folic acid supplements during the first 3 months of your pregnancy?	58	96.7	2	3.3
Did you use any other supplements/herbal products during pregnancy?	14	23.3	46	76.7
Did you or your baby experience any health issues related to the medication or herbal products you used during pregnancy?	0	0	60	100

DISCUSSION

Pregnancy is a critical period for the safety of both the mother and fetus. Medication use during pregnancy may be required because of pre-existing chronic conditions, newly emerged symptoms, or pregnancy-induced diseases (Mitchell et al., 2011). Medication management in pregnancy is based on the principle of providing an effective treatment for the mother and minimising potential harm to the fetus or newborn. In this respect, healthcare providers, including pharmacists, have a pivotal role in ensuring the rational use of medication during this period (Lynch et al., 2017; Ceulemans et al., 2020). In addition, women's awareness, attitudes and practice also have a significant impact on the safety of medication use during pregnancy (Lupattelli et al., 2014; Petersen et al., 2015; Devkota et al., 2017; Terzioğlu Bebitoğlu et al., 2022). In the current questionnaire-based pilot study, we assessed the attitudes and perceptions of pregnant or lactating Turkish women regarding medication use during pregnancy, who applied to a community pharmacy in Istanbul, Türkiye.

The majority of participants (70%) were between 25 and 35 years of age, which is the most common period of fertility in women. Differences were noted in the age ranges of women participating in similar studies in Türkiye (Olukman, Parlar, Orhan, 2006; Kahraman, Şen Aytekin, Sandalcı, Alparslan, 2023; Çobanoğlu, 2020; Terzioğlu Bebitoğlu et al., 2022; Albayrak et al., 2024). The age range of our study population may be partly related to the high education level of most women. On the other hand, some participants had pre-existing chronic conditions such as T2DM, hypertension, hypothyroidism, asthma, thalassaemia, and kidney failure, as well as pregnancy-induced diseases such as gestational diabetes and hypertension. In addition, some women experienced common pregnancy-induced conditions such as nausea and vomiting and gastroesophageal reflux, while others experienced various acute conditions, including flu, cold, bacterial infection and headache, toothache, or joint pain.

Medication-related risks vary depending on the stage of pregnancy, with the first trimester being particularly crucial due to the potential for adverse effects on fetal development, including miscarriage, organ malformations, and functional impairments. We determined that the majority (65%) of the women believed that certain medications were harmful during pregnancy. On the other hand, 31.7 % of the women considered that all medications were unsafe, particulary in the first 3 months or during the entire pregnancy. In contrast, other studies have reported a much higher percentage of pregnant women who believed that taking medications either in the first or other trimesters is dangerous or probably detrimental (Wolgast, Lindh-Astrand, Lilliecreutz, 2019; Alhajri et al., 2022). Overall, this difference from our findings may be related to the variations in the education levels of the women who participated in these studies, which was also suggested previously (Nordeng et al., 2010; Zaki & Albarraq, 2014).

Medication use during pregnancy must be carefully supervised by healthcare professionals. In this study, the majority of participants (90%) consulted their physicians before using any medication during pregnancy, whereas some others received advice from pharmacists, nurses or non-professionals (friends/relatives). Most of the women involved in this study adhered to the prescribed treatment regimen for their pre-existing chronic (83.3%) and pregnacy-induced diseases (75%), by taking the medicine consistently and in the recommended dosage. Similarly, a previous study showed that most pregnant women (64.1%) adhered to the prescribed treatment (Wolgast et al., 2019). Thus, it can be speculated that pregnant women ensure a high adherence to the treatment regimen given by the physician. Notably, in the current study, none of the participants had a habit of self-medication while some women with pre-existing chronic (16.7%) or pregnancy-induced diseases (25%) stopped taking their medication thinking that it might harm their baby. In contrast to our findings, a high percentage of women (64.2% and 72.4%) were reported to have a habit of self-medication in previous studies (Abasiubong et al., 2012; Devkota et al., 2017). This discrepancy may be related to differences in the demographic characteristics of the participants, particularly their education level, highlighting the importance of strategies aimed at educating pregnant women. In the current study, approximately 46.6% of the pregnant women had a high education level, which may explain the high adherence to the treatment regimen given by the physician as well as the lack of self-medication habit. It is noteworthy that the level of education is low in other studies conducted in Türkiye, which reported that self-medication and the tendency to buy drugs from a community pharmacy without consulting a physician are high among pregnant women (Alptekin & Koruk,2020; Kahraman et al., 2023).

Insulin, methyldopa, levothyroxine, salbutamol and iron were used for pre-existing chronic and pregnancy-induced diseases, which are the most common medications prescribed for pregnant women (Undela, Joy, Gurumurthy, Sujatha, 2021). Concerning pregnancy-induced acute conditions, which were reported by half of the women who participated, metoclopramide and trimethobenzamide were preferred for nausea and vomiting, while antacids were used for gastroesophageal reflux. Headache, toothache, joint pain, and flu/cold were the most common acute conditions reported by the participants during their pregnancy. For these acute conditions, pregnant women used medications containing paracetamol under the physician's supervision to alleviate their symptoms. In addition, penicillin antibiotics were preferred for treating bacterial infection. Indeed, these are commonly prescribed medications in pregnant women, consistent with previous studies (Nordeng et al., 2010; Wolgast et al., 2019). Notably, 50% of the women experienced pregnancy-induced conditions, and 64.3% of the women with acute conditions (i.e., flu/cold) did not use any medication but instead attempted to take supplements. Furthermore, a small percentage of pregnant women who experienced acute conditions such as headache, toothache, joint pain, or flu/cold used paracetamol-containing medications based on a pharmacist's recommendation, which is comparable to findings from other studies (Wolgast et al., 2019; Ceulemans et al., 2020).

Almost all women who participated in the study were receiving iron and folic acid supplements (96.7%) during the first trimester of pregnancy according to their physician's recommendations. Moreover, fish oil, vitamin D, calcium, multivitamins, and fennel were also determined to be used by pregnant women as other supplements/herbal products, mostly under the physician's recommendation. Notably, none of the pregnant women applied to a community pharmacist for supplement recommendation.

This study has some limitations such as a small sample size and the collection of data by self-reporting, which may be subject to reporting bias. Additionally, the survey was conducted among participants who applied to a community pharmacy in

Istanbul; hence, their knowledge and attitudes on medication use may not be representative of pregnant women in other regions of the country.

CONCLUSION

Overall, most women who participated in this study used medication with high adherence to the treatment regimen and had high confidence in the advice from a physician. This positive attitude and perception is likely to be linked to the higher educational levels of most participants. However, the tendency to avoid medication use due to concerns about fetal harm, and the low rate of consultation with pharmacists are notable findings. Given the pivotal role of community pharmacists in ensuring the appropriate and safe use of medications, more effort is needed to encourage pregnant women to obtain information regarding medication use during pregnancy from these healthcare providers.

Ethics Committee Approval: This study was conducted in accordance with the approval of the Istanbul University Faculty of Dentistry Clinical Research Ethics Committee (30/07/2024, No:747).

Informed Consent: Written consent was obtained from the participants.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study: B.S.U.D., B.D..; Data Acquisition: N.Ö.S., B.D.; Data Analysis/Interpretation: : B.S.U.D., B.D., N.Ö.S., D.K.D., İ.K., E.G.K.; Drafting Manuscript: B.S.U.D., B.D., N.Ö.S., D.K.D., İ.K., E.G.K.; Critical Revision of Manuscript: B.S.U.D., D.K.D.; Final Approval and Accountability: B.S.U.D., B.D., N.Ö.S., D.K.D., İ.K., E.G.K.

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Original Article

Paediatric oral dosage forms: Why do caregivers need drug modifications and how?

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ABSTRACT

Background and Aims: A major obstacle to developing appropriate medications is the lack of understanding of what is acceptable for paediatric patients and the types of alterations that can be made to the dosage form. This study aimed to identify problems encountered when administering oral dosage forms to paediatric patients and solutions identified by patients' caregivers.

Methods: A questionnaire was developed by the research team based on relevant literature and their experiences. The first part involves questions to determine the demographic characteristics of the paediatric patient and their caregivers. The second section includes 11 statements about the problems experienced by caregivers regarding the child's medication use. The last part consists of 4 closed-ended questions and one open-ended question to evaluate the solutions offered by the patient's caregivers.

Results: A total of 419 caregivers participated in the study. In particular, it has been revealed that children experience problems regarding the taste, smell, and size of medicines. When the caregivers' solutions are evaluated, it was observed that the most common method is convincing or forcing the child to take, followed by dosage form alterations such as breaking, crushing, and dividing the form.

Conclusion: This is the first study to evaluate problems encountered when administering oral dosage forms to paediatric patients in Türkiye. The results show that the absence of age-appropriate medicines forces caregivers to alter their dosage forms. This study also highlights the necessity of considering user preferences in the dosage form design, which is an essential parameter of the target product quality profile that ensures patient compliance.

Keywords: Acceptability, Age-appropriate, Alteration, Paediatrics, Dosage forms

INTRODUCTION

The active pharmaceutical ingredient (API) is a primary consideration for determining dosage, clinical effects, and adverse drug responses in pharmacology and clinical paediatrics. However, formulation is crucial as it determines whether the dose can be given to the paediatric patient (Liu, Ghaffur, Bains, & Hamdy, 2016; Liu et al., 2015). Pharmaceutical drug product design should consider patients' needs and preferences to simplify drug administration and resolve medicine-related problems (Drumond, van Riet-Nales, Karapinar-Çarkit, & Stegemann, 2017). Drug product design that is patient-centric can be characterised as "the process of identifying the comprehensive needs of individuals or the target patient population and utilising the identified needs to design pharmaceutical drug products that provide the best overall benefit to risk profile for that target population over the intended duration of treatment" (Stegemann, Ternik, Onder, Khan, & van Riet-Nales, 2016; Walsh, Ranmal, Ernest, & Liu, 2018).

The incidence of medication errors is notably higher in children than in adults. A study in Korea that analysed data from 1989 to 2012 found that medication errors occurred three times more frequently in children than in adults (Woo, Kim, Chung, & Park, 2015). Similarly, Stratton et al. (2004) reported a higher rate of medication errors in paediatric patients (67%) than in adult patients (56%) (Stratton, Blegen, Pepper, & Vaughn, 2004). Doherty and McDonnel (2012) highlighted that 43.3% of errors in paediatric patients were due to prescribing mistakes, while 34.5% were due to practise errors (Doherty & Mc Donnell, 2012). The FDA studies conducted between 1993 and 1998 identified the most common errors as inappropriate dos-

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ing (41%), incorrect drug administration (16%), and incorrect drug delivery (16%) (Phillips et al., 2001). Sears et al. (2013) found that the most frequent errors in paediatric patients were incorrect timing (45.2%) and incorrect dosing (22%) (Sears, O'Brien-Pallas, Stevens, & Murphy, 2013). Özkan et al. (2013) revealed that nurses in Türkiye most commonly made timing (10.6%) and dosing (10.3%) errors when administering medications to children (Özkan, Kocaman, & Ozturk, 2013). Adams et al. (2013) investigated the medication administration practises of families and caregivers in Tanzania and concluded that the taste and formulation of medicines significantly influenced children's adherence to treatment regimens (Adams et al., 2013). Additionally, Venables et al. (2015) conducted a focus group study with healthcare professionals in the UK and identified that the taste and oral sensations of dosage forms were key factors affecting children's compliance with oral medication (Venables, Batchelor, Stirling, & Marriott, 2016).

While the pharmacists' roles in paediatric drug usage have been evaluated, several studies have addressed this issue. Pirhan and Özçelikay (2005) focused on evaluating pharmacists' roles in paediatric drug usage (Pirhan, 2005). Nahata and Taketomo emphasised the importance of having clinical pharmacists in paediatric wards to increase patient safety (Nahata & Taketomo, 2017). The American Academy of Paediatrics (AAP) emphasises the role of multidisciplinary teams—including doctors, nurses, pharmacists, laboratory staff, and information specialists—in reducing medication errors in children. Stucky (2003) highlighted the critical role of clinical pharmacists, physicians, and nurses, especially in intensive care and oncology services, to minimise paediatric medication errors (Stucky, 2003). The Joint Commission (JCI), in its 2008 report "Prevention of Medication Errors in Paediatric Patients," suggested integrating pharmacy personnel into paediatric services and assigning pharmacists and technicians with paediatric expertise to neonatal/paediatric intensive care units and paediatric oncology services (D'Errico et al., 2021). Sanghera et al. (2007) reported from a different perspective that the error rate in paediatric patients was four times higher than in adults. Clinical pharmacists made 2,449 recommendations for paediatric patients, of which 99.2% were approved by physicians.

Due to the scarcity of research focused on the appropriate formulations for paediatric patient group, the drug product needs were initially not well understood (van Riet-Nales, de Jager, Schobben, Egberts, & Rademaker, 2011; van Riet-Nales, Schobben, Egberts, & Rademaker, 2010). Therefore, pharmaceutical scientists may face considerable difficulties in developing formulations suitable for paediatric use (Nunn & Williams, 2005; Stegemann et al., 2016). As an integral component of developing these medications, the pharmaceutical industry must demonstrate that novel paediatric formulations are acceptable to target age groups while considering user needs (Kozarewicz, 2014). This prerequisite is essential for ensuring the best possible treatment compliance and the therapy's effectiveness and safety (Ranmal, Cram, Tuleu, 2016). When attempting to find an acceptable solution, two questions arise: "Which dosage form should be chosen for each target age group?" and "How should it be formulated after the dosage form is determined?" (Liu et al., 2016; Liu et al., 2015)

Given the significant impact of formulation preferences, medication palatability, and home administration practises on paediatric medication adherence, understanding parent/caregiver behaviours and involving them in initiatives to improve administration options and promote responsible use are crucial (Adams et al., 2013). Any alterations to the dosage form not authorised by the label can potentially compromise effectiveness or, in extreme cases, endanger the patient because the responsibility for patient care often lies with the drug administrator rather than the pharmaceutical manufacturer (Schiele, Quinzler, Klimm, Pruszydlo, & Haefeli, 2013). Concerns about high off-label prescription rates and inappropriate modifications to drug products are widespread globally (van Riet-Nales et al., 2010). Regulatory support and frameworks play a crucial role in overcoming these challenges, transforming paediatric formulation development into a vital aspect of drug development. Both the European Medicines Agency (EMA) and the FDA mandate detailed formulation development strategies in Paediatric Investigation Plans (PIPs) and Paediatric Study Plans (PSPs), respectively (Ranmal et al., 2016). According to the EMA, an age-appropriate paediatric medication is one whose pharmaceutical design makes it suitable for use in the target age group(s), encompassing factors such as composition, dosage form, dosing frequency, and packaging (EMA, 2013). Ensuring patient acceptance of formulations defined as "the overall ability and willingness of the patient and its caregiver to use and administer the medicine as intended is highly prioritised (EMA, 2013; Kozarewicz, 2014)." The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Product Development Guidelines (Q8 (R2)) emphasise that pharmaceutical products should be designed to meet patient needs and intended performance standards ((ICH), 2009). Therefore, alongside technical challenges in pharmaceutical development and manufacturing, considering patient needs is crucial in defining the Quality Target Product Profile (QTPP) and selecting suitable dosage forms (Walsh et al., 2018).

A significant barrier to developing appropriate medications is the limited understanding of what constitutes acceptability for paediatric patients, how to evaluate a new product's acceptability, and how caregivers modify dosage forms to enhance acceptability. The lack of research on challenges in administering oral pharmaceuticals to paediatric patients and caregivers' solutions to these problems, particularly in Türkiye, motivates this study. In this regard, this study aimed to reveal problems encountered when administering oral pharmaceutical dosage forms to paediatric patients, especially concerning the taste and shape of the dosage form as acceptability criteria and solu-

tions found by patients' caregivers to overcome problems based on the shape and taste of medicines.

MATERIALS AND METHODS

According to the study's aim, a Turkish questionnaire was administered to caregivers of paediatric patients and comprised three parts. The first part collected demographic data about the patients and caregivers. The second section included 11 statements assessing caregivers' challenges with child medication use. To determine the common problems encountered by paediatric patients, firstly, relevant literature (ICH, 2019; Schiele et al., 2013; Stegemann et al., 2016; Venables et al., 2016; Zajicek et al., 2013) was evaluated by all research team members. Accordingly, a statement tool was created, which included 15 statements. After evaluating these statements by the research team, which also considered their pharmacy experiences, 11 were selected as appropriate. A pilot study was then conducted with eight individuals to test the face validity of the statements. Because of the pilot study, it was observed that the questionnaire had face validity.

To evaluate the frequencies of the selected problems, statements were graded with a 4-point Likert-type scale (Never "1", Always "4"). The last part of the questionnaire includes four closed-ended questions and one open-ended question to evaluate the solutions offered by the patient's caregivers.

Sample size and data collection

The study population comprised male and female individuals aged between 18 and 65 who applied to community pharmacies for paediatric patients in Van and Istanbul city centres. The minimum sample size was calculated using the Cochran sample size formula at a 95% confidence level with a margin of error of 0.10 equal to 88. To increase the reliability of the results obtained, we attempted to reach the maximum possible number of individuals. After obtaining informed consent, the researchers applied the questionnaires face-to-face and online via Google Forms. Two researchers administered the survey face-to-face to the caregivers of patients eligible for the study sample in pharmacies where they were interned during the research period. To increase the sample size, individuals who participated in the face-to-face survey were asked to share the online survey link with appropriate individuals. In addition, all researchers shared the survey link with individuals who met the study criteria and asked them to share it.

The study was conducted between 04.03.2020 and 15.04.2020 with the ethical approval of the Van Governorship Provincial Health Directorate (73040253-044-E.438 and the Van-Bitlis-Hakkari Chamber of Pharmacists with number 2019/1626 and the World Medical Association (WMA) Declaration of Helsinki Ethical Principles in Medical Research on Human Volunteers.

Data analysis

The data obtained from the questionnaire were subjected to descriptive statistical analysis, including frequencies, percentages, and histograms, using the IBM SPSS 22.0 (IBM Electronics, USA) package programme.

RESULTS

419 caregivers of patients participated in the questionnaire. A total of 314 participants (74.9%) were female, and 105 (25.1%) were male. Most participants were in the 25-50 age range (81.1%). Among the participants, 140 were primary school graduates, 153 were high school graduates, 111 had a university degree, and 15 had postgraduate degrees. When the information about the children to whom the caregivers participating in the study administered the medication was evaluated, it was determined that 57% of the children were girls and 43% were boys.

Only 9 caregivers of the patients stated that the child had an eating/chewing disorder, and 49 of them indicated that the child had chronic disease. Chronic ailments reported by caregivers include asthma, allergic rhinitis, allergies, and diabetes.

The percentage of caregivers' responses to the statements prepared for the problems experienced by children regarding medication use are presented in Table 1.

In light of the information presented in Table 1, when the sum of the "often" and "always" responses given to the statements was considered, it was revealed that children experienced problems, especially regarding the taste, smell, and size of the medicine.

When the patient's caregivers were asked which form of solid medicine the child had the most difficulty swallowing, 40.6% of the caregivers answered that it was in rectangular form. The bar diagram of the answers is shown in Figure 1.

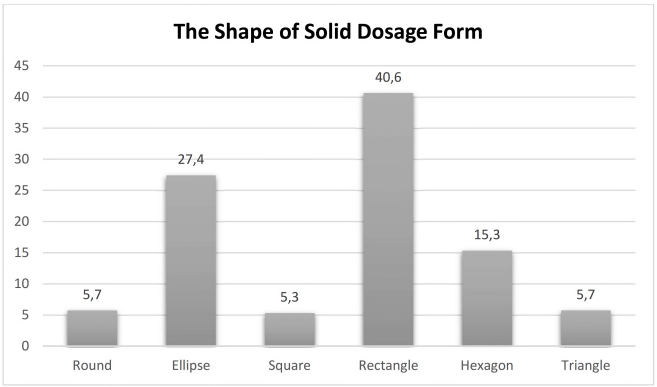

Figure 1. Percentages of the answers given by the caregivers of the patient to the question of which form of solid medicine the child had the most difficulty swallowing.

Table 1. Percentage of responses from caregivers of patients to statements about problems experienced by children regarding medication use

Statements	Response Percentages (n=419)			
	1 (Never)	2 (Rarely)	3 (Often)	4 (Always)
Does not want to swallow/drink—does not like the taste of the medicine	9.8%	32.7%	31.3%	26.3%
Does not want to swallow/drink because he/she does not like the smell of the medicine	20.5%	28.2%	26.5%	24.8%
Does not want to swallow/drink because he/she does not like the colour of the medicine	48.4%	18.6%	16.5%	16.5%
The dimensions of the medicine make it difficult to swallow	13.6%	30.5%	33.9%	22.0%
Difficulty swallowing due to the form of the medicine	18.4%	39.4%	26.5%	15.8%
Medicine is very intense and is difficult to consume	21.7%	38.7%	23.2%	16.5%
It is difficult to drink because the medicine is too	22.2%	37.5%	22.2%	18.1%
The medicine makes the child become nauseous	31.7%	28.4%	19.6%	20.3%
The medicine stuck to the child's throat	53.7%	30.1%	11.2%	5.0%
The medicine is caught in the child's throat	56.1%	30.1%	10.5%	3.3%
Coughing while taking the medicine	53.0%	32.0%	9.3%	5.7%

The caregivers of the patients were also asked which flavour syrups the child took more easily, and the answers are presented in Figure 2.

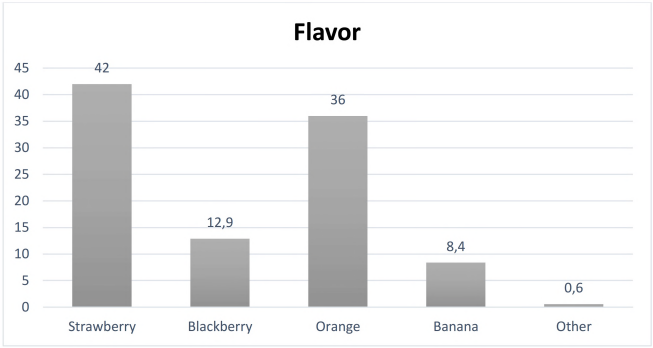

Figure 2. Percentages of the answers given by the caregivers of the patients to the question of which flavor syrup the child took more easily

Another critical finding obtained in the study was the solution suggestions offered by the patients' caregivers so that the patients could take the medicine when it was difficult to swallow/take. The recommendations and information about the number of respondents giving similar responses are presented in Table 2.

In Table 2, it can be seen that the most common method used by caregivers, other than convincing or forcing them to take medicine, is trying to give it by breaking it, crushing it into powder, or dividing it into two. When asked who the caregivers consult regarding these solution suggestions, it was revealed that they consult physicians and pharmacists the most. This was followed by other healthcare professionals, caregivers, friends, and the other groups, and a bar diagram for the results is presented in Figure 3.

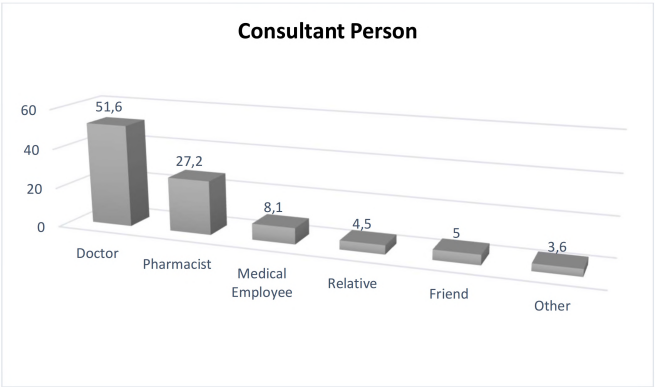

Figure 3. The graph of the people whom the caregivers refer to for solution suggestions

Finally, the caregivers of the patients were asked what they did when they could not find a solution. Responding to this question, of the patients' caregivers, 255 stated that they had consulted a doctor, 118 stated that they had consulted a phar-

Table 2. Solution suggestions offered by the patient caregivers.

Suggestions	Number of responses
I try to encourage them to take medicine by playing games with them, distracting their attention.	27
I try to persuade people by talking (saying that if they take medicine, they will get better, or I will take them to the park, I will give them candy, or I will buy them their favourite toys).	68
I make them take medicine with the help of an apparatus (dropper, injector, etc.)	26
I try to make them take medicine with plenty of water.	30
After taking the medicine, I try to give them water immediately.	6
I give medicine by dissolving it in water.	21
I give medicine by adding it to a drink she or he likes.	27
I give medicine by adding it to a food she or he likes.	26
I try to give medicine little by little.	1
I try to give medicine by breaking it up, crushing it into powder, or dividing it into half.	43
I give medicine by force (such as covering their noses and pouring medicine into their mouths)	60

macist, and 46 said that they had stopped giving the medicine. The bar diagram of these responses is shown in Figure 4.

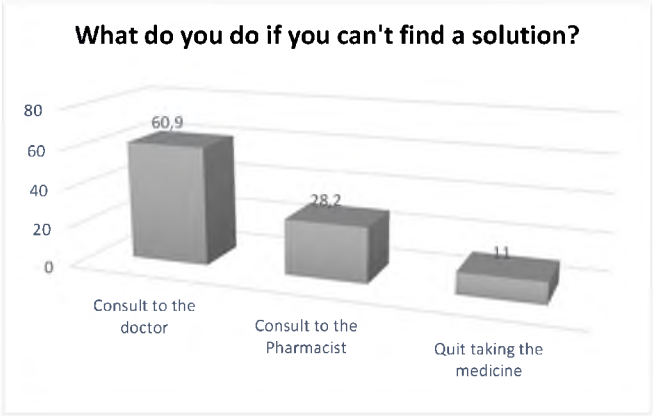

Figure 4. Percentage of the answers given by the caregivers of the patients to the question of what they did if they could not find a solution

DISCUSSION

The design of patient-oriented drug formulations has become one of the main topics that researchers are interested in and working on with technological developments in recent years. However, drug formulation designs targeting paediatric and geriatric populations are particularly challenging because of variations in design parameters. One of the obstacles hindering the development of age-appropriate paediatric medications is the lack of understanding of the dosage forms that are acceptable for the paediatric population. Additionally, there is a shortage of studies on patient populations that reveal their preferences and behaviours.

The oral route is the most commonly used of all administration methods because it is the easiest and most convenient (Kim & De Jesus, 2023). The overall acceptability of an oral paediatric medication is influenced by the choice of oral dose form and formulation features, such as tablet size or oral suspension palatability (van Riet-Nales et al., 2013; van Riet-Nales et al., 2014). Nevertheless, this route of administration poses the greatest challenge in designing age-appropriate formulations for paediatric populations. Since API is typically not dispersed evenly throughout the tablet, the EMA does not recommend solid oral dosage forms that are split or crushed to obtain the target dose unless the method has been validated (Shah et al., 2010; Zajicek et al., 2013; Zhao, Zidan, Tawakkul, Sayeed, & Khan, 2010). However, the absence of age-appropriate formulation force caregivers and medical professionals to alter dosage forms. These alterations include breaking, crushing, or dissolving the pills, taking the contents out of the capsules, and mixing them with meals. These changes may compromise the active ingredient's purity and affect the drug's stability and/or absorption (Juárez-Hernández & Carleton, 2022).

Within the scope of this study, the patients' caregivers evaluated the difficulties children experienced in the oral administration of solid and liquid dosage forms. The most frequently mentioned problems by caregivers of the patients were "She/he does not want to take medicine because she/he does not like the taste of the it.", "The medicine is difficult to swallow because of its large size." and "She/he does not want to take medicine because she/he does not like the smell of the it." These three problems have been among the factors that negatively affect compliance with medication in the literature (Bergene, Nordeng, Rø, & Steinsbekk, 2019; Marconati, Raut, Burbidge, Engmann, & Ramaioli, 2018; Schiele et al., 2013). Venables et al. (2015) evaluated children's compliance with medicine treatment at the point of the dosage form, and it was stated that one of the most critical factors reducing compliance was the taste of the form (Venables et al., 2016). Similarly, Adams et al. (2013) revealed that the type of dosage form, especially its taste, affects adherence to medication in children, and in concordance with study findings, sweet-tasting medicines are pre-

ferred by paediatric patients (Adams et al., 2013). Breitkreutz and Boos (2007) stated that the taste and size of the medicine are essential factors in the difficulties experienced by paediatrics and geriatrics group in oral medicine use (Breitkreutz & Boos, 2007). In addition, Klingmann (2017) revealed that small solid dosage forms are preferred by the paediatric group (Klingmann, 2017). Hence, the findings are in parallel with literature that is independent of socioeconomic and geographic conditions. The respondents emphasised the struggle they face when giving ellipse and rectangular shapes of solid medicines, which are mainly used in antibiotic-type medications used for many conditions.

Because liquid oral dosage forms are easier to swallow and have adjustable doses, they are frequently used when giving medications to children (EMA, 2013). However, issues with poor flavour and unfavourable ingredients could make them less suitable (Nunn & Williams, 2005; Walsh et al., 2014). Oral solid dosage forms, including tablets and capsules, are considered to be better than liquids in terms of logistics, production costs, stability, and the capacity to cover undesirable tastes (Nunn & Williams, 2005). Studies conducted in the past few decades have demonstrated that newborns and early children can swallow tiny pills measuring 2-4 mm and may even favour solid formulations over liquid formulations (Drumond et al., 2017; Klingmann, 2017; Wargenau, Reidemeister, Klingmann, & Klingmann, 2022). However, in clinical settings, solid monolithic formulations are rarely found at suitable doses and sizes for common paediatric diseases (Lajoinie, Henin, Kassai, & Terry, 2014). Children have been observed to find it challenging to swallow typically sized solid monolithic formulations, although studies suggest that this skill can be improved with guidance (Patel, Jacobsen, Jhaveri, & Bradford, 2015).

It has been observed that children mostly prefer strawberry fruit. Taste masking is essential in the design of pharmaceutical dosage forms. Most active ingredients are tasteless or can be bitter, and some may even be extremely salty. With the taste masking process, the unpleasant taste of the medicine can be reduced or eliminated physiologically (Coupland & Hayes, 2014). After drug dissolution in saliva, it remains in the oral cavity until swallowing. If the active substance has an unpleasant taste, this may adversely affect patient compliance. The results obtained in this study guide the choice of sweetener and indicate that strawberry and orange flavours are favourable among others (Figure 2).

Considering the solutions caregivers have found for the difficulties encountered while administering oral medication to their children, the study findings revealed that the most common method used by caregivers, aside from convincing or forcing their children to drink the medication, is to modify the dosage form. This involves breaking the slurry into a powder or dividing it into two parts. This practise is observed both in Türkiye and in the UK (Alessandrini et al., 2021). Schiele et al. (2013) also emphasised that dosage modifications, if not allowed in the patient leaflet, will diminish the effectiveness of the medicine and, in some occasions, can cause harm to the patient (Schiele et al., 2013). Zajiscek et al. (2013) emphasised the negative effect of dosage form alterations and called attention to the differences between the adult and paediatric age groups regarding bioequivalence and responsiveness of clinical data (Zajicek et al., 2013). In summary, crushing or breaking of dosage forms seems to facilitate drug intake; but it may cause insufficient dosage, adverse effects on bioavailability, and antagonistic effects (Akram & Mullen, 2012).

It has been revealed that caregivers of patients mostly consult physicians for the solutions they benefit from. In fact, 52% of caregivers of patients consult physicians, and 27% consult pharmacists about their solution suggestions. Fewer people consulted with caregivers, friends, or other healthcare professionals. 61% of the patient's caregivers who could not find any solution stated that they consulted a doctor and 28% consulted a pharmacist.

Ultimately, our findings align with the current literature and highlight the significant influence of taste and shape on patient preferences, even when the medicine is intended for therapeutic use. Furthermore, the results of this study illuminate the challenges caregivers face in administering medication and addressing acceptance issues without appropriate guidance.

This study has two main limitations. The first concerns were the data collection process, which was affected by the onset of COVID-19 and the resulting restrictions, which led to a lower than expected rate of face-to-face surveys and forced a switch to online surveys. Despite this, the sample size remained adequate, so the analysis results were not compromised. The second limitation is that only the syrup and tablet forms of oral dosage were evaluated. Future studies will segment the survey by age group and include other oral dosage forms, such as lozenges and chewable tablets.

CONCLUSION

This study aimed to enhance the treatment of paediatric patients by identifying the challenges faced by caregivers when orally administering medications. This is the first detailed evaluation of these issues in Türkiye, highlighting the importance of considering user preferences in dosage form design, which is a key aspect of the Quality Target Product Profile (QTPP) that is crucial for patient adherence. Notably, caregivers tend to prefer consulting physicians over pharmacists for formulation development, indicating the need for attention during formulation development and patient counselling.

The findings contribute significantly to the literature and provide preliminary data for future research. Pharmacists should have a thorough understanding of paediatric medications and to offer comprehensive counselling to caregivers. Furthermore,

incorporating children's preferences regarding oral dosage forms during the formulation research phase is vital for creating more child-friendly medications. This patient-centered approach recognizes that children have unique physiological characteristics and are not simply smaller adults. Therefore, paediatric formulations must meet regulatory safety and efficacy standards while allowing for dosage adjustments across various age groups.

Ethics Committee Approval: The study was conducted with the permission decisions of the Van Governorship Provincial Health Directorate numbered 73040253-044-E.438 and the Van-Bitlis-Hakkari Chamber of Pharmacists with number 2019/1626 and the World Medical Association (WMA) Declaration of Helsinki Ethical Principles in Medical Research on Human Volunteers.

Informed Consent: Informed consent was obtained from the participants

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study: Ö.A.D., M.A.; Data Acquisition: F.İ., G.T.; Data Analysis/Interpretation: M.A., Ö.A.D.; Drafting Manuscript: Ö.A.D., M.A.; Critical Revision of Manuscript: Ö.A.D., M.A.; Final Approval and Accountability : Ö.A.D., M.A., F.İ., G.N.T.

Conflict of Interest: The authors have no conflict of interest to declare.

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Original Article

Development and validation of UPLC-MS/MS method for estimation of Saxagliptin in bulk and tablet dosage forms

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ABSTRACT

Background and Aims: Saxagliptin is an antidiabetic drug used to treat type 2 diabetes mellitus. During the manufacture of bulk and dosage forms, assay of the drugs is important in determining the percentage purity. Hence, this study aimed to develop and validate a simple, rapid, and selective method based on ultra-performance liquid chromatography-Tandem mass spectrometry (UPLC-MS/MS) analysis for the determination of saxagliptin in bulk and tablet dosage forms.

Methods: Chromatographic separation was performed using a Waters Acquity UPLC BEH C_{18} column (2.1 X 50 mm, 1.7 µm) with a mobile phase consisting of a mixture of acetonitrile: 0.1% formic acid (60:40, v/v) at a flow rate of 0.120 mL/min. Separation was performed in 3 min run time. The analyte was ionised and detected by tandem mass spectrometry, which is performed in positive ion and multiple reaction monitoring modes.

Results: Linearity was established in the 10–150 ng/mL with r^2=0.9980. The results were observed to be well within the limits when validation was performed as per the ICH guidelines.

Conclusion: The proposed method can be applied successfully for the analysis of saxagliptin in bulk and tablet formulations.

Keywords: Saxagliptin, UPLC-MS/MS, Method development, Validation.

INTRODUCTION

Saxagliptin, chemically known as 1S, 3S, 5S-2[(2S)-2-amino-2-(3-hydroxy-1-adamantyl) acetyl]-2-azabicyclo [3.10] hexane-3-carbonitrile, is an anti-diabetic drug (Figure 1). It is used by patients with type 2 diabetes as second-line therapy when first-line treatment (metformin) cannot control the disease (Mengistu, Ole & Alemayehu, 2021). It is a highly selective, reversible, and competitive dipeptidyl peptidase-4 inhibitor, increasing the secretion of insulin and decreasing the secretion of glucagon (Darshan, 2011). Inhibition of plasma DPP-4 activity can be achieved by administering saxagliptin to type 2 diabetes mellitus patients once daily before breakfast (Kulsa & Edelman, 2010; Anderson, Hayes &Stephens, 2016).

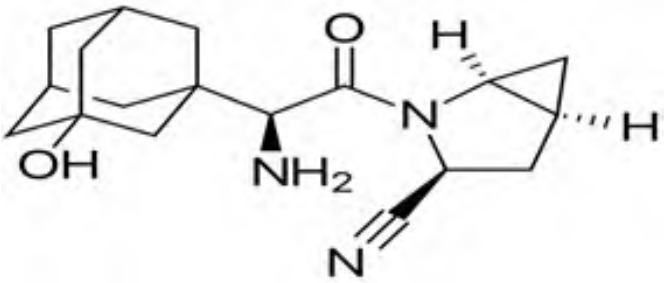

Figure 1. Chemical Structure of saxagliptin

As per the available literature, methods have been reported for the quantification of saxagliptin alone (Lais, Ana, Andrea & Clarice, 2015; Maha, Omar, Miriam & Mariam, 2015; Sridhar et al., 2014) or in combination with other anti-diabetic drugs (Saiful, Taleb, Sukalyan, Abdul & Rafiquzzaman, 2016; Faroqui & Kakde, 2016; Sanchay, Patel, Gaikwad & Jadhav, 2019; Singh, Bansal, Maithani & Chauhan, 2018; Mahnoor & Roshan, 2022; Rageeb et al., 2020; Ghawate & Chopade, 2019; Darshak, Ujashkumar, Jayvadan, Darshana & Pavan, 2021; Rahul & Ganesh, 2019; Amit & Bhuvnesh, 2021; Vijaya, Fainaz, Riya & Tejaswini, 2018; Padmaja, Sivagami, Chandrasekar & Niranja, 2018; Sarada, Narendra & Pragati, 2017; Rohini & Nagaraju, 2018; Vijaya & Narendra, 2020; Abdul-Azim, Ehab & Marwa, 2012; Narender & Shanmuga, 2021) using liquid chromatography techniques. The method reported by Lais et al. (2015) employed the RP-HPLC technique with a PDA detector. A run time of 12.5 min was required for regular sample anal-

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ysis. The method reported by Maha, Omar, Miriam & Mariam (2015) quantified saxagliptin using the RP-HPLC technique with a UV detector, followed by the elucidation of the structures of major degradation products using LC-MS. However, this method has less sensitivity (25-400 µg/mL) and a longer run time of 10 min. The RP-HPLC method reported by Sridhar et al., (2014) utilised the PDA detector for the assay of saxagliptin and tandem mass spectrometry for the characterisation of degradation products. This method has a run time of 30 min, which is not suitable for the routine analysis of saxagliptin. All methods reported for the assay of saxagliptin along with other drugs have longer run times and are therefore not appropriate for the regular analysis of saxagliptin samples.

The proposed work describes a UPLC-MS/MS technique for estimating saxagliptin in bulk and tablet formulations within a short run time of 3 min. This method is sensitive with a low LOQ value (10 ng/mL). The method was successfully applied for the quantification of saxagliptin in tablet form after its validation following the ICH guidelines.

MATERIALS AND METHODS

Chemicals and Reagents

The working standard for saxagliptin was procured from Vivan Life Sciences (Mumbai, India). Research Lab Fine Chem Industries (Mumbai, India) supplied HPLC-grade methanol, acetonitrile, and water. Analytical grade Formic acid was purchased from Merck India (Mumbai, India).

UPLC-MS/MS Instrument and Conditions

A Waters ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a Waters Acquity UPLC BEH C_{18} column (2.1 X 50 mm, 1.7 µm) was utilised for the present work. A mobile phase consisting of a mixture of acetonitrile:0.1 formic acid (60:40, v/v) was used in isocratic mode at a flow rate of 0.120 mL/min. A Quattro Premier XE™ triple quadrupole mass spectrometer (Waters, Milford, MA, USA) was used to detect the analyte. The data were acquired in the positive ion mode. The desolvation gas temperature was kept at 400 ºC, capillary voltage of 3 KV, source temperature was kept at 120 ºC, and cone gas flow rate was set at 102 L.h-1. The collision energy was maintained at 25 V. The multiple reaction monitoring (MRM) mode was used to monitor the ions by detecting the transition of the precursor ion to the product ion of saxagliptin from m/z 316.40 to 179.86. Mass Lynx software version 4.1 was employed for analysing the acquired data.

Preparation of Standards

A standard stock solution was prepared by dissolving the required amount of working standard saxagliptin with the diluent (HPLC grade methanol: water, 50:50, v/v) to obtain a concentration of 1000 µg/mL. The stock solution was diluted appropriately using the mobile-phase solvent to obtain the required concentrations of the standard solution.

Preparation of Samples

The tablet dosage form of saxagliptin (Brand name: Onglyza; label claim: 5 mg) was weighed and powdered, and a powder equivalent to 5 mg of saxagliptin was taken. It was solubilised in 50 mL of diluent. The solution was then filtered, and the filtrate was diluted to a concentration of 10 ng/mL with the mobile-phase solvent.

Method Validation

The developed method for estimating saxagliptin using UPLC-MS/MS was validated according to ICH guidelines (ICH Q2 (R1), 2005). The validation parameters were accuracy, precision, linearity, limit of detection, limit of quantification, robustness, and stability.

The linearity of the method was evaluated by measuring the peak area response of standard solutions at concentrations of 10, 25, 50, 75, 100, 125, and 150 ng/ml. A graph was plotted based on standard concentrations on the abscissa and peak areas on the ordinate. Recovery studies were conducted to assess the accuracy of the proposed method. Blank solutions were spiked with the working standards at three different levels (50%, 100%, and 150%, and triplicate measurements were performed at each level. A working standard solution of saxagliptin was injected into the instrument six times on the same day to assess intra-day precision and on different days for inter-day precision. The limits of detection and quantification of the method were assessed at S/N (signal to noise) ratio of 3:1 and 10:1, respectively. The flow rate of the mobile phase was altered from 0.12 mL/min to 0.14 mL/min, and the composition of the organic phase in the mobile phase was changed from 60% to 62% to evaluate its robustness. Three measurements were performed at a concentration of 100 ng/mL for each of the altered conditions, and the %RSD was calculated.

Assay of marketed tablet formulation:

The tablet formulation - was analysed, employing the proposed method to quantify the amount of saxagliptin. Three replicate injections of the sample solutions were analysed to calculate the %assay of tablet formulation.

Table 1. Literature survey

Parameter	Lais et al.	Maha et al.	Sridhar et al.	Present method
Technique	RP-HPLC-PDA	RP-HPLC-UV	RP-HPLC-PDA	LC-MS/MS
Column	Waters XBridge C_{18} (250x4.6 mm, 5 µm)	Symmetry C_{18} (150x4.6 mm, 5 µm)	Zorbax Eclipse plus (150x4.6 mm, 5 µm)	Waters Acquity UPLC BEH C_{18} (2.1 X 50 mm, 1.7 µm)
Mobile phase	0.1% phosphoric acid (pH 3):methanol (70:30, v/v)	Potassium dihydrogen phosphate buffer:acetonitrile:methanol (40:30:30, v/v/v)	10 mM ammonium formate:methanol (gradient elution)	Acetonitrile: 0.1% formic acid (60:40, v/v)
Flow rate (mL/min)	1.0	1.0	0.5	0.12
Retention time (min)	8	2.5	19	1
Run time (min)	12.5	10	30	3
Concentration range	15-100 µg/mL	25-400 µg/mL	50-150 µg/mL	10-150 ng/mL

RESULTS AND DISCUSSION

Method Development

As reported in the available literature, few methods for estimating saxagliptin in bulk and pharmaceutical dosage forms. All of these methods have longer run times and are not appropriate for routine analysis of saxagliptin (Table 1). Hence, the objective of this work was to develop a rapid and sensitive method for determining saxagliptin using UPLC-MS/MS.

During method development, the chromatographic and mass spectrometry conditions were optimised. An electrospraying-ion source was employed to record the mass spectrum of saxagliptin in both positive and negative ionisation modes. The presence of an amino group in the structure of saxagliptin imparts a basic nature, resulting in a higher response observed in the positive ion mode. During multiple reaction monitoring, the transition from the precursor ion to the product ion occurred from m/z of 316.40 to m/z 179.86 (Figure 2).

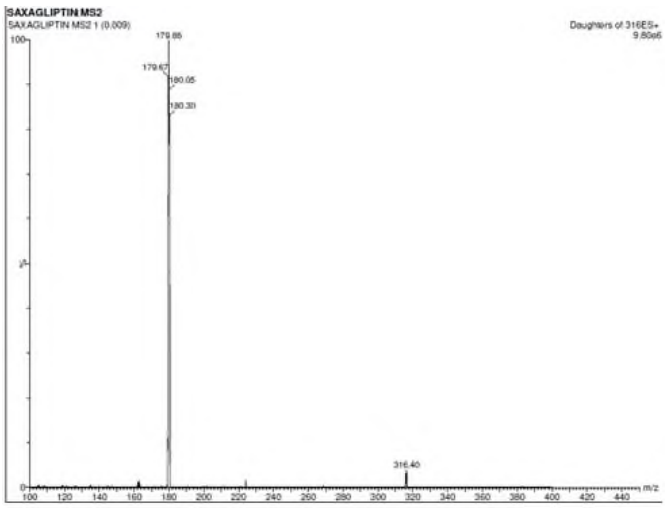

Figure 2. Product ion Mass Spectra

During chromatographic analysis, the column, mobile-phase composition, and flow rate were optimised to achieve good response and peak shape within a short run time. In combination with acetonitrile, various concentrations of acetic acid and formic acid were tested at altered ratios on Zorbax XDB-Phenyl (75 x 4.6 mm, 3.5 µm), Kromasil 100-C¬18 (100 x 4.6 mm, 5 µm), and Waters Acquity UPLC BEH C18 (2.1 X 50 mm, 1.7 µm) columns. Finally, a mixture of acetonitrile and 0.1% formic acid (60:40, v/v) as the mobile phase on a Waters Acquity UPLC BEH C18 column (2.1 X 50 mm, 1.7 µm) at a flow rate of 0.12 mL/min produced an acceptable response and satisfactory peak shape within 3 min of run time (Figure 3).

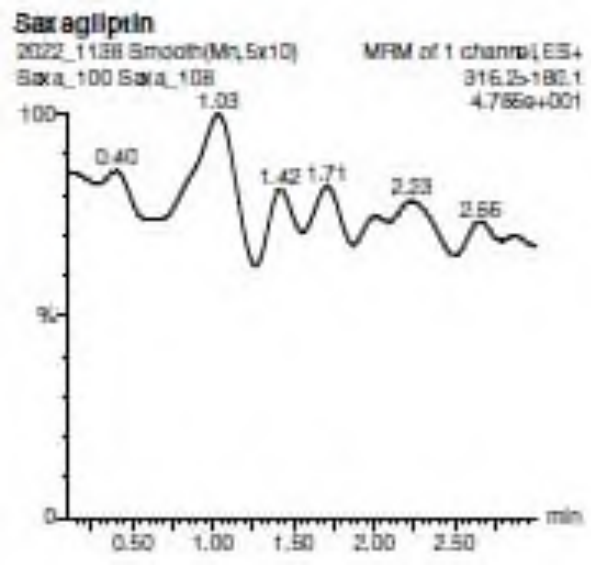

Figure 3. (a) Multiple Reaction Monitoring Chromatogram of blank

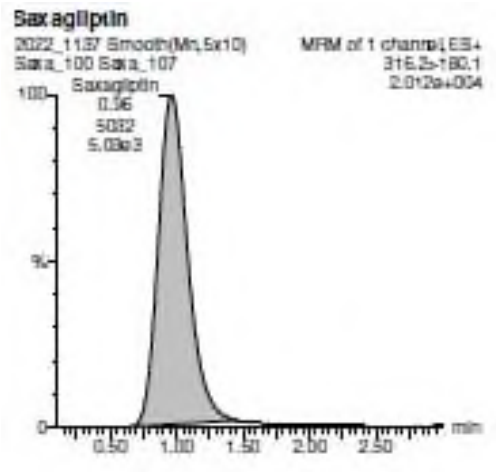

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Figure 3. (b) Multiple Reaction Monitoring Chromatogram of analyte at LOQ level-10ng/mL

Validation Results

The calibration curve showed good linearity over the concentration range of 10 ng/mL to 150 ng/mL with R2>0.99. The linear equation established is y=388.92x+652.71 (Table 2) (Figure 4) The percentage recovery of the analyte was found in the range of 98.20% to 98.55%, proving the accuracy of the proposed method (Table 3.)

Table 2. Linearity data

S.No	Concentration (ng/ml)	Mean Peak area (n=3)
1	10	3771
2	25	14612
3	50	21296
4	75	28842
5	100	40463
6	125	49113
7	150	58642
Range (ng/ml)		10-150
R^2		0.9980
Slope		396.588
Y-intercept		- 22.269

R^2 – Regression coefficient

The % RSD of the peak area and retention times were calculated and were found to be between 1.16% and 1.45% for the peak area and between 0% and 0.54% for the retention time (Results are shown in Table 4).

The limit of detection and limit of quantification were determined to be 3 and 10 ng/mL, respectively, proving the method to be highly sensitive.

The robustness of the proposed method was demonstrated by the %RSD values in the range of 0.07% to 1.5% for peak area and 0.1% for retention time.

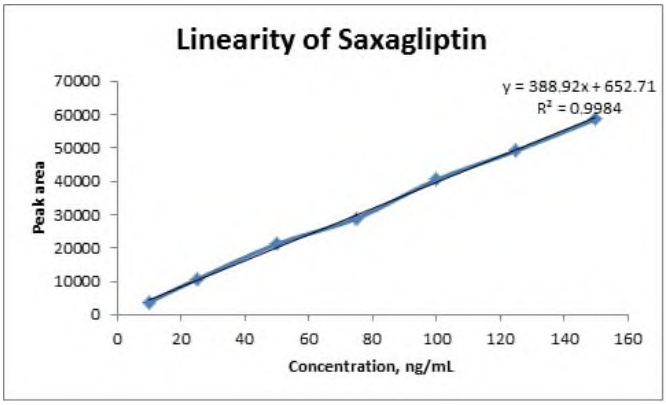

Figure 4. Linearity graph

The analyte present in the standard solution remained stable for 24 h at the auto-sampler temperature with a %stability value of 101.60%.

Assay of commercial formulation:

The percent purity of the tablet formulation was determined to be in the range of 98.60%–101.47% (Table 5).

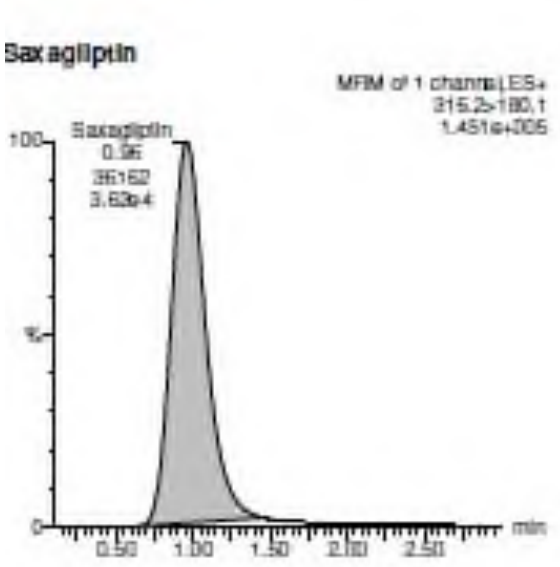

Figure 5. MRM chromatogram of a saxagliptin tablet formulation

Table 3. Accuracy data

Concentration (ng/mL)	Amount recovered	Mean % recovery	% RSD
50	49.10	98.20	0.78
100	98.30	98.30	1.15
150	147.83	98.55	0.55

RSD–Relative standard deviation

Table 4. Precision data

Concentration, ng/mL	Intra-day precision				Inter-day precision			
	Peak area	%RSD	RT	%RSD	Peak area	%RSD	RT	%RSD
50	22663	0.42	0.96	0	22585	0.48	0.96	0
	22637		0.96		22478		0.96	
	22489		0.96		22368		0.96	
100	40857	0.99	0.96	0	40568	0.55	0.96	0
	40675		0.96		40147		0.96	
	40092		0.96		40488		0.96	
150	61135	0.34	0.96	0	61536	0.59	0.96	0
	61008		0.96		61154		0.96	
	61410		0.96		61878		0.96	

RSD–Relative standard deviation

Table 5. Precision data

S.No.	Label claim	Amount found	% Assay	%RSD
1	5 mg	5.07	101.4	1.51
2		4.93	98.6	
3		5.05	101.4	

RSD–Relative standard deviation

CONCLUSION

An LCMS/MS method was developed and validated for quantifying saxagliptin in bulk ?drug and tablet formulations. It is a sensitive, accurate, precise, and linear quantification method in the concentration range of 10–150 ng/mL. Hence, the developed method is recommended for the regular analysis of saxagliptin in bulk? drug and tablet form. The shorter run time (3 min.) allows the analysis of more samples per day.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study: M.N.; Data Acquisition: M.N.; Data Analysis/Interpretation: S.D.; Drafting Manuscript: M.N.; Critical Revision of Manuscript: S.D.; Final Approval and Accountability: M.N., S.D.

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Original Article

Modified curdlan-based hydrogels containing ornidazole for vaginal delivery

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ABSTRACT

Background and Aims: This study aimed to prepare and characterize, as well as compare the potential of biopolymer-based hydrogels for topical administration of ornidazole, a commonly used drug against vaginal infections. Hydrogels were successfully prepared using curdlan (Crd), carboxymethyl curdlan (CMCrd), hydroxypropyl methyl cellulose (HPMC), and xanthan gum (XG) as biopolymers, which were used alone or blended. In addition, carboxymethylation of Crd, a natural polysaccharide polymer that is attractive in the pharmaceutical field, was carried out in-house.

Methods: The structure of the synthesised CMCrd was analysed by Fourier-transform infrared spectroscopy(FT-IR). The physicochemical, mechanical, and mucoadhesive properties of hydrogels were evaluated, then the drug release patterns from the hydrogels were examined in a simulated vaginal environment.

Results: The hydrogels exhibited a uniform appearance and were pH-compatible with the vaginal environment. The viscosity, spreadability, and drug release characteristics were dependent on the polymer type and total amount of polymer present in the hydrogels. The texture profile analysis results indicated that all formulations exhibited appropriate mechanical characteristics (hardness, compressibility, cohesiveness, and elasticity) for vaginal administration, while also demonstrating mucoadhesive properties and good stability. Carboxymethylation improved mucoadhesion of Crd.

Conclusion: The results obtained indicate that the hydrogels developed in this study can be considered promising candidates for the local treatment of vaginal infections.

Keywords: Hydrogel, Polymer, Curdlan, Ornidazole, Vaginal infection, Texture analysis

INTRODUCTION

Vaginal infections, including those caused by yeast, bacteria, and parasites, are highly prevalent among women of reproductive age worldwide. These infections can lead to vaginitis, and delayed treatment may result in serious clinical consequences, including pelvic inflammatory disease and infertility (Palmeira-de-Oliveira R, Palmeira-de-Oliveira A & Martinez-de-Oliveira, 2015; Ravel, Moreno, & Simón, 2021). Several products formulated as solutions, semisolids, foams, and vaginal tablets are used to treat vaginal infections. However, the successful vaginal delivery of drugs either for systemic or local effects faces challenges due to several factors, including the pH of the vagina, physiological changes in the vaginal epithelium, and the presence of a mucus barrier, which can lead to poor absorption. Furthermore, the natural self-cleaning of the vagina, which acts as a defence mechanism against external pathogens, limits the contact time of locally delivered drugs at the target site. Consequently, local drug administration frequently results in a reduction in the efficacy of the treatment, a decrease in patient compliance, and the necessity for repeated administration (Swingle, Riccardi, Peranteau, & Mitchell, 2023). The design and development of patient-friendly formulations with mucoadhesive properties can overcome the problems related to the delivery of therapeutic agents *via* the vaginal route, thereby increasing treatment efficacy.

Hydrogels are widely preferred for vaginal application in the treatment or prevention of infections due to their ease of application, good spreading ability over a large surface area, and low production costs. Moreover, polymeric hydrogels exhibit mucoadhesive properties, thus allowing a higher drug retention time in vaginal tissue and possibility for controlled drug release

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(Gosecka & Gosecki, 2021). To date, a variety of natural, semi-synthetic, and synthetic biopolymers, including hydroxypropyl methylcellulose and chitosan, have been used to fabricate hydrogels containing antimicrobial drugs for vaginal administration. Biopolymers exhibit biocompatible, biodegradable, and non-toxic properties, rendering them suitable for use in drug delivery, as evidenced by numerous studies (Ahuja, Singh & Kumar,, 2013; Al-barudi, Sinani & Ulker, 2024; Osmałek et al., 2021).

In this study, polymeric hydrogels were developed for the vaginal delivery of ornidazole, an antibiotic currently used in clinical practice for the treatment of infections caused by both anaerobic bacteria and protozoa (Vaghani, Patel & Satish, 2012). Curdlan (Crd), carboxymethyl curdlan (CMCrd), hydroxypropyl methylcellulose (HPMC), and xanthan gum (XG) were used as biopolymers in the preparation of hydrogels. HPMC is a hydrophilic polymer derived from cellulose and has a wide range of applications in drug delivery, including its use in formulations to control release of drugs over extended periods of time. It is extensively studied to form hydrogel-based systems due to its gelling properties in aqueous environments. Moreover, HPMC exhibits good adhesiveness to mucosal surfaces (Caramella, Rossi, Ferrari, Bonferoni & Sandri, 2015). On the other hand, (XG) is a naturally occurring, non-toxic anionic biopolymer. This heteropolysaccharide exhibits viscous properties even at very low concentrations. Furthermore, XG exhibits high stability in acidic and alkaline environments, rendering it safe for use in pharmaceutical formulations for a variety of applications, including the formulation of vaginal hydrogels (Jadav, Pooja, Adams, & Kulhari, 2023). Crd is a biopolymer with a well-known linear (1→3)-β-glucan structure derived from *Agrobacterium* species, which exhibits unique rheological and thermal gelling properties. Several studies have demonstrated the suitability of this biopolymer for use in the food, cosmetic, and pharmaceutical industries. Furthermore, the bioactive properties of Crd, including antimicrobial and anti-inflammatory effects, have been identified (Lin et al., 2021). Nevertheless, its poor solubility in water represents a significant limitation for its utilisation, particularly in the context of drug delivery applications. Consequently, modification approaches have been employed to enhance its water solubility. The introduction of hydrophilic carboxymethyl groups into the Crd structure *via* carboxymethylation results in the formation of a Crd derivative (*i.e.* CMCrd) with good aqueous solubility. Moreover, carboxymethylation can enhance the physicochemical and bioactive properties of native Crd, making it a potentially advantageous polymer for drug delivery applications (Chen & Wang, 2020).

Hydrogels containing ornidazole were prepared using the aforementioned biopolymers in various amounts, either alone or blended. CMCrd was synthesised in-house and subjected to characterisation. The physiochemical, mechanical, and mucoadhesive properties of the hydrogels were evaluated. In addition, the drug release patterns and kinetics from hydrogels were determined. The potential of using the hydrogels of ornidazole as alternative dosage forms was discussed. To the best of our knowledge, this is the first study to evaluate the textural and mucoadhesive properties of Crd-based hydrogels in simulated vaginal fluids and, to explore their potential use in the local treatment of infections.

MATERIAL AND METHODS

Materials

Ornidazole was kindly gifted from Deva İlaç, Türkiye and HPMC (Benecel™ K4M) was gifted from Ashland, Türkiye. Crd was obtained from Kirin Company, Japan. CMCrd was synthesised in-house. XG was purchased from Jungbunzlauer, Austria. Methylparaben and propylparaben were from Doğa İlaç, Türkiye. Porcine mucin type II was obtained from Sigma-Aldrich, Germany. All other chemicals were of analytical grade.

Quantification of ornidazole

The analysis of ornidazole was carried out using high-performance liquid chromatography (HPLC), as previously described in the literature, with some modifications (Baloğlu et al., 2006). The drug quantification was performed using a C18 column with a particle size of 5 µm (VP-ODS C18, 250 mm x 4.6 mm). A mixture of phosphate buffer (pH 4.5):acetonitrile:methanol (55:15:30, v/v/v) was used as the mobile phase. The analysis was conducted at a flow rate of 1.0 mL min^{-1}, wavelength of 318 nm, oven temperature of 40°C, and injection volume of 20 µL. The correlation coefficient (r^2) was found to be 0.999 (n=3). None of the other ingredients in the formulation interfered with the ornidazole peak.

Synthesis and characterisation of carboxymethyl curdlan

The CMCrd polymer was synthesised in-house as previously described (Sessevmez, Sinani, Okyar, Alpar & Cevher, 2023). Briefly, Crd in 2-propanol was stirred at room temperature for 30 min. Sodium hydroxide solution (30% w/v) was added, stirred for a further 90 min, and sodium chloroacetate was added. After stirring at 55°C for 5 h, the product was obtained by filtration. Subsequently, the product was subjected to a series of washings with methanol:acetic acid (7:3, v/v), methanol:water (4:1, v/v), and methanol and acetone. Thereafter, the final product was dissolved in water and subjected to dialysis (12-14 kDa, Visking®, Serva, Germany) against purified water at 4°C for 4 days. CMCrd was freeze-dried and stored in a desiccator at room temperature until further use. The successful synthesis of CMCrd was confirmed by FT-IR (Spectrum 100 FT-IR, Perkin Elmer), and the spectra were recorded over a scan range of 400-4000 cm^{-1} at room temperature.

Preparation of ornidazole-containing hydrogels

Hydrogels were prepared using varying amounts of polymers, either alone or in combination, as detailed in Table 1. Firstly, CMCrd, XG, or HPMC and 150 mg ornidazole were dispersed in 10 mL of purified water. Subsequently, 20% (w/w) propylene glycol and 10% (w/w) glycerine were added under constant stirring. Finally, methylparaben (0.03% w/w) and propylparaben (0.01% w/w) were added to all the formulations. As gelling of Crd requires heating above 55°C, Crd-containing hydrogels were prepared by the same method via heat treatment (Jin, Zhang, Yin & Nishinari, 2006).

Physicochemical characterisation of hydrogels

Visual inspection

The physical appearance of each hydrogel formulation was visually observed at room temperature against a dark background, and homogeneity, transparency, clarity, and colour were assessed.

Viscosity and pH

The viscosity of the formulations was evaluated using a viscometer (Brookfield DV-II+ Pro Viscometer, USA) as specified in the manufacturer's instruction manual (Brookfield, 2015). The pH values of the hydrogels were determined using a calibrated digital pH meter (InfoLab pH 720, Germany).

Spreadability

To evaluate the spreadability of the hydrogels, 0.5 g of each formulation was placed in a pre-marked 1 cm diameter circle on a glass plate. A second glass plate was placed on top of the first, and a 500 g weight was applied for 5 min. At the end of the period, the increase in diameter resulting from the spread of the gels on the plate was determined (Bachhav & Patravale, 2009).

Content uniformity

To quantify the ornidazole content in the formulations, the hydrogels were first extracted with ethanol and then centrifuged at 4000 rpm for 20 min. The supernatant was filtered through a 0.45 µm membrane filter, and the ornidazole content of the sample was quantified by HPLC using a previously described method.

Texture profile analysis

Texture profile analysis (TPA) was performed to investigate the mechanical properties of hydrogels using Texture Analyser (Stable Micro Systems, UK) equipped with a 5-kg weighted load cell (Cevher, Taha, Orlu & Araman, 2008). Hydrogel samples from each formulation (25 g) were collected, and air bubbles were removed using an ultrasonic water bath. A cylindrical analytical probe with a diameter of 10 mm was used to compress each sample twice at a speed of 2 mm s^{-1} and at a specified depth of 15 mm, with a delay period of 15 s allowed between the two compressions. Each sample was analysed in triplicate at 37±0.5 °C. Hardness (the maximum peak force during the first compression cycle, F), compressibility (the work required to deform the gel in the first pass of the probe and calculated the value as Area 1:3), adhesiveness (the work required to overcome the attractive forces generated between the sample and the surface of the probe and calculated the value as Area 3:4), cohesiveness (the ratio of the area under the force–time curve obtained on the second compression cycle to that of the first compression cycle after a defined recovery time and calculated the value as Area 4:6/Area 1:3), and elasticity (the rate at which a deformed sample returns to its original, undeformed shape upon removal of stress and calculated the value as the ratio of the distance between anchors 4:5 divided by the distance between anchors 1:2) were determined using the force-time graphs. A typical force-time graph with the annotated properties of two-cycle texture profile analysis (TPA) is shown in Figure 1.

Mucoadhesion studies

The mucoadhesion of hydrogels was tested using TA-XT*Plus* texture analyser (Stable Micro Systems, UK) as described earlier (Cevher et al., 2008). A filter membrane disc wetted with 10 µL of 8 % (w/v %) mucin dispersion in pH 5.5 phosphate buffer saline (PBS) was attached to the probe and used as an *in vitro* simulated mucosal membrane (de Araújo et al., 2019; Şenyiğit et al., 2014). The probe was lowered onto the surface of each sample (25 mg) at a constant speed of 0.1 mm s^{-1} and contact force of 0.1 N for 120 s. The probe was then moved vertically upward at a constant speed of 0.1 mm s^{-1}. All measurements were performed at 37 ± 0.5 °C. The mucoadhesion work was determined using the equation given below. Each experiment was performed in triplicate. A typical force-time graph with the annotated properties of the mucoadhesion test is shown in Figure 2.

$$Work\ of\ mucoadhesion\left(\frac{mJ}{cm^2}\right) = \frac{AUC}{\pi r^2} \qquad (1)$$

AUC: Area under the curve between anchors 1:2

πr^2: the artificial mucosal surface in contact with hydrogel

Fourier transform infrared (FT-IR) spectroscopy

The FT-IR spectra of pure ornidazole alone and hydrogel samples containing the drug were recorded by scanning at a wavelength range of 400-4000 cm^{-1} at room temperature using a Spectrum 100 FT-IR spectrophotometer (Perkin Elmer).

Table 1. Polymeric composition of hydrogels

Formulation (w/w%)	Crd	CMCrd	HPMC	XG
Crd-1	4.25	-	-	-
Crd-2	4.5	-	-	-
Crd-H1	3.25	-	1	-
Crd-H2	3.5	-	1	-
CMCrd-H1	-	1	1	-
CMCrd-H2	-	1.5	1	-
H1	-	-	2	-
H2	-	-	3	-
H3	-	-	4	-
XG1	-	-		3.5
XG2	-	-		4
XG3	-	-		5

Note: Each hydrogel formulation contains 150 mg ornidazole, propylene glycol (20% w/w), glycerine (10% w/w), methylparaben (0.03% w/w), and propylparaben (0.01% w/w).

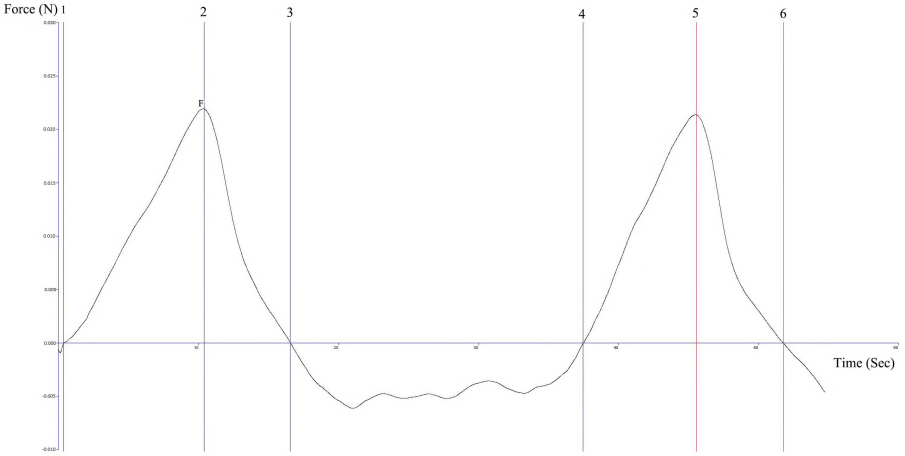

Figure 1. Typical force-time graph with annotated properties for two-cycle texture profile analysis (TPA)

Microstructure analysis

Samples of ornidazole-containing hydrogels were lyophilised using a freeze dryer (Virtis (SP Scientific, USA)), and the surfaces and cross sections of the lyophilised hydrogels and ornidazole were examined using a scanning electron microscope (Phenom ProX, Phenom-World B.V.) at 10 kV and 70 Pa.

In vitro drug release and kinetics

The drug release from hydrogels was determined by dialysis-based testing with some modifications (Enggi et al., 2021). Each formulation (1 g) was placed in dialysis tubes and subjected to drug release studies in 100 mL of pH 4.5 lactic acid buffer at 37°C to simulate vaginal fluid (Singh et al., 2017). At predetermined time intervals, 2 mL of sample was taken and replaced with an equal volume of fresh medium. The withdrawn samples were analysed by HPLC, as previously described. Studies were conducted in triplicate for each formulation.

To investigate drug release kinetics, data obtained from drug release studies were fitted to zero-order, first-order, Higuchi, and Korsmeyer-Peppas models (Sethi et al., 2020). The kinetic model was selected based on the largest r^2 value obtained in each linear regression analysis.

Short-term stability studies of hydrogels

Hydrogels were subjected to a short-term stability study at 25 ± 2°C and 65 ± 5 % relative humidity according to the

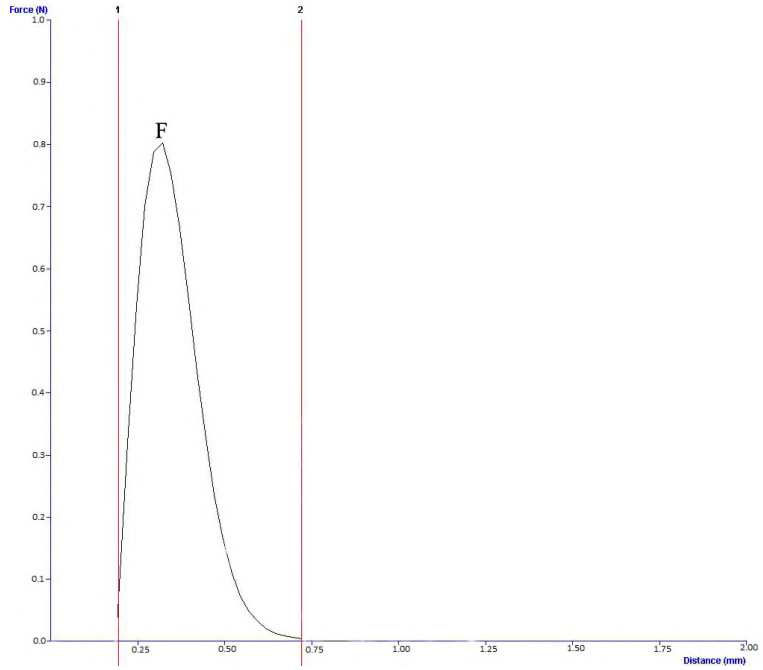

Figure 2. Typical force-time graph with the annotated properties of mucoadhesion test

storage conditions stated in the International Conference on Harmonisation (ICH) guideline (ICH Q1A (R2), 2003) for 1 month. At the end of the study, the physical appearance and drug content of the hydrogels were examined.

RESULTS AND DISCUSSION

Structural characterisation of carboxymethyl curdlan

The structure of CMCrd synthesised in-house was confirmed by FT-IR analysis (Figure 3). The spectra are dominated by a broad band of approx. 3323 cm^{-1} assigned to the stretching vibration modes of the OH groups. This band tended to shift to a higher wavenumber upon chemical modification. The peak at 1627 cm^{-1} observed in the spectrum of Crd is attributed to the presence of water in the structure of Crd (Jin et al., 2006). On the CMCrd spectrum, a new peak at 1583 cm^{-1} resulting from the stretching vibration of the carboxylate $RCOO^-$ group was observed. In particular, the absorption band at 1404 cm^{-1}, corresponding to the symmetric vibration of $RCOO^-$, became more intense after the addition of the carboxymethyl group, indicating that CMCrd was successfully synthesised. The FT-IR spectra are similar to previous reports (Jin et al., 2006; Rafigh et al., 2016). In our previous study, the carboxymethylation of Crd synthesised by the same method was confirmed by ^{13}C NMR in our previous study (Sessevmez et al., 2023).

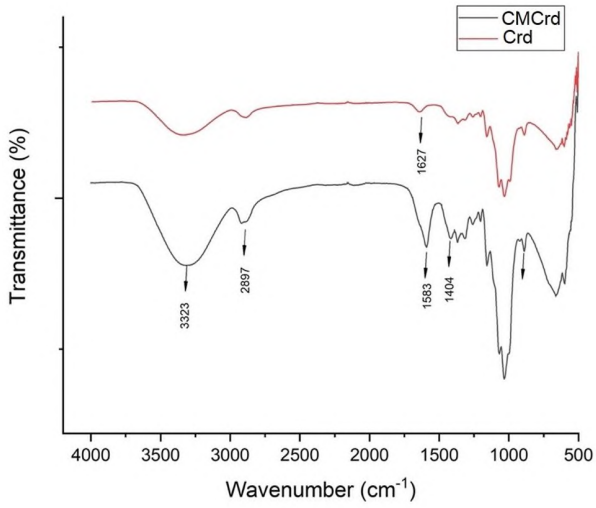

Figure 3. FT-IR spectrum of CMCrd and Crd

Preparation and physicochemical characterisation of hydrogels

Different classes of excipients, including gelling agents, humectants, and preservatives, are used in the composition of hydrogel formulations. Among them, several polymers can have function both as gelling and mucoadhesive agents. They also can prolong the retention time of hydrogels at mucosal sites (Caramella et al., 2015; Cook & Brown, 2018). In this study, hydrogels containing ornidazole as the active ingredient, propylene glycol

and glycerine as humectants, and methylparaben and propylparaben as preservatives were successfully prepared using different biopolymers from natural sources. Hydrogels comprising CMCrd, XG, and HPMC were spontaneously formed in an aqueous solution at defined concentrations given above. The carboxymethylation of Crd resulted in a notable improvement in its water solubility, enabling the successful preparation of hydrogels in purified water at room temperature. In contrast, the preparation of Crd hydrogels involved heat treatment to achieve gelation. It is acknowledged in the literature that the introduction of carboxymethyl groups into the structure of various polysaccharides can alter their physicochemical properties and consequently enhance their water solubility (Jin et al., 2006). In our previous study, we synthesised a carboxymethyl derivative of pullulan, a natural non-derivatised biodegradable polysaccharide, with the objective of enhancing its solubility in water at room temperature. It was utilised in the formulation of hybrid nanoparticles with chitosan derivatives as vaccine carriers (Sessevmez et al., 2023). Also, the carboxymethylation of the cationic polysaccharide chitosan resulted in an improvement of the polymer's water solubility and enhancement of its bioactivity. Consequently, carboxymethyl chitosan has emerged as a versatile polymer for the delivery of antimicrobial drugs (Zhang et al., 2023) and gene therapy (Sinani, Durgun, Cevher & Özsoy, 2023). In another study, carboxymethyl laminarin demonstrated enhanced *in vitro* bioactivity compared with its unmodified form, sulphated or aminated derivatives (Malyarenko, Usoltseva, Rasin & Ermakova, 2023). Thus, that the utilisation of CMCrd could be more advantageous than that of its native polymer for several drug delivery applications.

The physicochemical parameters of the prepared hydrogels, including their physical appearance, pH, viscosity, spreadability, and drug content, are presented in Table 2. The hydrogels were observed to be colourless and free of air bubbles. The XG formulations exhibited an opaque appearance, in contrast to the transparent nature of the other hydrogels. The high drug content in each formulation indicated that ornidazole was homogeneously dispersed within the hydrogels. Healthy vaginal pH is moderately acidic, with a typical range between 3.8 and 4.5, and infection can cause an imbalance in vaginal pH by increasing the pH levels (Machado, Palmeira-de-Oliveira A, Martinez-de-Oliveira & Palmeira-de-Oliveira R, 2017). Ideally, the pH of the formulation should be maintained at a level that ensures the stability of the drug and should be as close as possible to the pH of the application area to avoid irritation. The pH values of all hydrogels ranged from 4.0 to 5.5, which is consistent with the pH values of other semi-solid commercial vaginal products already in clinical use (Machado et al., 2017). While the pH range of hydrogels demonstrates satisfactory pH compatibility with vaginal environments, ornidazole remains stable in acidic environments (pH < 6) (Pyka-Pająk, 2023).

Viscosity is another factor that can influence the clinical performance of hydrogels due to its effect on drug release as well as applicability of hydrogels and contact time with the vaginal epithelium. Although a low viscosity improves spreadability, a reduction in residence time at the application site which can decrease treatment outcomes can be observed. On the other hand, vaginal hydrogels should maintain their viscosity when subjected to increased shear rates during application or when diluted with vaginal fluids (Machado et al., 2017). Thus, the optimal viscosity of vaginal hydrogels is difficult to define. The hydrogels prepared in this study exhibited a viscosity range of 6200–350000 cP. As expected, the hydrogel viscosity was influenced by the type and total amount of polymers in the formulation and increased in a concentration-dependent manner, in agreement with previous studies on many polymers (Cook & Brown, 2018; Mikušová, Ferková, Žigrayová, Krchňák & Mikuš, 2022). The blending of Crd and HPMC in hydrogels prepared with a total amount of solid polymers of 4.25-4.5% resulted in an increase in hydrogel viscosity compared to hydrogels prepared with Crd alone at the same solid polymer content. Nevertheless, composite CMCrd-HPMC hydrogels demonstrated higher viscosity than hydrogels containing Crd and HPMC alone at all the concentrations studied.

Spreadability is another parameter that indicates the ability of hydrogels to spread over a surface upon application and demonstrates how easily the topical formulation can be spread to administer a standard dose. As shown in Table 2, the spreadability values varied between 4.5 and 6.5 cm. However, the results revealed that the spreading ability and viscosity of hydrogels are correlated. Increasing the polymer concentration led to a decrease in spreadability, as expressed by the smaller diameter of the spreading circle, which can be further explained by the increased hydrogel viscosity, i.e., increased viscosity leads to a lower spreading ability. Similar results have been reported in other studies (Arpa et al., 2020; Cook & Brown, 2018).

Texture characterisation

Texture profile analysis enables evaluation of the texture properties of semi-solid products and prediction of their behaviour upon application. It is essential that vaginal gel formulations exhibit the requisite hardness, compressibility, adhesiveness, cohesiveness, and elasticity properties to ensure that they provide maximum benefits to the patient. The mechanical properties of the hydrogels were evaluated using texture profile analysis and were calculated from the resultant force-time curve (Table 3) (Cevher et al., 2008).

Hardness is measured as the maximum peak of the first compression cycle and is expressed as the maximum compressive force. The hardness of hydrogels must be evaluated to assess the necessary force required for the deformation of hydrogels. Low hardness values are preferred for ease of application to the desired site (Cevher et al., 2008). The hardness values of

Table 2. Physical observation, pH, viscosity, spreadability, and drug content of hydrogels

Formulation	Physical evaluation		pH (±SD)	Viscosity (cP) (±SD)	Spreadability (cm±SD)	Drug content (%±SD)
	Appearance	Clarity				
Crd-1	Transparent/Clear		4.00±0.08	8500±170	4.7±0.1	98.5±1.4
Crd-2	Transparent/Clear		4.13±0.05	12000±235	4.2±0.3	98.3±1.6
Crd-H1	Transparent/Clear		4.12±0.02	35270±423	3.7±0.2	98.8±2.9
Crd-H2	Transparent/Clear		4.18±0.03	39140±282	4.0±0.3	99.0±1.3
CMCrd-H1	Transparent/Clear		5.33±0.05	64803±362	5.2±0.9	98.8±1.9
CMCrd-H2	Transparent/Clear		5.50±0.08	66406±292	4.7±0.3	100.0±0.1
H1	Transparent/Clear		4.14±0.00	6200±38	5.0±0.5	99.2±1.8
H2	Transparent/Clear		4.13±0.00	14000±120	4.4±0.3	99.8±3.6
H3	Transparent/Clear		4.11±0.00	51650±145	3.9±0.1	99.7±0.7
XG1	Opaque/Clear		4.57±0.06	9133±75	4.9±0.7	100.8±1.6
XG2	Opaque/Clear		4.69±0.18	190000±365	4.8±0.5	98.7±1.6
XG3	Opaque/Clear		4.77±0.07	350000±315	4.1±0.3	99.3±0.2

Data presented as average ± SD (n = 3).

Table 3. Mechanical properties of ornidazole-containing hydrogels (n=3)

Formulation	Hardness (N ± SD)	Compressibility (mJ ± SD)	Adhesiveness (mJ ± SD)	Cohesiveness (± SD)	Elasticity (± SD)
Crd-1	0.024±0.002	0.154±0.136	0.035±0.013	0.677±0.007	1.041±0.092
Crd-2	0.043±0.021	0.065±0.004	0.056±0.022	0.708±0.027	1.032±0.088
Crd-H1	0.025±0.003	0.059±0.001	0.008±0.007	0.762±0.057	1.057±0.007
Crd-H2	0.038±0.022	0.059±0.002	0.011±0.004	0.880±0.029	0.998±0.000
CMCrd-H1	0.030±0.000	0.069±0.001	0.011±0.002	0.848±0.045	1.019±0.027
CMCrd-H2	0.030±0.000	0.069±0.001	0.017±0.007	0.859±0.033	1.039±0.038
H1	0.024±0.003	0.070±0.002	0.023±0.004	0.952±0.044	0.966±0.006
H2	0.024±0.000	0.058±0.003	0.029±0.010	1.019±0.075	1.000±0.000
H3	0.050±0.035	0.061±0.000	0.070±0.004	1.025±0.036	1.030±0.021
XG1	0.067±0.005	0.138±0.006	0.062±0.013	0.768±0.013	0.981±0.010
XG2	0.078±0.012	0.151±0.004	0.069±0.006	0.780±0.029	0.968±0.027
XG3	0.091±0.015	0.163±0.008	0.127±0.012	0.780±0.004	0.947±0.035

the prepared hydrogels exhibited a range from 0.024 to 0.091 N, contingent on the polymer type and its concentration. The H2 formulation exhibited the lowest hardness value, while the XG3 formulation had the highest hardness. The XG-based hydrogels also resulted in higher hardness values than Crd-based hydrogels. It was observed that for all hydrogels, an increase in the polymer concentration was accompanied by an increase in hardness, as expected.

A similar trend was observed when the compressibility of hydrogels was investigated. All hydrogels exhibited low compressibility values in the range of 0.058–0.163 mJ (mJoule, N.mm), and the highest values were measured for the XG hydrogels, ranging from 0.138 to 0.163 mJ. As compressibility indicates the necessary work for deformation of hydrogels and influences the removal of the formulation from the container and spreading at the application side, low compressibility values are favourable (Arpa et al., 2020). Overall, the low compress-

ibility values of all hydrogels indicate that they are convenient for vaginal application.

Furthermore, a product intended for mucosal application should possess good adhesive properties, as this can enhance the localisation of the drug at the application site, thereby improving clinical efficacy (Caramella et al., 2015). In texture profile analysis, adhesiveness is commonly expressed as the work required to overcome attraction forces between the sample and probe surface (Cevher et al., 2008). In all formulations, adhesiveness increased with increasing polymer content, with values ranging from 0.008 to 0.127 mJ. The composite CMCrd-HPMC hydrogels exhibited lower adhesiveness compared to Crd or HPMC hydrogels alone. This may be attributed to the lower total amount of polymers present in the formulation: 2% for CMCrd-H1 and 2.5% for CMCrd-H2, in contrast to 4.25% for Crd1, 4.5% for Crd2, 3% for H2, and 4% for H3, all expressed as weight-to-weight percentages. In accordance with previous investigations on the adhesiveness of different polysaccharide-based hydrogels, it can be concluded that a direct correlation exists between the total polymer quantity and the adhesiveness of the hydrogels (Mikušová et al., 2022).

While adhesion describes the ability of a material to bind to a surface, cohesion indicates the strength with which the hydrogel coheres under external shear. The degree of cohesion in a hydrogel depends on the strength of the intermolecular attractive forces within the hydrogel network. The lowest values for cohesiveness were observed in Crd-based hydrogels. HPMC hydrogels exhibited higher cohesion, which was found to depend slightly on the polymer concentration in a linear fashion. Similarly, previous studies have indicated that hydrogel cohesiveness tends to increase at high HPMC concentrations (Karavana, Güneri & Ertan, 2009). The blending of Crd with HPMC resulted in enhanced cohesiveness compared to hydrogels containing Crd alone. Additionally, the HPMC hydrogels demonstrated superior cohesiveness compared to Crd or XG hydrogels. The cohesiveness of Crd-HPMC and CMCrd-HPMC blend hydrogels was found to be comparable.

In addition to the aforementioned characteristics, an ideal vaginal hydrogel should exhibit appropriate elasticity to respond to physiological and mechanical effects upon application. Elasticity refers to the rate at which a deformed sample returns to its former state after the applied force is removed (Rençber et al., 2017). The hydrogel elasticity ranged from 0.947 to 1.057. All elasticity values were found to be comparable among the formulations, with no significant differences observed in the elastic properties of the hydrogels due to varying polymer content. Comparable elasticity values were observed for clotrimazole-containing hydrogels intended for vaginal administration (Rençber et al., 2017).

It is noteworthy that the mechanical characteristics of hydrogels may be influenced by the properties of the polymer, including its molecular weight, structure, and concentration. The results of texture profile analysis conducted in this study for the developed hydrogels indicate that none of the formulations exhibited excessive hardness or compressibility that would hinder their application. On a more comparative basis with widely used HPMC and XG hydrogels, the mechanical properties of the novel Crd and CMCrd hydrogels support their use as vaginal products.

Mucoadhesion studies

Mucoadhesion studies are widely conducted to investigate the ability of hydrogels to adhere to mucosal surfaces. Hydrogels with mucoadhesive properties are advantageous for vaginal drug delivery. They can enhance therapeutic outcomes by increasing the residence time of the drug in the vaginal mucosa and by reducing hydrogel leakage. Thus, they allow sufficient drug release from the formulation at the vaginal mucosa, thereby enhancing the local action of the drug. The mucoadhesiveness of hydrogels can be affected by several factors related to the polymer characteristics, including the type, structure, molecular weight, and concentration of the polymer within the hydrogel (Palmeira-de-Oliveira et al., 2015). Furthermore, it is essential that the same mucoadhesion testing method and experimental conditions are employed to perform a comparative evaluation of the results (Bayer, 2022). The mucoadhesion data of the hydrogels prepared in this study were obtained using Texture Analyser and is presented in Figure 4. As anticipated, alterations in the polymer content of hydrogels resulted in changes in mucoadhesion.

HPMC is commonly used in vaginal hydrogels because of its mucoadhesive properties (Cook & Brown, 2018). The mucoadhesion of the hydrogels increased with increasing concentration of HPMC. However, the incorporation of HPMC with Crd did not enhance the mucoadhesive properties of the Crd hydrogels. The work of mucoadhesion was found to be lower for Crd-HPMC blend hydrogels than for Crd hydrogels alone. Furthermore, the mucoadhesive properties of Crd hydrogels were found to be superior to those of HPMC hydrogels. Crd polymer exhibits better mucoadhesive properties in a simulated vaginal environment than HPMC. To the best of our knowledge, no previous reports have described the mucoadhesion properties of Crd for vaginal application. In previous studies, Crd derivatives were combined with chitosan to form nanocarriers for the nasal administration of antigens. One of these studies (Zhang et al., 2018) reported that curdlan sulfate–O–linked quaternised chitosan nanoparticles, prepared via polyelectrolyte complexation, probably had mucoadhesive capacity and could be used as a mucoadhesive agent. Also, the results from our earlier studies indicated that chitosan-CMCrd composite nanoparticles exhibit optimal properties for nasal immunisation of protein antigens, suggesting that these nanocarriers have mucoadhesive properties (Sessevmez et al., 2023). A comparison of the mucoadhesion between the CMCrd-HPMC and Crd-HPMC

blend hydrogels revealed almost a 2-fold increase in the work of mucoadhesion for the former, indicating a positive impact of carboxymethylation on the mucoadhesive properties of the hydrogels. In line with these results, it was also reported that the use of carboxymethylated guar gum enhanced the mucoadhesive properties of native guar gum (Giri & Singh, 2020), whereas in another study, carboxymethylation increased the mucoadhesive strength of gellan gum (Ahuja et al., 2013).

The work of mucoadhesion was found to be inversely proportional to the polymer concentration in XG hydrogels. A similar trend was previously observed by our group. It was observed that mucoadhesion decreased with increasing XG content in the formulation (Cevher et al., 2014). A similar fashion was observed by other researchers who evaluated vaginal gel for treating mixed vaginal infections. They showed that a reduction in the XG content of vaginal gels, either alone or in combination with other polymers (*e.g.* HPMC or sodium alginate), enhanced bioadhesive strength (Ahmad, Alam, Khan, Khar & Ali, 2008). The inherit mucoadhesive properties of XG have been already acknowledged in the literature (Jadav et al., 2023). In this study, the XG hydrogels demonstrated superior mucoadhesiveness to HPMC hydrogels. It is noteworthy that the mucoadhesion results were comparable to those obtained for the Crd-based hydrogels.

The results obtained from the mucoadhesion tests in this study provide a comparative evaluation of the mucoadhesive properties of Crd-based hydrogel formulations and hydrogels prepared with widely used polymers, namely HPMC and XG. Furthermore, these findings contribute to a deeper understanding of the mucoadhesive characteristics of Crd-based pharmaceutical formulations, particularly those intended for vaginal administration.

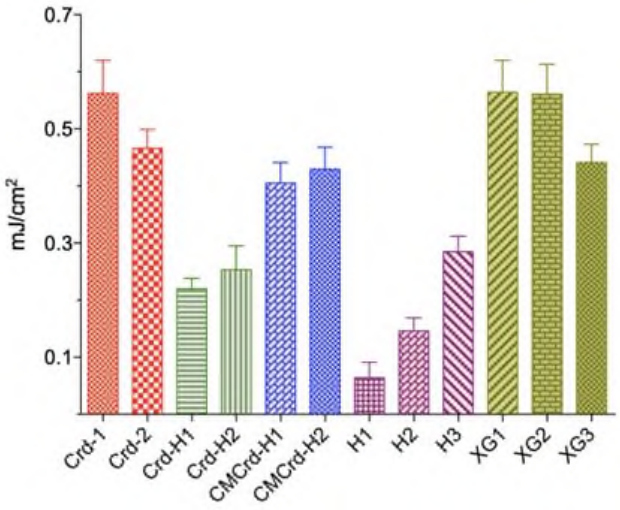

Figure 4. Work of mucoadhesion of ornidazole-containing hydrogels (n=3)

Fourier transform infrared (FT-IR) spectroscopy analysis of ornidazole-containing hydrogels

The FT-IR studies were conducted to assess potential interactions between the drug and other components in the hydrogels. As shown in Figure 5, the FT-IR spectrum of ornidazole reveals bands due to O–H stretch at 3109 cm^{-1}, C–H stretch at 3086 cm-1, asymmetric NO2 stretch at 1539 cm^{-1}, symmetrical NO2 stretch at 1363 cm^{-1} and 1273 cm^{-1}, C–H-dependent stretching at 1190 cm^{-1} to and stretching at 831 cm^{-1} is attributed to C–N and NO2. All of these peaks were identified in the FT-IR spectrum of ornidazole-containing hydrogels, confirming the presence of the drug in the formulation without any significant interaction with other components (Vaghani et al., 2012).

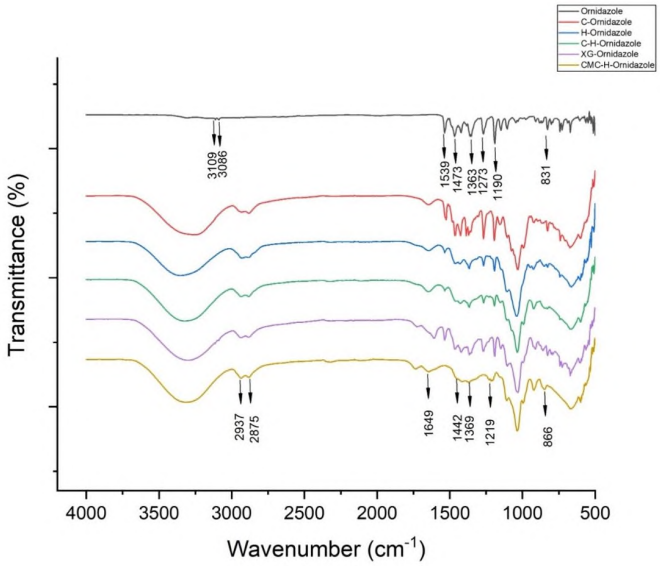

Figure 5. FT-IR spectra of ornidazole-containing hydrogels

Microstructure analysis

SEM is a widely used technique to observe surface morphology and characterise the hydrogel structure at the micrometer scale (Antonietti, Caruso, Göltner & Weissenberger, 1999). SEM images of ornidazole and hydrogels are presented in Figure 6.The surfaces of all hydrogels appeared heterogeneous. HPMC hydrogels exhibited a random circular or ellipsoidal pore shape, whereas Crd-HPMC hydrogels displayed smaller and denser pores. Zhang et al. (Zhang, Nishinari, Williams, Foster & Norton, 2002) observed that gels containing HPMC and Crd exhibited greater porosity and smaller pore sizes than gels containing only HPMC. Furthermore, increasing Crd amount in the formulations resulted in an increase in porosity and a reduction in pore size in the hydrogels. Blending Crd with HPMC may increase the viscosity and gel strength, which could lead to the formation of more compact structures. In contrast, the Crd hydrogel exhibited a dense, heterogeneous, and slightly rough

surface. The observed compact network structure can be attributed to hydrogen bonds (Tao et al., 2021). It is pertinent to highlight that the XG hydrogels exhibit greater porosity than the other hydrogels. The SEM images revealed distinct differences in surface morphology between the hydrogels and ornidazole.

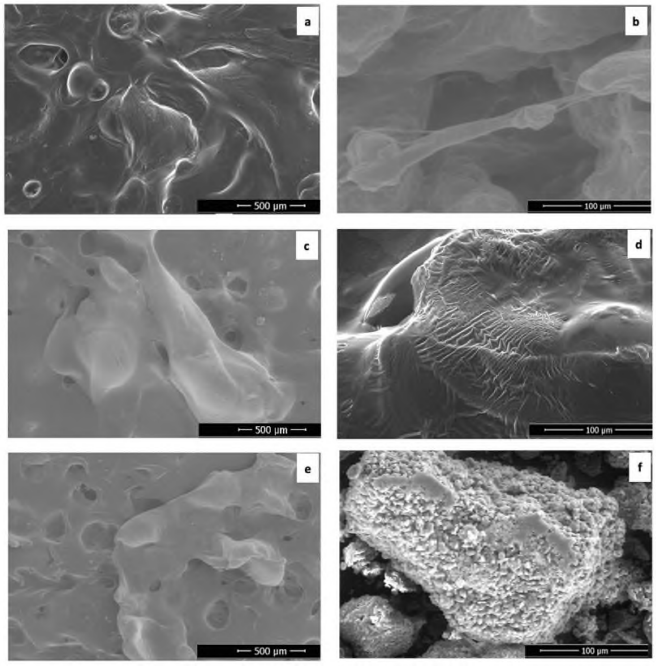

Figure 6. SEM images of ornidazole-containing hydrogels (a) HPMC, (b) Crd, (c) Crd-HPMC, (d) CMCrd-HPMC, (e) XG, and (f) pure ornidazole

In vitro drug release and release kinetics

A drug release profile characterised by controlled release can provide a favourable approach to improve the success of therapy with antimicrobial drugs (Osmałek et al., 2021). Overall, the drug-release pattern of hydrogels depends on the type and amount of polymer present in the hydrogel (Figure 7). For all hydrogels, fast drug release was observed during the first hour. Crd-based hydrogels released most of the ornidazole content within 2 h. The blending of HPMC and Crd (C-H1 and C-H2) resulted in a slight decrease in drug release, indicating that it is possible to alter drug release by modifying the hydrogel composition. In a previous study, it was reported that the release of tetracycline hydrochloride from Crd-phosphorylated Crd hydrogels crosslinked with 1,4-butanediol diglycidyl ether was also influenced by the composition of the hydrogels, and the equilibrium of drug release was achieved after 3.5 h in PBS pH 6.8 (Suflet, Popescu, Prisacaru & Pelin, 2021). CMCrd-based hydrogels exhibited faster drug release due to the increased solubility of CMCrd in the aqueous environment, which results in greater swelling and consequently loosening of the polymer network, allowing faster drug release. Among the various hydrogels studied, the XG- and HPMC-based hydrogels exhibited a more sustained drug release profile, with approximately 75-85% of ornidazole content released within 6 h.

The drug release data of ornidazole-containing hydrogels were fitted using various release kinetic models, including zero-order, first-order, Higuchi and Korsmeyer-Peppas models (Table 4). Application of the Korsmeyer-Peppas kinetic model also enables identification of the release mechanism by calculating the n exponent (Baloğlu et al., 2006; Sethi et al., 2020). Kinetic modelling of drug release from hydrogels showed that the drug release data best fit to the first-order kinetic model. The first-order kinetics describes drug release from the system as proportional to the amount of drug remaining in the system over time. Thus, according to this model, drug release decreases with decreasing concentration gradient (Barradas, Senna, Cardoso, de Holanda e Silva & Elias Mansur, 2018). A recent paper reviewing the modelling of drug release from hydrogel-based systems outlined that first-order equations are widely employed to describe drug release from hydrogels (Caccavo, 2019). Hydrogels composed of natural polymers have been reported to show drug release according to first-order model (Khan & Ranjha, 2014; Khanum, Ullah, Murtaza & Khan, 2018).

Short-term stability study

Stability studies were performed to evaluate the ability of hydrogels to remain stable at 25±2 °C and 65±5% relative humidity conditions, as specified in the relevant ICH guidelines (ICH Q1A (R2), 2003). At the end of the study, the physical appearance and percentage of drug content of the hydrogel formulations were evaluated. As illustrated in Figure 8, no alterations were observed in the percentage drug content of hydrogels at the end of the stability study. Furthermore, the physical appearance of the hydrogels after one month was comparable to that observed at the beginning of the study. It can be concluded that the polymeric hydrogels developed in this study exhibit good stability when stored at room temperature.

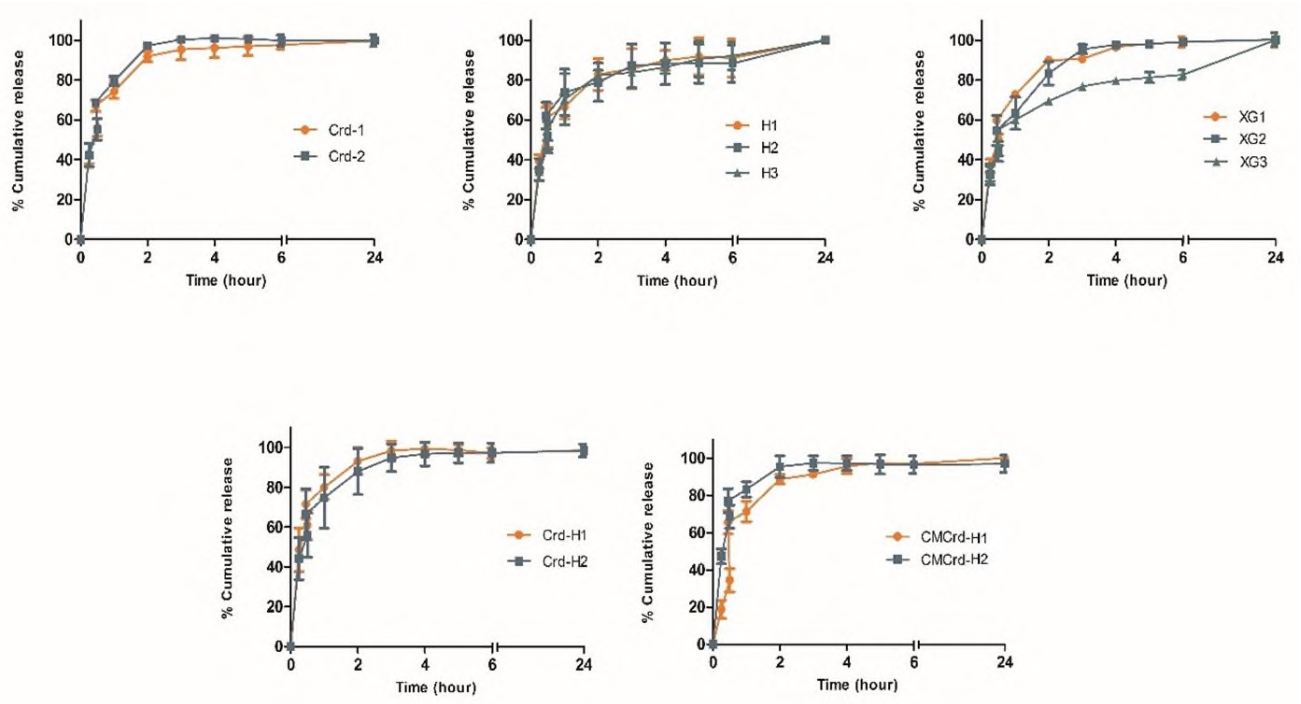

Figure 7. Drug release profile of ornidazole from hydrogels (mean ± SD, n = 3)

Table 4. Kinetic models of ornidazole release from hydrogels

Formulation	Zero order	First order	Higuchi	Hixson–Crowell	Korsmeyer-Peppas	
	r^2	r^2	r^2	r^2	r^2	n
Crd-1	0.824	0.970	0.897	0.937	0.723	0.508
Crd-2	0.806	0.979	0.884	0.981	0.672	0.536
Crd-H1	0.784	0.992	0.878	0.958	0.715	0.439
Crd-H2	0.739	0.924	0.858	0.875	0.705	0.462
CMCrd-H1	0.806	0.918	0.898	0.885	0.798	0.356
CMCrd-H2	0.811	0.964	0.900	0.940	0.799	0.710
H1	0.746	0.928	0.866	0.902	0.839	0.422
H2	0.702	0.945	0.827	0.906	0.789	0.696
H3	0.746	0.918	0.862	0.866	0.796	0.556
XG1	0.754	0.984	0.870	0.936	0.761	0.569
XG2	0.797	0.978	0.904	0.933	0.818	0.457
XG3	0.783	0.910	0.885	0.878	0.773	0.407

r^2: correlation coefficient, n: diffusional exponent

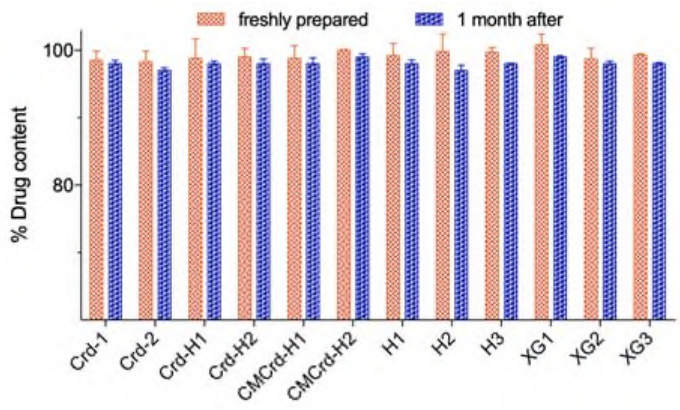

Figure 8. Drug content in hydrogels initially and after one month

CONCLUSION

In this study, hydrogels of ornidazole were prepared using Crd, CMCrd, and their combinations with HPMC, as well as XG alone. The physicochemical characterisation studies, mechanical and mucoadhesive properties and drug release characteristics were determined. The preparation process was straightforward, and the results demonstrated that all hydrogels exhibited favourable properties (*i.e.* physicochemical and textural properties) for use as vaginal formulations for the treatment of infections. In particular, the mucoadhesive properties of the Crd-hydrogels and CMCrd-HPMC hydrogels were comparable to those of the XG hydrogels and superior to those of the HPMC hydrogels in simulated vaginal environment. Consequently, these vaginal hydrogels can be considered promising mucoadhesive systems capable of prolonging the retention time of drug at the application site. They can be alternative formulations for the local treatment of vaginal infections. It is important to mention that the use of vaginal delivery systems that can provide therapeutically relevant levels of antimicrobial drugs in the vaginal tissue can overcome issues related to the use of high-dose drugs required for oral therapy. Moreover, reduced administration frequency and increased patient compliance could be potential positive outcomes. Nevertheless, further studies are required to evaluate the biological activity and safety of these biopolymer-based hydrogels.

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Original Article

Traditional and complementary medicine use and associated factors in COVID-19 pandemic

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ABSTRACT

Background and Aims: The aim of this study was to determine the attitude and behaviour of patients who applied to a family health centre during the COVID-19 pandemic regarding traditional and complementary medicine (TCM) applications and the factors related to their usage.

Methods: A questionnaire consisting of sociodemographic information and frequency, behaviours regarding the use of TCM during the pandemic was administered to individuals over the age of 18 who were admitted to family health centres, located in two socioeconomically different districts of Istanbul. The data were analysed in the SPSS 22, and the p-value was set as 0.05.

Results: A total of 352 participants were recruited. The use of TCM during the pandemic period was 36.3% in Üsküdar and 26.8 % in Sancaktepe, and traditional herbal product use was high in both districts at 87% and 90.9%, respectively. The use of non-prescription vitamins and supplements were about 1.7 times greater in Üsküdar. In total, 73.7% of the participants stated that they used at least three products, and 57% of them used 5 products. The reasons for using TCM were similar in both districts; however, chronic disease and old age were reported only in the Üsküdar district. A total of 25% of the total participants stopped their current medications when they started the use of TCM, and 51.7% did not receive information about the side effects of TCM. The use of TCM in those with university education was higher than that in the other groups.

Conclusion: TCM use was high during the COVID-19 pandemic, especially with herbal products and supplements.

Keywords: COVID-19, Herbal product, Pandemic, Traditional and complementary medicine

INTRODUCTION

In the last 20-30 years, there has been a dramatic increase in traditional and complementary medicine (TCM) practises in our country as well as all over the world for the prevention or treatment of diseases (Pokladnikova & Telec, 2020; de Moraes Mello Boccolini & Siqueira Boccolini, 2020). However, the regulation of TCM varies from country to country, and no common approach has been adopted yet. The use of herbal products is among all TCMs. Depending on current data, general conclusions regarding the therapeutic value of herbal preparations are difficult to reach. In addition, herbal products may cause serious liver and kidney damage, and the possible interactions with the drugs may adversely affect already given treatments (Valdivia-Correa, Gómez-Gutiérrez, Uribe & Méndez-Sánchez, 2016; Erdem & Eren, 2009).

In recent years, legal regulations have been formulated on TCM in Europe, and some rules have been determined to inform consumers. In Turkey, herbal products are classified as food supplements or traditional herbal medicinal products, similar to European Union regulations (Resmi Gazete, 2010). Although medicinal plants and phytotherapy are frequently used by healthcare professionals and patients in our country, this practise has not been fully institutionalised or systematised.

In addition to herbal products, many applications such as homoeopathy, meditation, massage, reiki, and chiropractic, are also used in other countries (Sousa, Hortale & Bodstein, 2018). Leech, cupping, and prayer are also common in Turkey be-

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cause of its cultural aspects (Şimşek et al, 2017). Traditional medicine, which was widely used during the epidemic in the past, has come to the forefront of the treatment of COVID-19 because currently, there is no proven antiviral treatment. Although data on the potential effects of dietary therapy and herbal medicines against SARS-CoV-2 have been presented in the current literature, there is not enough supporting evidence (Ang et al, 2020; Panyod, Ho, Sheen, 2020; Zhang et al, 2020). There is a lot of non-scientific information on the use of complementary and traditional medicine applications, especially herbal nutritional supplements, for the purpose of protection from COVID-19 infection and treatment of COVID-19 infection, in internet resources and social media. In the research of the Ministry of Industry and Technology on the socioeconomic development ranking of the districts in Istanbul, Üsküdar was shown as the first-level district and Sancaktepe district as the second (SEGE, 2017).

The aim of this study was to investigate traditional and complementary medicine use and related factors during the COVID pandemic in two districts of Istanbul with different socioeconomic levels by administering a questionnaire to patients who applied to the family health centre.

MATERIALS AND METHODS

Study Design and Participants

In this cross-sectional descriptive study, family health centres located in two socioeconomically different districts of Istanbul (Üsküdar and Sancaktepe) were included. Districts were selected according to socioeconomic levels and accessibility by the researchers. The Socio-Economic Development Ranking of Districts Research 2022 report, conducted by the Ministry of Industry and Technology, was used to determine the socioeconomic development ranking. According to this report, Üsküdar district was in the 1st stage and was ranked 16th for socioeconomic development, with a development score of 3,045. Hoverer, Sancaktepe district was in the second stage and ranked 95th, with a development score of 1,275. For the development score, demographic variables of the districts, employment and social security variables, education, health, finance, competitiveness, innovation, and quality of life variables were used. In addition, according to the development ranking for 39 districts of Istanbul, Üsküdar is 11th and Sancaktepe is 34th (SEGE,2022).

After obtaining permission from the Ministry of bnHealth for the study, ethical approval was obtained from the Zeynep Kamil Women's and Children's Diseases Education Hospital Clinical Trials Ethics Committee (Approval no: 2021/34).

The research was conducted on individuals over the age of 18 who applied to both family health centres between 14 September and 16 October 2020.

Survey Items

The survey consisted of three parts and was structured by the researchers using relevant literature (Konakci, Uran & Erkin,2020; Cuellar, Aycock, Cahill & Ford, 2003; Mbizo et al.2018).

In the first part, independent variables (age, gender, marital status, educational status, income level, chronic disease history and self-assessment of their health status) were included to determine the sociodemographic and socioeconomic characteristics of the participants.

The second part of the survey included questions regarding TCM use attitudes and behaviours during the epidemic period. TCM usage, reasons for using TCM, consultancy for TCM, their behaviour regarding the drugs they are currently using when they started using TCM, their information about the side effects of the products they use for TCM, the source of the TCM products they use, places where they apply, and if they had any benefit or not were asked.

A table consisting of two parts was added in the last section of the survey. In the first part of the table, the most commonly used traditional herbs included in the list of medicinal herbs published by the Pharmaceuticals and Medical Devices Agency of Türkiye (TITCK) were included. In the second part, other TCM approaches and their usage (acupuncture, yoga, prayer, music therapy, relaxation techniques, daydreaming, massage, reflexology, spa, homoepathy, reiki, leech therapy, cupping, cupping, bioenergy, hypnotherapy, mesotherapy, ozone therapy) were included. Data were collected by two researchers who took the necessary precautions with personal protective equipment. A questionnaire prepared by the researchers after the literature review was applied to individuals over the age of 18 who were admitted to both family health centres (FHC). The use of traditional and complementary medicine in the pandemic period in two FHC populations and related factors were evaluated. The final questionnaire revision was made after pilot implementation. The participants were informed, and those who agreed to participate were given gloves in disposable packages and asked to fill in the questionnaire under observation. All necessary precautions were taken by the two researchers using personal protective equipment.

Statistical Analysis

The statistical analysis was conducted using SPSS V.22 software with descriptive and inferential methods. The descriptive methods included frequency distribution tables and graphs, and the inferential methods included χ^2 tests for associations between the sociodemographic and other variables. $P<0.05$ statistical significance was set for all analyses.

RESULTS

A total of 352 participants (270 from Üsküdar and 82 from Sancaktepe) were included in the study. Participants were between the ages of 30 and 49 years (42.3%), and the majority were women (67.6%). 45.7% of the participants were high school graduates, and 12.5% were secondary school. Many respondents described their health status as good (60.2%), however 30.1% of them had a chronic disease (Table 1).

The use of TCM during the pandemic period was 34.1% (n=120), comprising 36.3% in Üsküdar and 26.8 % in Sancaktepe. Traditional herbal products were mostly used in TCM in both Üsküdar and Sancaktepe districts (87% and 90.9% respectively). Participants using herbal products were asked to indicate what products they used. The use of traditional and non-traditional herbal products, juices, and herbal teas was similar between the two districts. However, non-prescription vitamins and supplements use differed in Üsküdar and Sancaktepe, 54% and 31.8%, respectively, and it was about 1.7 times higher in Üsküdar (Figure 1).

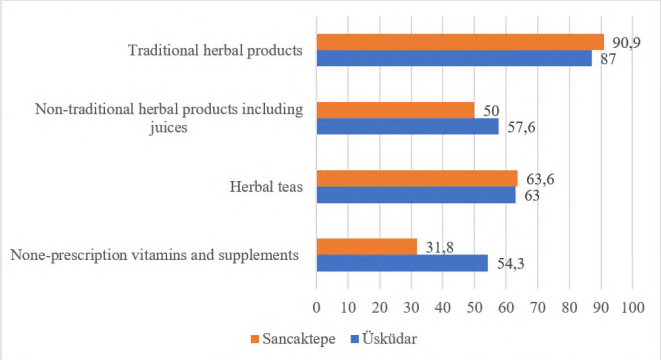

Figure 1. Distribution of herbal products and other dietary intake as therapeutic or preventive

In total, 73.7% of the participants stated that they used at least three products, and 57% of them 5 products. TCM methods, other than herbal products and dietary practises were similar in the Sancaktepe and Üsküdar districts at 65.2% and 54.5%, respectively. In all these methods, mostly the prayer method (41.2 %) was applied and hypnotherapy, reiki and homoeopathy were the least, with 0.9% of each (Figure 2).

Herbal product use was 87.0% in Üsküdar and 90.9% in Sancaktepe. It was the most widely used method in both districts. (Figure II). The distribution of herbal products used by the districts is shown in Table 2. The most commonly used herbal products were herbal teas (63.2%), mint (61.4%), and garlic (47.4%), respectively (Table 2).

Among the 5 most applied methods, the biggest difference was seen in the relaxation method between the districts, which was higher in Üsküdar than in Sancaktepe. Hovewer prayer and dreaming were more common in Sancaktepe (Figure 3).

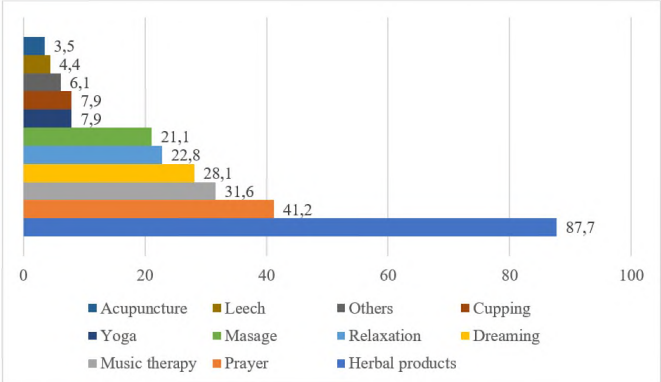

Figure 2. Distribution of TCM methods other than herbal therapy and dietary practises (Others: Reflexology, homoepathy, cupping, mesotherapy, ozone, hypnotherapy etc.)

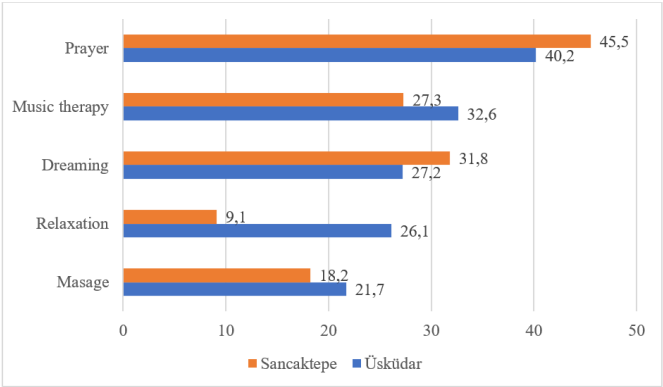

Figure 3. Distribution of the 5 most applied TCM methods by district, excluding herbal treatment and other dietary applications

The reasons for the use of TCM were similar in both districts instead of the presence of chronic disease and old age, which are reported only in the Üsküdar district (13.3 %). The most frequently reported reason was to strengthen the immune system in both Üsküdar and Sancaktepe (80.6 % and 63.6 % respectively) (Table 3).

When the behaviours related to the use of TCM during the COVID period were evaluated, consultations with physicians or other healthcare professionals did not differ in both districts (with an avarage of 47.5% and 10.8 %). However, respondents who obtained information via the internet were more common in Üsküdar (26.5% and 13.6% in Üsküdar and Sancaktepe, respectively), and consultation with neighbours/relatives was more in Sancaktepe (26.5% and 13.6% in Üsküdar and Sancaktepe, respectively). A 25% (n=30) of the total participants stopped their current medications when they started using TCM, and 51.7% (n=62) did not receive information about the side effects of TCM applications, they used without much difference in between the two districts.

Table 1. TCM use according to sociodemographic characteristics of respondents

Sociodemographic characteristics		TCM						p
		Not Used		Used		Total*		
		n	%	n	%	n	%	
Gender	**Male**	78	68.4	36	31.6	114	32.4	0.549
	Female	154	64.7	84	35.3	238	67.6	
Age	**18-29**	79	61.2	50	38.8	129	36.6	0.109
	30-49	97	65.1	52	34.9	149	42.3	
	50 -	56	75.7	18	24.3	74	21.0	
Marital status	**Married**	140	69.3	62	30.7	202	57.4	0.139
	Single	92	61.3	58	38.7	150	42.6	
Education status	**Primary School**	62	79.5	16	20.5	78	22.2	**0.01**
	Secondary School	13	29.5	31	70.5	44	12.5	
	High School	96	59.6	65	40.4	161	45.7	
	University	43	62.3	26	31.7	69	19.6	
Household Income	**Higher**	56	60.2	37	39.8	93	26.4	0.288
	Intermediate	155	68.9	70	31.1	225	63.9	
	Lower	21	61.8	13	38.2	34	9.7	
Health status	**Higher**	138	65.1	74	34.9	212	60.2	0.336
	Intermediate	86	65.6	45	34.4	131	37.2	
	Lower	8	88.9	1	11	9	2.6	
Chronic disease	**Yes**	69	65.1	37	34.9	106	30.1	0.903
	No	163	66.3	83	33.7	246	69.9	
Residence	**Üsküdar**	172	63.7	98	36.3	270	76.7	0.143
	Sancaktepe	60	73.2	22	26.8	82	23.3	
Total		232	65.9	120	34.1	352	100.0	

Note. n=Number, %= Percentage, *=Column %

Table 2. Distribution of traditional herbs and herbal teas used during the epidemic period according to districts**

Herbal products *	Üsküdar	Sancaktepe	Total
	n (%)	n (%)	n (%)
Herbal teas (green tea, sage, chamomile tea, linden)	58 (63.0)	14 (63.6)	72 (63.2)
Mint	58 (63.0)	12 (54.5)	70 (61.4)
Garlic	42 (45.7)	12 (54.5)	54 (47.4)
Ginger	39 (42.4)	10 (45.5)	49 (43.0)
Turmeric	37 (40.2)	9 (40.9)	46 (40.4)
Thyme	33 (35.9)	5 (22.7)	38 (33.3)
Black cumin	25 (27.2)	7 (31.8)	32 (28.1)
Dead nettle	9 (9.8)	4 (18.2)	13 (11.4)
St. John's wort	7 (7.6)	1 (4.5)	8 (7.0)
Echinacea	4 (4.3)	1 (4.5)	5 (4.4)
Aromatherapy	5 (5.4)	0 (0)	5(4.4)

* More than one option was marked. 114 people responded.
****Herbs included in medicinal herb list*: https://www.titck.gov.tr/dinamikmodul/112

Table 3. Reasons for TCM use during COVID pandemic by districts

Reasons for TCM use	Üsküdar		Sancaktepe		Total	
	n	%	n	%	n	%
Strengthen the immune system						
Yes	79	80.6	14	63.6	93	77.5
No	19	19.4	8	36.4	27	22.5
For supportive treatment in COVID-19 disease						
Yes	10	10.2	2	9.1	12	10.0
No	88	89.8	20	90.9	108	90.0
Presence of chronic disease, old age						
Yes	13	13.3	0	0	13	10.8
No	85	86.7	22	100.0	107	89.2
Physical and psychological relaxation						
Yes	15	15.3	3	13.6	18	15.0
No	83	84.7	19	86.4	102	85.0
Doctor's recommendation						
Yes	10	10.2	2	9.1	12	10.0
No	88	89.8	20	90.9	108	90.0
Total	98	81.7	22	18.3	120	100.0

The participants received herbal products mostly from herbalists (53.3%, n=56) and obtaining from the internet was only declared in Üsküdar (5.6 %). They stated that they usually had non-herbal TCM applications at home (80.4%, n=45), similar in the two districts (Table 4).

TCM use did not differ according to gender, age, marital status, income status, health status, and presence of chronic disease ($p>0.05$). However, a significant difference was found according to education level ($p=0.008$). The use of TCM in those with university education was higher than the other groups (40.4%, n=69, $p<0.05$) (Table 5).

Among all TCM applications, herbal tea use was significantly higher in those with higher education levels ($p<0.05$) (Table 1).

DISCUSSION

Many studies have presented different results regarding TCM use and behaviours in different countries and regions of the same country. It is emphasised that these different results may be due to differences in the sociodemographic characteristics of the participants. This is the first study in Turkey on the use of TCM and related factors according to different socioeconomic characteristics during the COVID pandemic.

In this study, TCM use was 34.1%, and traditional herbal products were mostly used during the COVID period, followed by herbal teas and traditional herbal products. Similar to our data from a study conducted in Adana, Turkey, 39.3% of respondents used TCM, and herbal products were mostly preferred (Karataş et al,2021). In a study conducted in Hong Kong, TCM was used by 44.0% of respondents during the pandemic, similar to our study (Lam, Koon, Chung & Cheung, 2021). The fact that people prefer to use herbal products may be due to the consideration of being healthier and more natural, and many allopathic drugs do not have negative effects besides being less costly. In recent studies, it has been seen that phytotherapy is widely used in TCM applications because herbal products and supplements are easily accessible and can be purchased online. People may believe that these products are safer than prescribed drugs because they perceive that nature-based products are safer for consumption (Chali, Hasho & Koricha, 2021; Umeta Chali et al, 2021; Bahall,2017). However, the use of herbs can lead hepatotoxicity and nephrotoxicity and herb-drug interactions (Quintieri, Palatini, Nassi, Ruzza & Floreani, 2008; Akbulut & Bayramoglu, 2013.

Additionally, important changes in the composition of herbal products depending on the source and package, misidentification of the plant, overdilution, mislabelling, active ingredient imbalance, changes in collection procedures, and inadequacy of explanations to patients are among the reasons that may cause problems with phytotherapy (Akbulut & Bayramoglu, 2013; Palabaş Uzun & Koca, 2020). The majority of the partcipitants who used herbal products, obtained these products from herbal shops in our study. Medicinal plants can be obtained from herbalists who have long experience in the preparation and use of plants for years (Palabaş Uzun & Koca, 2020; Kasole, Martin & Kimiywe, 2019). Herbal drugs must be kept under modern hygienic standards to prevent pathogenic microorganisms from microbial load (Govender, du Plessis-Stoman, Downing & van de Venter, 2006). The expiry dates and storage standards of these products should be paid much attention in herbal shops.

Table 4. TCM use behavior during COVID pandemic by districts

TCM use behavior	Üsküdar		Sancaktepe		Total	
	n	%	n	%	n	%
Consultation for the use of TCM (n=120)						
Physician	46	46.9	11	50.0	57	47.5
Healthcare professional (pharmacist, dietitian, physiotherapist)	11	11.2	2	9.1	13	10.8
Internet/Media/newspaper	26	26.5	3	13.6	29	24.2
Advice of neighbors/relatives	15	15.3	6	27.3	21	17.5
Stopping currently used medications (n=120)						
Yes	25	25.5	5	22.7	30	25
No	73	74.5	17	77.3	90	75
Getting information about the side effects TCM (n=120)						
Yes	48	49.0	10	45.5	58	48,3
No	50	51.0	12	54.5	62	51.7
TCM herbal product source (n=105)						
Herbal shop	48	53.9	8	50.0	56	53.3
Pharmacist	36	40.4	8	50.0	44	41.9
Internet	5	5.6	0	0	5	4.8
The place where TCM (non-herbal product) application is made (n=56)						
Home	34	81.0	11	78.6	45	80.4
The places where it applies	8	19.0	3	21.4	11	19.6

Table 5. Distribution of different TCM applications by education levels

TCM		Education levels										p
		≤Primary		Secondary		High school		University		Total		
		n	%	n	%	n	%	n	%	n	%*	
Herbal teas	**Yes**	6	8.3	6	8.3	38	52.8	22	30.6	72	63.2	**0.007**
	No	10	23.8	6	14.3	23	54.8	3	7.1	42	36.8	
Vitamins and supplements	**Yes**	6	10.5	5	8.8	31	54.4	15	26.3	57	50.0	0.503
	No	10	17.5	7	12.3	30	52.6	10	17.5	57	50.0	
Traditional herbs*	**Yes**	2	14.3	1	7.1	10	71.4	1	7.1	14	12.3	0.436
	No	14	14.0	11	11.0	51	51.0	24	24.0	100	87.7	
Other TCM applications	**Yes**	8	19.0	5	11.9	24	57.1	5	11.9	42	36.8	0.210
	No	8	11.1	7	9.7	37	51.4	20	27.8	72	63.2	
Total		16	14.0	12	10.5	61	53.5	25	21.9	114	100	

Note. n=Number, %= Percentage, *=Column %

**Herbs included in medicinal herb list*: https://www.titck.gov.tr/dinamikmodul/112

Apart from herbal treatment and other dietary applications, the most commonly used TCM method in our study was prayer. Similarly, a study conducted in Iran it is emphasised that prayer used as frequently as herbal products among TCM methods (Dehghan, Ghanbari, Ghaedi Heidari, Mangolian Shahrbabaki & Zakeri, 2022).

As there is no sufficient evidence of any antiviral agent against COVID-19, traditional medicine, which has been widely used during the pandemic in the past, has become prominent. Data on the potential effects of dietary therapy and herbal medicines against SARS-CoV-2 have been studied in the literature, but there is not enough supporting evidence (Ang et al,2020; Paudyal, Sun, Hussain, Abutaleb & Hedima, 2022).

We included two districts with different socioeconomic characteristics in this study.

TCM use was higher in Üsküdar, which has a better socioeconomic level than Sancaktepe. Studies based on the overall socioeconomic status (SES) index (SEGE,2022), revealed that the use of TCM is higher in those with high SES similar to previous studies (Xin et al, 2020; Shih, Liao, Su, Yeh & Lin, 2012).

In this study, we also examined the effect of socioeconomic status on TCM choices.

Traditional herbal product use was most common in both districts, whereas non-prescription vitamins and supplements were about 1.7 times more common in Üsküdar district, which has a better socioeconomic status than Sancaktepe. Among the other TCM methods, prayer and dreaming, which have no economic cost, were high in Sancaktepe, while relaxation and massage were high in Üsküdar. These data demonstrate that socioeconomic characteristic affect choices in TCM.

The participants with university education tended to use more TCM, and herbal teas were mostly preferred during the COVID-19 pandemic. We believe that education could promote the understanding of traditional medicine and tend to do more research and awareness to facilitate the use of TCM, as reported in previous findings conducted in Turkey and other countries (Karataş et al, 2021; Lam et al, 2021; Al-Naggar, Bobryshev, Abdulghani & Osman, 2013; Zakaria, Mohd Noor & Abdullah, 2021; Ghaedi, Dehghan, Salari & Sheikhrabori, 2017; Liu et al,2017). However, some studies have shown that an increase in education level reduces the use of TCM, and they also suggest that TCM use decreases because of learning possible side effects (Li et al,2020; Fakeye, Adisa & Musa, 2009). There are also some studies showing that there is no relationship between education level and TCM use (Hori, Mihaylov, Vasconcelos & McCoubrie, 2008; Lim, Sadarangani, Chan & Heng, 2005). Considering these data, it can be seen that the relationship between education level and TCM use may differ.

Another important finding of the study is that more than 50% of those who use herbal products consume five or more products. Combining multiple herbs may also increase the risk of allergic reactions, adverse reactions, and cross-reactions with other pharmaceuticals and supplements. In addition, many dietary supplements potentially increase the risk of interaction even more are packaged in proprietary combinations and can be purchased without a prescription (Falzon & Balabanova, 2017).

In this study, most respondents used TCM without consulting a physician, similar to other studies (Pokladnikova & Telec, 2020 ; Zhang et al,2020; Zakaria et al,2021). Information via the internet was approximately twice as common in Üsküdar as in Sancaktepe, while the rate of seeking friends/relative advice was approximately twice as common in Sancaktepe as in Üsküdar. This result may be related to high socioeconomic level of Üsküdar revealed in socioeconomic status (SES) index (SEGE,2022) and more access of participants to the Internet and computers.

It is also noteworthy that more than 50% of herbal product users does not have any information about the side effects of the products they use.

The herbal products are usually used together with conventional medicine in other studies (Al-Naggar et al, 2013; MacLennan, Wilson & Taylor, 2002; Xue, Zhang, Lin, Da Costa & Story, 2007). In a study conducted in Turkey, participants reported that the use of drugs and herbal products was more effective than the use of drugs alone (Nur,2010).

In contrast to these studies, 25% (n=30) of the participants discontinued their current drug for COVID while using TCM. The use of TCM by discontinuing drug treatment may be due to the lack of an effective antiviral drug therapy for COVID-19.

In this study, we selected two districts with socioeconomic differences. One of these districts was in the first status, and the other was in the second status. It would be good to compare it with the third- and fourth-level regions as well.

Due to the research method, it was not possible to fully represent the populations of both districts. In addition, it is possible that there are temporal differences in the use of TCM by individuals during different periods of the pandemic. Although masks, distance, and personal hygiene rules were followed during the research process, the respondents' answers to the questionnaire may have been lower due to personal concerns.

CONCLUSION

The use of TCM during the COVID-19 pandemic is high, especially in herbal and supplement products. There are differences in the use of TCM, the selection of TCM to use, and some behaviours in districts with different socioeconomic status. The use of TCM increased as education level increased, and those with higher education levels consumed mostly herbal tea.

The fact that more than half of the participants using herbal products used 5 or more products may cause possible interaction and liver/kidney toxicity. It is necessary to raise public awareness of this issue and to prevent misuse. It should not be emphasised that TCM applications pose great risks to the health of individuals unless they are performed by competent individuals.

Ethics Committee Approval: Ethical approval was obtained from the Zeynep Kamil Women's and Children's Diseases Education Hospital Clinical Trials Ethics Committee (Approval no: 2021/34).

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Original Article

Drug-drug interactions with venetoclax in acute myeloid leukemia

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ABSTRACT

Background and Aims: Venetoclax is an important treatment option, especially in patients who are unfit for acute myeloid leukemia treatment. However, because venetoclax is metabolized by CYP3A4, it can lead to many drug-drug interactions (DDIs). DDIs may make a drug less effective, cause unexpected side effects, or increase the action of a particular drug. This study aims to examine venetoclax-related DDIs in this vulnerable patient population and to illuminate possible interventions for both patients and clinicians.

Methods: This observational study was performed between November 2018-December 2022 in the Department of Hematology, Erciyes University Faculty of Medicine. The study involves 60 patients and uses Lexi-interact® to determine potential DDIs (pDDIs) in all patients and uses Lexi-interact® to take into account category D and category X interactions.

Results: Forty-seven (78.4%) patients experienced drug interactions. The most common drug interactions were with azole antifungals, most commonly with posaconazole in category D (31.6%). Clarithromycin and diltiazem were found in more than 20% of patients. Carbamazepine, phenytoin and cladribine were found as contraindicated (category X) drugs.

Conclusion: The study shows that at least 78.4% of the patients treated with venetoclax were at risk of DDIs. Dose reduction of venetoclax is necessary when used with azole antifungals. Due to the extremely high occurrence of DDIs, pharmacists have a significant role in drug interaction management in the multidisciplinary team.

Keywords: Acute myeloid leukemia, Interaction, Posaconazole, Venetoclax

INTRODUCTION

Acute myeloid leukemia (AML) remains challenging in the elderly and/or unfit patients, with the long-term prognosis being generally poor (Juliusson et al., 2009). Venetoclax is a BH3 mimetic and small molecule inhibitor of the anti-apoptotic B cell-lymphoma-2 (BCL-2) (Vervloessem et al., 2017). The US Food and Drug Administration (FDA) and European Medicines Agency (EMA) have approved venetoclax in combination with azacitidine, decitabine, or low-dose cytarabine for patients over 75 years of age or who have comorbidities that preclude the use of intensive induction chemotherapy. Drug interactions become even more important in elderly patients due to the use of multiple drugs (Delafuente, 2003).

Venetoclax has many drug-drug interactions (DDIs). As stated in Venclyxto's summary of product characteristics (SmPC; AbbVie Inc, North Chicago, IL), cytochrome P-450 (CYP450) 3A4 is the primary enzyme responsible for the metabolism of venetoclax, as well as an enzyme that causes drug interactions. The SmPC states, "Caution should be exercised when using Venclyxto with inhibitors of the CYP3A4 family (such as voriconazole, posaconazole) [or the] concurrent use of venetoclax with strong CYP3A4 inducers such as carbamazepine, phenytoin" (EMA, 2002).

As a BCL-2 inhibitor, venetoclax is a substrate of the drug efflux transporter p-glycoprotein in vivo and in vitro (Agarwal, Tong, Bueno, Menon, & Salem, 2018). Pharmacists play a significant role in drug management. In such a particularly sensitive patient population, a multidisciplinary team should be involved with regard to such situations as DDIs, side effect prevention, and dose adjustment (Ma, 2014). In addition, pharmacists play an essential role in terms of the benefits and risks of treating patient and their caregivers by increasing patient participation and shared decision making (Rocque et al., 2018).

This article aims to study all potential DDIs (pDDIs) ob-

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served in patients treated with venetoclax. The study also emphasizes the importance of multidisciplinary work involving pharmacists.

MATERIALS AND METHODS

This retrospective study performs a systematic analysis of all aspects of patient treatment to detect potential clinically significant interactions (CSIs) and involves patients with acute myeloid leukemia (AML) using venetoclax between January 1, 2018-December 31, 2022. The study has included 60 patients with AML who were followed up in the Hematology Department of the Medical Faculty of Erciyes University. The Erciyes University Clinical Research Ethics Committee approved this study in accordance with the Declaration of Helsinki (Decision No. 2022/711). The study obtained patient information retrospectively from the patients' electronic medical records. The following data were collected: age and gender of the patient, drugs prescribed, and adverse drug reactions (ADRs). The study's clinic routinely checks the digoxin serum concentrations in patients using digoxin. This only applies to digoxin. Digoxin serum concentrations are routinely monitored in patients' electronic medical records.

The study determined the pDDIs using Lexi-interact® to classify CSIs as category D or category X. Table 1 shows the classification of drug interactions by severity (Marion, 2022). Some interventions (e.g., dose modification, close monitoring, alternative treatments) are required to minimize toxicity in order to achieve beneficial results. The benefit-to-harm ratio of using multiple drugs should be determined. In Lexi-interact®, category D interactions are interactions with proven clinical significance, while category X interactions are interactions where the risk outweighs the benefit and thus their concomitant use is contraindicated (Marion, 2022).

Table 1. *Severity of Drug Interactions*

Severity of Drug Interaction	Up to Date
Contraindicated	X = Contraindicated
Major	D = Consider therapy modification
Moderate	C = Monitor Therapy
Minor	B = No action needed
None	A = No known interaction

The study performs a descriptive analysis of the variables. Central tendency and dispersion (*SD*) measures are used for the quantitative variables. Frequency distributions have been calculated for the qualitative variables. The statistical analyses are performed using SPSS® v. 25.0.

RESULTS

This retrospective study includes 60 patients, of whom 36 (68.7%) are male. The patients' mean age is 65.50 ±16.9 years. At least one drug was able to interact with venetoclax in 47 (78.4%) patients. The number of pDDIs ranged from one to three, totaling 73 pDDIs (Table 2).

Table 2. *Distribution of Patients According to the Number of pDDIs with Venetoclax*

Number of pDDIs with venetoclax	Number of patients $N = 60$ n (%)
0	13 (21.6%)
1	25 (41.6%)
2	18 (30%)
3	4 (6.8%)

Table 3 shows the distribution of patients according to drugs that were able to interact with venetoclax. The most frequent is posaconazole (31.6%), and this interaction is able to increase venetoclax's toxicity. More than 10% of patients also had pDDIs with carbamazepine (5%) or phenytoin (1.6%), which were able to decrease venetoclax effectiveness, and concomitant use with the degree of interaction X is contraindicated. Drug interactions in category X have also been detected with cladribine.

DISCUSSION

This study is critical for patients with hematological malignancies who also receive treatment with sensitive CYP3A substrates, such as venetoclax. Venetoclax is a new drug approved for use in AML in 2018. Studies are ongoing regarding the use of venetoclax in many other diseases/conditions. Venetoclax is metabolized by CYP3A4 and requires more attention in terms of its drug interactions. As one of the consequences of DDIs in this study, as the drug serum concentration increases, the dose may need to be reduced. If the dose is not reduced, the toxicity of the drug increases as the serum concentration increases, and the drug may need to be discontinued. As a result, situations occur such as the interruption of patient treatment and discontinuation of a costly treatment. The most common side effects are hematological and gastrointestinal toxicity.

This study saw the most common interactions with azole antifungals, diltiazem, and clarithromycin. The most common drug interactions occurred with posaconazole in category D (31.6%). One of the reasons for this is that patients had been given prophylactic antifungals (Maertens et al., 2018). Azole antifungals lead to increased concentrations of venetoclax, because azole antifungal agents inhibit CYP3A4 to varying degrees. CYP3A4 is the primary enzyme responsible for metabolizing venetoclax; therefore, adding azole antifungals reduces its metabolism (Agarwal et al., 2017). The steady dose of venetoclax should be 70 mg/day when used with posaconazole in patients with AML (Bhatnagar et al., 2021). When venetoclax is used concomitantly with a strong CYP3A4 inhibitor such as voriconazole,

Table 3. *Increase or Decrease in Drug Exposure or Effect*

Drugs able to interact with venetoclax	# of patients *N* =60 *n* (%)	Increase ↑ or decrease ↓ of drug exposure	Severity of drug interaction
Posaconazole	19 (31.6)	Venetoclax ↑	D
Fluconazole	17 (28.3)	Venetoclax ↑	D
Diltiazem	7 (11.6)	Venetoclax ↑	D
Voriconazole	6 (10)	Venetoclax ↑	D
Clarithromycin	6 (10)	Venetoclax ↑	D
Cladribine	3 (5)	Immunosuppressive effect ↑	X
Digoxin	3 (5)	Digoxin ↑	D
Carbamazepine	3 (5)	Venetoclax ↓	X
Amiadarone	3 (5)	Venetoclax ↑	D
Aprepitant	2 (3.2)	Venetoclax ↑	D
Carvedilol	2 (3.2)	Venetoclax ↑	D
Phenytoin	1 (1.6)	Venetoclax ↓	X

the venetoclax dose should be reduced by 75%. A 50% dose reduction is required with a moderate CYP3A4 inhibitor such as fluconazole (Venclexta, 2019). Rausch et al. (2021) showed that the platelet healing process was prolonged despite dose reduction. In a retrospective study of AML patients treated with venetoclax and hypomethylating agents (HMAs), concomitant use of posaconazole/voriconazole plus 100 mg/day of venetoclax or 200 mg/day of isavuconazole/fluconazole plus venetoclax was compared with azole-free plus full-dose venetoclax. As a result of another study, higher febrile neutropenia, infection risk, and increased hospitalization were observed in those using azole plus venetoclax (Chiney, Menon, Bueno, Tong, & Salem, 2018). Therefore, venetoclax should be used more carefully in patients receiving azole antifungals.

Venetoclax may increase digoxin concentrations, a substrate of p-glycoprotein, with concomitant use. Avoid concomitant use of venetoclax and digoxin if possible. If combined, administer digoxin at least 6 hours before venetoclax to minimize the potential for interaction (Venclexta, 2019; Chiney et al., 2018). Digoxin toxicity is more likely to develop at digoxin serum concentrations of 1.2 ng/mL or greater. The current study found one patient with an increased digoxin serum concentration observed at 1.8 ng/mL. The dose of digoxin was reduced in this patient. Little research has been done on this, and more is needed. Because of venetoclax's narrow therapeutic range, monitoring digoxin levels should be considered in patients who receive venetoclax in combination with digoxin.

This study has found cladribine, carbamazepine, and phenytoin to interact in category X. When considering the interaction of venetoclax with cladribine, this interaction appears contraindicated. However, treatment regimens are also found in which cladribine and venetoclax have been used together. For this reason, drug interactions need to be evaluated by a specialist clinical pharmacist (Kadia et al., 2021).

Clarithromycin and diltiazem increase the level of venetoclax by inhibiting CYP3A4. Clarithromycin is a potent CYP3A4 inhibitor, while diltiazem is a moderate CYP3A4 inhibitor. When venetoclax is used concomitantly with clarithromycin, the venetoclax dose should be reduced by 75%, while the dose of venetoclax should be reduced by 50% with diltiazem (Freise, Shebley,& Salem,2017). Much attention has been paid to the interaction of venetoclax with azole antifungals. Caution should also be exercised with other drugs that increase venetoclax's serum concentration. The patients receiving clarithromycin in the current study had been switched to a different and more appropriate antibiotic.

The use of venetoclax is contraindicated in patients taking carbamazepine and phenytoin. The mechanism for this is that these drugs reduce the level of venetoclax by inducing CYP3A4 (Venclexta, 2019). The drugs carbamazepine and phenytoin were replaced with appropriate antiepileptics in the patients in this study to avoid low response rates to venetoclax. Some patients were found with double and even triple drug interactions. No study could be found on what to do in these cases.

Awareness of patients' drug interactions with hematological malignancies is crucial for pharmacotherapy. This is related to the high incidence rate for drug interactions and the importance of the consequences of these interactions. Clinical pharmacy is a branch of pharmacy that involves the provision of patient care with the use of medications to optimize patients' health outcomes and includes promoting wellness and preventing disease. The practice of clinical pharmacy embraces the philosophy of pharmaceutical care. In this case, having pharmacists be part of the healthcare team and involved in drug manage-

ment is crucial. In addition, clinical pharmacists' knowledge of prescription monitoring, detection, and management of interactions makes the clinical pharmacist the most qualified personnel for fulfilling this task. Further studies are also needed to understand the importance and magnitude of drug interactions in patients with hematological malignancies.

Meanwhile, this study has some limitations. Due to being a retrospective study, the results of drug interactions could not be monitored beyond the information in the patients' files (e.g., digoxin level). The study also could not measure venetoclax levels to see the outcomes of drug interactions.

CONCLUSION

This study suggests that at least 78.4 % of patients treated with venetoclax are at risk of DDIs. This paper also identifies the drugs with the highest rate of pDDIs (category D) with venetoclax as azole antifungals (i.e., posaconazole, fluconazole, voriconazole), clarithromycin, and diltiazem. The study also observed that a high percentage of the patients had experienced DDIs, including contraindicated (category X) drug combinations. Increased digoxin levels were found in one patient, and the dose of digoxin had been reduced in this patient. Although preventing these DDIs is difficult at times, serious situations such as increasing or decreasing the level of side effects and drug ineffectiveness may occur due to the increase in the level of venetoclax. Therefore, a strong need exists for high awareness of DDIs in order to manage patient care well. This is more important in settings such as hematology that involve cytotoxic agents. As a multidisciplinary team member that treats patients with hematological malignancies, clinical pharmacists can contribute by conducting a systematic review of treatment, identifying interactions, and making recommendations such as optimizing drug therapy and reducing drug-related problems in these patients.

Ethics Committee Approval: The Erciyes University Clinical Research Ethics Committee approved this study in accordance with the Declaration of Helsinki (Decision No. 2022/711).

Peer-review: Externally peer-reviewed.

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Original Article

Mitragyna speciosa Korth. downregulates macrophage inflammatory responses by inhibiting TLR-4 and increasing IL-10 production

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ABSTRACT

Background and Aims: *Mitragyna speciosa* Korth. is a tropical plant native to Asia with various medicinal properties. This study examined the immunotherapeutic potential of *M. speciosa* methanolic extract (MSME) against lipopolysaccharide (LPS)-stimulated activation of macrophages via the expression of *Toll-like receptor 4 (TLR-4)* and *CD14* and downstream signalling cascades leading to the activation of *nuclear factor kappa B (NF-κB)*, which potentially affects macrophage immune responses.

Methods: The expression of *TLR-4/CD14* and *NF-κB* genes in macrophages was determined in total RNA by qRT-PCR. Subsequently, the macrophage phagocytic activities and secretion of immune mediators such as reactive oxygen species (ROS) and cytokines were evaluated by fluorescent latex beads uptake assay, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), and cytometric bead array, respectively, in LPS-activated RAW264.7 cells.

Results: MSME significantly reduced macrophage-mediating inflammation by inhibiting *TLR-4/CD14* signalling and subsequently suppressed the *NF-κB* expression. Inhibition of *TLR-4* by MSME attenuated macrophage phagocytic activity, which consequently reduced the production of ROS and pro-inflammatory cytokines such as IL-6, MCP-1, and TNF-α. Interestingly, MSME significantly increased the production of IL-10, which supports the anti-inflammatory properties of *M. speciosa.*

Conclusion: Our findings suggest the therapeutic potential of MSME through the suppression of macrophage inflammatory responses mediated by IL-10 secretion.

Keywords: *Mitragyna speciosa*; Macrophages; Anti-inflammatory; IL-10, TLR-4.

INTRODUCTION

Mitragyna speciosa Korth. (*M. speciosa*) locally known as Kratom, belongs to the Rubiaceae family and has a rich history of medicinal use for centuries (Jansen & Prast, 1988). The leaves have medicinal value in treating chronic pain, as morphine substitutes in the treatment of addiction, and as an energy booster. *M. speciosa* has also been featured in folk remedies aimed at addressing various ailments, such as coughing, diarrhoea, diabetes, and hypertension (Assanangkornchai, Muekthong, Sam-angsri, & Pattanasattayawong, 2007). *M. speciosa* possesses noteworthy therapeutic attributes, including anti-inflammatory (Tohar, Shilpi, Sivasothy, Ahmad, & Awang, 2019), antioxidant (Parthasarathy et al., 2009), antibacterial (Juanda, Andayani, & Maftuch, 2019), and anti-diabetic (Zailan, Sarchio, & Hassan, 2022), antidepressant (Buckhalter et al., 2021), anti-pain and analgesic (Kruegel et al., 2019), and antipyretic effects (Annas, Mastura Shaik Mossadeq, & Abdul Kadir, 2020). Plant leaves have yielded a plethora of bioactive phytochemicals, including alkaloids like mitragynine, speciogynine, paynantheine, and 7-hydroxymitragynine, along with flavonoids, polyphenols, flavonoids, saponins, triterpenoid saponins, glycoside derivatives, roseoside, vogeloside, and epivogeloside (Firmansyah, Sundalian, & Taufiq, 2020; Kafo, Mahayidin, et al., 2023; Zailan et al., 2022).

Mitragynine is a well-characterised alkaloid found in *M. speciosa* that has emerged as a potential reservoir for natural anti-inflammatory agents (Shaik Mossadeq et al., 2009). It has demonstrated anti-inflammatory properties by impeding the secretion of inflammatory mediators, including nitric oxide (NO), interleukin (IL)-6, tumour necrosis factor (TNF)-α, and IL-1β (Kafo, Elsalami, et al., 2023; Sornsenee, Chimplee, & Romyasamit, 2023). In addition, *M. speciosa* extract was found to alleviate the inflammatory response of macrophages through the attenuation of phagocytosis activity and downregulation of

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proinflammatory cytokine gene expression (Kafo, Elsalami, et al., 2023).

Macrophages are vital components of the immune system and have diverse functions, particularly antigen presentation, phagocytosis, wound repair, and embryonic tissue remodelling. Macrophages are classified into two phenotypes, M1 and M2. M1 is a classical macrophage activation that typically mediates pathogen elimination through phagocytosis and the production of inflammatory mediators such as NO, reactive oxygen species (ROS), and cytokines (Ahmad, Jantan, Kumolosasi, Haque, & Bukhari, 2018). M2 macrophages undergo alternative activation in response to anti-inflammatory stimuli and play a pivotal role in processes related to tissue restitution, wound healing, and the resolution of inflammatory responses. The M2 functional repertoire includes the secretion of anti-inflammatory cytokines, notably IL-10 and transforming growth factor-beta (TGF-β) (Dalmas, Tordjman, Guerre-Millo, & Clément, 2012). Moreover, M2 macrophages actively participate in tissue remodelling, angiogenesis, and immune response modulation, highlighting their multifaceted involvement in maintaining tissue homeostasis and resolving inflammatory conditions (Atri, Guerfali, & Laouini, 2018). Dysregulation in the equilibrium of macrophage polarisation between M1 and M2 phenotypes is frequently correlated with various pathological states or chronic inflammatory manifestations, including conditions like rheumatoid arthritis and systemic lupus erythematosus (SLE). The modulation of distinct macrophage phenotypes plays a pivotal role in governing the onset, progression, and resolution of inflammatory disorders (Dalmas et al., 2012). Consequently, the directional differentiation of macrophages into either the M1 or M2 phenotype has emerged as a prospective therapeutic strategy for managing inflammatory disorders. Notably, prevalent approaches to mitigating inflammation involve augmentation of M2 polarisation and/or attenuation of M1 polarisation (Y. Wang, Smith, Hao, He, & Kong, 2019).

M. speciosa potentially mitigates inflammatory responses by attenuating excessive NO production in RAW264.7 cells (Kafo, Elsalami, et al., 2023; Tohar et al., 2019). Additionally, Mitragynine isolated from *M. speciosa* has been shown to suppress prostaglandin E2 (PGE2) production by inhibiting *cyclooxygenase-2 (COX-2)* expression in lipopolysaccharide (LPS)-stimulated macrophages (Utar, Majid, Adenan, Jamil, & Lan, 2011). The current study complements our previous investigation that elucidated the impact of MSME on macrophages (Kafo, Elsalami, et al., 2023). In the current phase, we assessed the immunotherapeutic effects and mechanisms of *M. speciosa* methanolic extract (MSME) on LPS-stimulated macrophage immune response. This study encompasses the assessment of *Toll-like receptor 4 (TLR-4)* and *CD14* expression, the downstream signalling cascade through NF-κB, and the evaluation of macrophage activities.

MATERIALS AND METHODS

Mitragyna speciosa methanolic extract (MSME)

M. speciosa leaves were collected from Kedah, Malaysia, and the plant sample (KM 0024/22) was authenticated by the Institute of Bioscience (IBS) at the Universiti Putra Malaysia (UPM). The extraction of *M. speciosa* and characterisation of its bioactive compounds were described in detail in our previous study (Zailan et al., 2022). Briefly, a quantity of 100 g of the powdered leaves were extracted in 100 mL of methanol (v/v) using an Ace Soxhlet Extractor 6730 and Condenser 6740 (Quick Fit, England) for 4 h at 60°C. The resulting extract was concentrated using a rotary evaporator. The dried extract was dissolved in methanol and further diluted in dH20 (the maximum concentration of methanol was estimated as ≤ 0.1% in the cell culture).

RAW264.7 cell culture

RAW264.7, a murine-derived monocyte-macrophage cell line from the American Type Culture Collection (ATCC® TIB-71™) was cultivated in Dulbecco's Modified Eagle Media (DMEM) (Capricorn, Germany) containing 10% Foetal Bovine Serum, 1% glutamine, and 1% Penicillin-Streptomycin at 37°C in a 5% CO_2 incubator.

Lipopolysaccharide (LPS) stimulation and MSME treatment

RAW264.7 cells were stimulated with LPS (E. coli O111: B4, Merck, Germany) 1 µg/mL following our previous study (Kafo, Elsalami, et al., 2023). The evaluation of the cytotoxicity of MSME was performed in our previous study in which the working concentrations of MSME (25, 50, and 100 µg/mL) on RAW264.7 cells were determined based on IC20 (≥80% cell viability) to limit the degree of cell death due to the toxicity of the extract (Kafo, Elsalami, et al., 2023). Dexamethasone (10 µM) (Solarbio, China) is an immunosuppressive drug used as a positive control, and its concentration was determined in a previous study (George, Shyni, Abraham, Nisha, & Raghu, 2021). In contrast, 0.1% (final concentration) methanol was used as the cell-only control.

Evaluation of phagocytosis by latex beads

Red fluorescent latex beads (2.0 µ, carboxylate-modified polystyrene) (Sigma-Aldrich, Cat. no: L3030, Louis, USA) were used to evaluate the phagocytic capacity of RAW264.7 cells, as described by (Feng et al., 2021). RAW264.7 cells (1×10^5 cells/well) were treated with MSME or dexamethasone, along with the presence or absence of LPS. After 24 h of incubation, the culture medium was replaced with a suspension of 0.1% latex beads and incubated for 30 min. The cells were

washed three times with phosphate buffer saline and fixed with 10% paraformaldehyde (Solarbio, China), followed by imaging using a fluorescent microscope (Avio Vert A1, Germany).

Measurement of Reactive Oxygen Species (ROS)

The pre-treated cells were stained with 20 µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Invitrogen, Carlsbad, CA) for 30 min at 37°C. Flow cytometry (BD FACS Aria) was used to quantify the generation of ROS (Liu, Chen, Zheng, Yu, & Wei, 2022). The percentage of ROS-expressing cells was determined by H2DCFDA+ cells in the FL-1 channel via comparison with the unstained control.

Cytokine production

The BDTM Cytometric Bead Array (CBA) Mouse Inflammation Kit (Becton Dickinson, Holdrege, NE, USA) was used to assess cytokine production, including IL-6, IL-10, MCP-1, and TNF, from the cell culture supernatant following the manufacturer's instructions. Each sample was acquired via flow cytometry using FACSDiva software (BD FACS LSR Fortessa) followed by data analysis using FCAP Array software.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted from macrophages using Trizol reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The RNA was then converted to cDNA by reverse transcription using a HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, China). Second, a gDNA wiper mixture was prepared by combining RNA with 2 µL of 5× gDNA wiper mix and RNase-free water to a final volume of 10 µL and incubating at 42°C for 2 min. Next, the first-strand cDNA synthesis mixture was prepared with 2 µL of 10× RT mix, 2 µL of HiScript III Enzyme Mix, 1 µL of Oligo (Dt)20 VN, 1 µL of Random hexamer, and 4 µL of RNase-free water. Finally, both mixtures were combined and incubated at 37°C for 15 min, followed by inactivation at 85°C for 5 s. qRT-PCR was performed using the SYBR Green master mix kit on an Eppendorf realplex4 qPCR Real-Time PCR Thermocycler, as outlined by (Srisuwan, Tongtawe, Srimanote, & Voravuthikunchai, 2014) and All-in-One qPCR primers including *TLR*-4 (5'-CTAAGGCCAACCGTGAAAAG-3' and 5'-ACCAGAGGCATACAGGGACA-3'), CD14 (5'- CCTCCAAGTTTTAGCGCTGC-3' and 5'-CAGCATCCCGCAGTGAATTG-3'), *NF-κB* (5'-GGACCTATGAGACCTTCAAGAG-3' and 5'-AGAAGTTGAGTTTCGGGTAGG -3'), and *β*-actin (5'- CTAAGGCCAACCGTGAAAAG-3' and 5'-ACCAGAGGCATACAGGGACA3') (Gene Copoeia, Rockville, MD, USA). The qPCR protocol included 40 amplification cycles, starting with an initial step at 95°C for 10 min, followed by denaturation at 95°C for 5 s, annealing at 65°C for 10 s (except for TLR-4, which annealed at 60°C), and extension at 72°C for 15 s. The primers were validated by Gene Copoeia (Rockville, MD, USA). In addition, a standard curve of each primer was generated to validate the primer efficiency. Gene expression levels of genes were normalised to β-actin and quantified using the $^{\Delta\Delta}$CT method to determine fold changes in gene expression ($2^{-\Delta\Delta}$CT). The results of each sample were expressed as $\log^2$ fold-change in gene expression (Liu et al., 2022).

Statistical analysis

The GraphPad Prism software (version 9.0) was used for data analysis. A one-way ANOVA was applied to assess variations in the treatment control and sample groups. The values presented are means ± standard error mean (SEM) of two or three independent experiments.

RESULTS AND DISCUSSION

MSME downregulates the expression of *TLR-4, CD14*, and *NF-κB* in LPS-stimulated macrophages

Macrophages play a pivotal role in orchestrating the inflammatory immune response. Concurrently, the liberation of the cytokines and mediators from macrophage cells relies on the synergistic interplay between *TLR-4* and *CD14* (Kawasaki & Kawai, 2014). This collaboration facilitates the recognition and binding of microbial components such as LPS (Elisia et al., 2018). The activation of macrophages initiates the stimulation of *NF-κB* and mitogen-activated protein kinases (MAPKs) through diverse signal transduction pathways. Subsequently, this activation triggers the expression *of inducible nitric oxide synthase (iNOS), COX-2*, and production of proinflammatory cytokines, such as TNF-α, IL-1*β*, and IL-6 (Ahmad et al., 2018; Kim et al., 2018). TLR ligands are responsible for the classical activation of macrophages, inducing a phenotypic shift towards the M1 proinflammatory state (Wang et al., 2019). However, dysregulated TLR signalling can perturb immune homeostasis, characterised by the sustained secretion of pro-inflammatory cytokines and chemokine. Such disruption can frequently be implicated in the pathogenesis of various inflammatory and autoimmune disorders, including SLE, sepsis, atherosclerosis, and asthma (Gao, Xiong, Li, & Yang, 2017). Consequently, the modulation of the functionality of these pattern recognition receptors holds the potential to serve as a pivotal determinant in treating these disorders and may contribute to polarising the macrophage activation to M2 (anti-inflammatory response).

In the present study, the effects of MSME on *TLR-4, CD14*, and *NF-κB* gene expression were evaluated using qRT–PCR. The expression of *TLR-4, CD14*, and *NF-κB* in macrophages

increased by stimulation of LPS. Figure 1 shows that MSME (100 µg/mL) significantly reduced *TLR-4* (by 2-fold changes; P=0.0076), *CD14* (by 1.6-fold changes; P=0.0356), and *NF-κB* (by 2.25-fold changes; P=0.0008) in a dose-dependent manner. In addition, the expression of *TLR-4, CD14*, and *NF-κB* is also significantly inhibited by dexamethasone (by 2.28, 1.8, and 2.3-fold changes, respectively). The suppression of TLR/NF-κB signalling activation in macrophages by bioactive natural compounds can downregulate inflammatory responses, which is useful for managing or treating inflammatory diseases (Kim et al., 2018; Liao et al., 2021; Limtrakul, Yodkeeree, Pitchakarn, & Punfa, 2015; Wang et al., 2022).

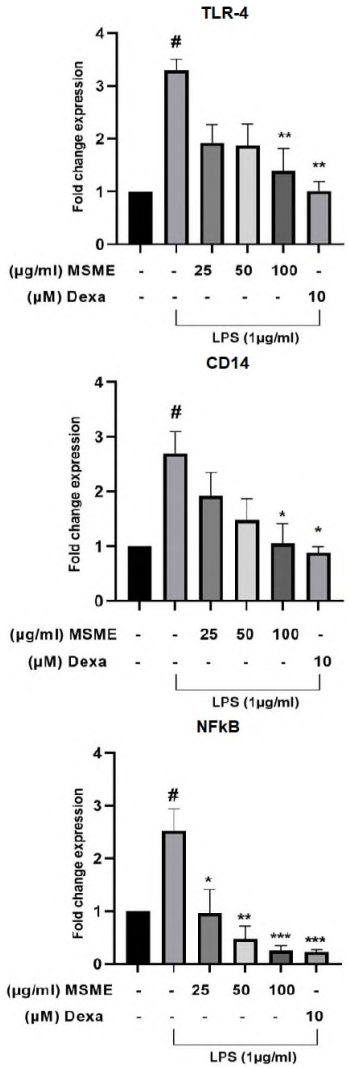


Figure 1. The effect of MSME on the expression of *TLR-4, CD14*, and *NF-κB* in LPS-stimulated RAW264.7 cells. Total RNA was isolated from RAW264.7 cells cultured with LPS (1 µg/mL), MSME, and/or dexamethasone (Dexa). *TLR-4, CD14*, and *NF-κB* expression levels were quantified using qRT–PCR. All data were normalised with the housekeeping gene β-actin. The presented values are the mean ± SEM from three independent experiments. #($p<0.05$) signifies statistical significance compared with the unstimulated control, whereas *($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$) indicate statistical significance compared with the LPS control.

MSME reduces macrophage phagocytic capacity

Phagocytosis is a cellular process characterised by the ingestion and internalisation of particles by phagocytic cells. It constitutes a multifaceted phenomenon encompassing diverse cellular behaviours, in which the orchestrated execution of this process is imperative for effective host defence mechanisms (Ryu et al., 2016). Phagocytosis is potentiated through the involvement of TLR-4, a pivotal component that plays crucial roles in initiating signal transduction pathways. These pathways ultimately result in the effective eradication and removal of pathogens, highlighting TLR-4's integral role in the immune system's capacity to combat microbial threats (Doyle et al., 2004). However, the regulation of TLR-4 receptors can affect phagocytosis activity.

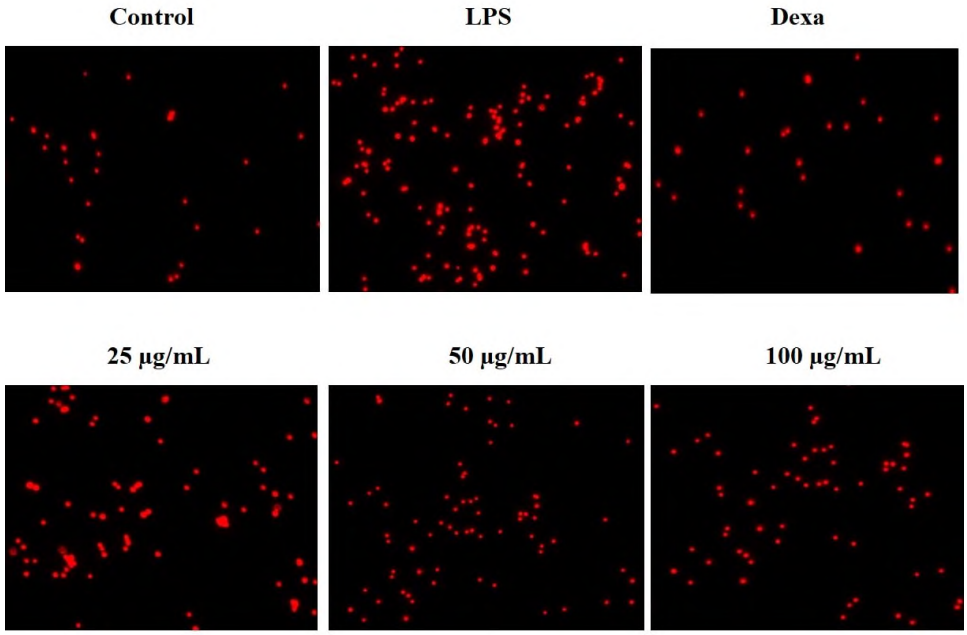
Latex beads are frequently employed in phagocytosis assays and function as representative particles that replicate the properties of pathogens or other foreign entities for experimental purposes (Gu et al., 2014). As indicated in Figure 2, stimulation of RAW264.7 cells with LPS increased the number of macrophages that engulfed the beads. Conversely, the number of cells treated with MSME exerted a reduction in engulfed beads. This finding suggests the attenuation of the phagocytosis activity of macrophages by MSME. Similarly, reduced capacity of macrophage phagocytic activity was also observed following treatment with dexamethasone.

Extracts derived from certain medicinal plants have exhibited the capacity to hinder the phagocytic activity of immune cells, which is consistent with the findings of this investigation. Specifically, the study elucidated those extracts from *Phyllanthus amarus* and *P. urinaria* exhibited a moderate inhibitory effect on E. coli uptake by monocyte cells (Jantan, Ilangkovan, Yuandani, & Mohamad, 2014). Moreover, the aqueous and methanolic extracts of *Ixora coccinea* have the ability to inhibit the phagocytic activity of neutrophils. This inhibitory effect is ascribed to the influence of these extracts on the activity and intracellular killing mechanism of neutrophils, thereby contributing to the modulation of immune cellular responses in inflammatory contexts (Wickramasinghe, Kumara, De Silva, Ratnasooriya, & Handunnetti, 2014).

MSME reduces ROS production in LPS-stimulated macrophages

Reactive Oxygen Species (ROS) are oxygen-containing molecules with chemical reactivity produced as inherent byproducts of cellular metabolism. ROS delicately influences various physiological and pathological processes in living organisms. Although they are necessary for various cellular functions, an imbalance between ROS production and the cellular antioxidant defence mechanisms can lead to oxidative stress, which is implicated in tissue damage, a range of diseases, and the ageing process (Finkel, 2011; Qi et al., 2013).

Upon exposure to bacterial LPS, macrophages exhibit a no-



Test	Phagocytosis rate
Control	+
LPS	++++
LPS + Dexamethasone 10 µM	+
LPS + MSME 25 µg/mL	+++
LPS + MSME 50 µg/mL	+++
LPS + MSME 100 µg/mL	++

Figure 2. MSME reduces macrophage phagocytic capacity by inhibiting the internalisation of fluorescent latex beads. RAW264.7 cells were cultured with MSME or dexamethasone in the presence or absence of LPS for 24 h. 0.1% latex beads were added for 30 min, and the cells were fixed with 10% paraformaldehyde. A representative field of macrophage phagocytic activity towards fluorescent latex beads was captured under 4x magnification from three independent experiments. The table shows qualitative phagocytosis scores.

table elevation of ROS production by 70% from RAW264.7 from RAW264.7 (p=0.0071). Our findings showed significant inhibition of ROS by MSME, suggesting antioxidant properties of MSME (Figure 3). This data was in line with a previous study that demonstrated the antioxidant activity of *M. speciosa* through its free radical-scavenging activity (Parthasarathy et al., 2009; Zailan et al., 2022). Therefore, ROS production may be suppressed by downregulation of *TLR-4* and *NF-κB*. As previously demonstrated by Meng et al., pre-treatment of macrophages with curcumin extract diminished excessive ROS generation. This inhibitory effect on the inflammatory response is posited to be contingent on the suppression of *TLR-4* activation, the prevention of *NF-κB* nuclear translocation, and the reduction in NADPH-mediated intracellular ROS production (Meng et al., 2013). Additionally, the methanol extract of *Caragana rosea Turcz* exhibits a dose-dependent reduction in ROS levels, which is achieved through the regulation of upstream *NF-κB* proteins and the TLR-4-mediated *NF-κB* signalling pathway (Meng et al., 2013).

MSME inhibits proinflammatory cytokine expression in LPS-stimulated macrophages

Cytokines play a crucial role in facilitating communication and networking among cells of the immune system in maintaining or re-establishing a state of equilibrium through the coordination of various types of cells such as haematopoietic, inflammatory, and lymphoid cells (Ahmad et al., 2018). Activated macrophages generate and sequentially release a diverse array of pro-inflammatory cytokines, including IL-6, IL-β, and TNF-α. Within the context of the inflammatory response, macrophages are the most highly responsive components of the innate immune system. They take on the roles of both initiators and detectors to regulate the course of both inflammatory and immunological reactions. To intensify and deliver this immune reaction, RAW 264.7 cells markedly enhance the production and transcription of TNF-α, IL-1β, IL-6, iNOS, and MCP-1 following LPS induction (Ahmad et al., 2018; Liu et al., 2022; Zailan et al., 2022).

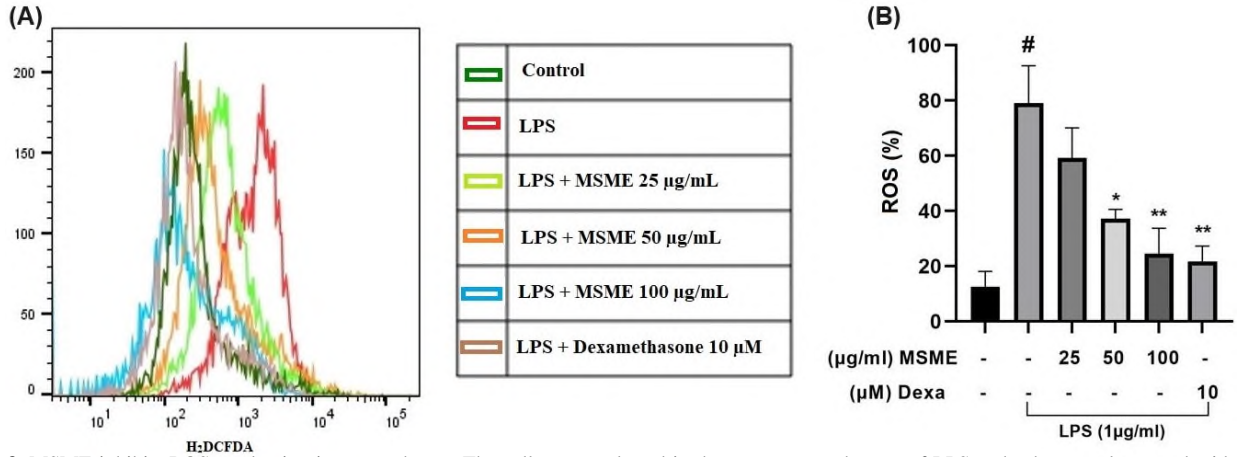


Figure 3. MSME inhibits ROS production in macrophages. The cells were cultured in the presence or absence of LPS and subsequently treated with MSME. Following a 24-h incubation, the cells were stained with 20µM of H2DCFDA and acquired on the flow cytometry. Cells expressing ROS+ were quantified according to fluorescence intensity. (A) Each overlayed histogram is representative of three independent experiments. (B) Bar graph presented as means ± SEM of ROS from three independent experiments. #(P<0.05) indicates statistical significance compared with the untreated control, whereas *(P ≤ 0.05), **(P < 0.01), and ***(P < 0.001) indicates statistical significance compared with LPS alone.

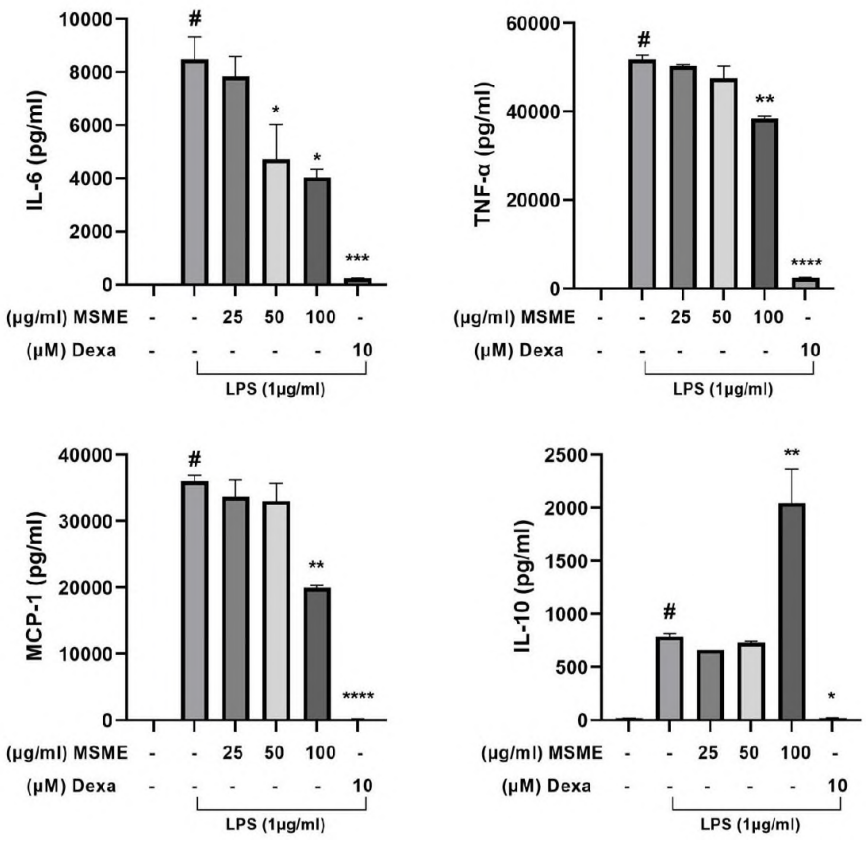


Figure 4. MSME reduces proinflammatory cytokine production in macrophages. RAW264.7 cells were treated with MSME or dexamethasone in the presence or absence of LPS. The production of secreted cytokines was evaluated using the BD Mouse Inflammatory Cytometric Bead Array Kit via flow cytometry. The data is represented as mean ± SEM from three independent experiments. #(p<0.05) indicates statistical significance in comparison with the unstimulated control. *(p<0.05), **(p<0.01), ***(p<0.001), and ****(p<0.0001) indicate statistical significance in comparison with the LPS stimulation without MSME treatment.

In this study, the secreted cytokines in the cell culture supernatants were quantified. Our findings showed that LPS-stimulated RAW264.7 cells with LPS significantly increased TNF-α, IL-6, IL-10, and MCP1, which principally indicated inflammatory responses (Figure 4). However, this phenomenon is counteracted by MSME treatment. It shows that MSME significantly inhibits the production of macrophage pro-inflammatory cytokines including TNF-α, IL-6, and MCP1 ($p<0.05$) in a dose-dependent manner. Expectedly, the production of these cytokines was also significantly inhibited by dexamethasone ($p<0.001$). These findings align with our earlier investigation demonstrating that MSME downregulates the expression of proinflammatory cytokines, including IL-6, TNF-α, and IL-1β (Kafo, Elsalami, et al., 2023). Similarly, fermented *M. speciosa* leaves exhibit anti-inflammatory properties by markedly suppressing the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Sornsenee et al., 2023). Furthermore, the methanol extract derived from the fruits of K*ochia scoparia* exerted inhibitory effects on LPS-induced proinflammatory cytokines, including PGE2 and TNF-α, as well as on the expression of iNOS and COX-2. These effects are attributed to the suppression of NF-κB activation (Shin et al., 2004). This supports the assertion that the observed inhibition of cytokine production is linked to the downregulation of cytokine gene expression. On the other hand, the secretion of the anti-inflammatory cytokine, IL-10, was significantly elevated by MSME (100 µg/mL; $p<0.001$) but not with dexamethasone treatment. The increase in IL-10 production showed the anti-inflammatory effect of the extract, which is in line with a study showing an increase in IL-10 levels by Moringa oleifera flower extract (Tan, Arulselvan, Karthivashan, & Fakurazi, 2015). However, the suppression of IL-10 by dexamethasone is associated with the mechanism by which glucocorticoids alleviate inflammation in allergic diseases (Fushimi, Okayama, Seki, Shimura, & Shirato, 1997; Tan et al., 2015). Dexamethasone exerted varying effects on LPS-induced TNF-α and IL-10 secretion. Unlike TNF-α which was inhibited in a dose-dependent manner, the dexamethasone effect on IL-10 was biphasic, whereby it increased IL-10 secretion at lower concentrations but inhibited it at higher concentrations (Franchimont et al., 1999).

CONCLUSION

The present study investigated the efficacy and mechanism of MSME in downregulating immune responses in an *in vitro* LPS-induced inflammation model in macrophages. The anti-inflammatory effect of MSME may be attributed to the up-regulation of IL-10, which concomitantly reduced the expression of pro-inflammatory cytokines (IL-6, TNF-α, and MCP-1) through the inhibition of *TLR-4* and *CD14* and the attenuation of *NF-κB* expression. In addition, a reduction in the phagocytosis capacity of macrophages and ROS production by MSME exerts the attenuation of inflammatory responses. Our findings suggest that MSME exhibits potent anti-inflammatory properties and is a potential candidate for the prevention and treatment of inflammatory diseases.

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Original Article

Sitagliptin does not improve isoprenaline-induced cardiac contractility in streptozotocin-induced diabetic rats

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ABSTRACT

Background and Aims: Sitagliptin, a dipeptidyl peptidase IV (DPP-IV) inhibitor, has been shown to have beneficial effects on the diabetic heart. Beta-adrenoceptor (β-AR)-mediated responses are impaired in diabetes. Our aim was to investigate the impact of sitagliptin on the diabetic rat heart in terms of β-AR-mediated responsiveness. In addition, we examined the expression of proteins associated with diastolic dysfunction and endoplasmic reticulum (ER) stress, as well as proteins involved in the β-AR signalling pathway.

Methods: Eight-week-old Sprague-Dawley rats were divided into control, diabetic, and sitagliptin-treated (10 mg/kg/day for 4 weeks) diabetic groups. Type 1 diabetes was induced by intraperitoneal injection of streptozotocin (STZ). Throughout the treatment period, the rats received sitagliptin orally. Cardiac β-AR responsiveness was assessed using *in vitro* papillary muscle experiments with a nonselective β-AR agonist, isoprenaline, and *in vitro* Langendorff heart preparation experiments with a β_3-AR selective agonist CL 316,243. Western blot experiments were conducted to assess the protein expression of SERCA2a, GRP78, β_3-AR, eNOS, and p-eNOS.

Results: Sitagliptin did not reduce blood glucose levels or reverse weight loss in diabetic rats. However, it improved the heart weight to body weight ratio, indicating a reduction in cardiac hypertrophy. Sitagliptin did not correct the isoprenaline-induced contractile response in the diabetic group, nor did it alter the β_3-AR mediated relaxation. Sitagliptin treatment also did not improve the downregulation of SERCA2a or the upregulation of GRP78. However, it reduced the upregulation of β_3-AR. The protein expression of eNOS and the ratio of p-eNOS to eNOS were similar among the groups.

Conclusion: This study indicates that sitagliptin treatment did not improve isoprenaline-mediated contractile responses or affect β_3-AR-mediated relaxation in the diabetic heart. However, the observed increase in β_3-AR protein expression in the diabetic heart treated with sitagliptin indicated a potential differential effect of the drug on this pathway compared to the β_1-AR signalling pathway. Further studies are needed to elucidate the precise mechanisms by which sitagliptin influences β_3-AR-mediated pathways.

Keywords: Beta adrenoceptor, Diabetes, Heart, Isoprenaline, Sitagliptin

INTRODUCTION

Dipeptidyl peptidase IV (DPP-IV) inhibitors represent a recent class of antidiabetic medications that enhance glycemic control in patients with type 2 diabetes. These inhibitors work by boosting insulin secretion from islet β-cells through increased availability of incretin hormones, thereby leading to decreased blood glucose levels (Gopal, Chahade, Kim, & Ussher, 2020). Studies in preclinical settings have demonstrated the positive impact of DPP-IV inhibitors on cardiac function (Zhou et al., 2018; Khodeer, Bilasy, Farag, Mehana, & Elbaz, 2019). However, clinical trials assessing cardiovascular outcomes have not yet shown this class to be superior to placebo in patients with type 2 diabetes and high cardiovascular risk (Scheen, 2018).

Sitagliptin, the first approved DPP-IV inhibitor, is a potent

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and highly selective member of this group (Lyseng-Williamson, 2007). Similar to other DPP-IV inhibitors, sitagliptin prevents the inactivation of incretin, thus promoting glucose-dependent insulin secretion. Experimental studies have indicated several cardiac benefits of sitagliptin. For instance, in Zucker Diabetic Fatty (ZDF) rats, 12 weeks of sitagliptin treatment improved ejection fraction and fractional shortening (Zhou et al. , 2018). Similarly, in Goto-Kakizaki rats, a model of type 2 diabetes, 20 weeks of sitagliptin treatment enhanced diastolic function (Ramírez et al., 2018). Furthermore, in streptozotocin (STZ) diabetic rats, 12 weeks of sitagliptin treatment ameliorated cardiac contraction and relaxation (Wu, Xu, Zhang, & Bao, 2019). The favourable effects of sitagliptin extend beyond the diabetic heart. In Dahl salt-sensitive rats fed a high-salt diet, 8 weeks of sitagliptin treatment improved diastolic dysfunction (Esposito et al., 2017). Sitagliptin also protects against isoprenaline-induced myocardial damage in rats (Ibrahim, Geddawy, & Abdel-Wahab, 2018). These findings show that sitagliptin may affect the heart through mechanisms beyond glucose regulation.

A notable characteristic of the diabetic heart is the diminished responsiveness of beta-adrenoceptors (β-AR) which is primarily associated with reduced β-AR expression (Dincer et al., 2001; Haley, Thackeray, Kolajova, Thorn, & DaSilva, 2015; Jiang et al., 2015). Changes in β-AR-mediated responses are critical because cardiac contractility is mainly regulated by β_1-ARs and to some extent by β_2-ARs. A third subtype, β_3-AR, is also noteworthy because it mediates a negative inotropic effect in the heart (Gauthier, Tavernier, Charpentier, Langin, & Le Marec, 1996). The role of this subtype is significant in conditions associated with catecholamine overstimulation, such as heart failure or diabetes (Moniotte & Balligand, 2003; Rozec, Noireaud, Trochu, & Gauthier, 2003).

The impact of sitagliptin on cardiac β-adrenergic responsiveness has not yet been investigated. Therefore, the present study aimed to determine whether sitagliptin treatment has a beneficial effect on cardiac β-adrenergic responsiveness independent of its metabolic benefits in the heart of STZ-induced diabetic rats.

MATERIALS AND METHODS

Animals

The study was approved by the local ethical committee of Ankara University (2014-24-161). Animal experiments were performed in accordance with the NIH Guidelines for Care and Use of Laboratory Animals. Eight-week-old male Sprague–Dawley rats (200-250 g) were purchased from Bilkent University and Gazi University. The rats were housed in the animal facility of the Faculty of Pharmacy, Ankara University, under a 12-h light/12-h dark cycle with free access to standard chow and water.

Induction of diabetes and sitagliptin treatment

Rats were randomly assigned to three groups: control (C, n=15), diabetic (D, n=20), and sitagliptin-treated diabetic (S, n=16). Diabetes was induced by intraperitoneal injection of STZ at doses of 35 or 40 mg/kg. After 72 h, glucose levels were measured using a glucose meter (VivaChek, Biotech, China) with blood samples taken from the tail. Rats with blood glucose levels (non-fasting) below 300 mg/dl received a second or third dose of STZ (40 or 45 mg/kg, respectively). In the preliminary study to determine the appropriate sitagliptin dose, STZ-diabetic rats were orally administered 10 and 30 mg/kg/day for 4 weeks, following protocols from previous studies. However, no significant decrease in blood glucose was observed at either dose (C, n = 6; D, n = 4; S (10 mg/kg), n = 5; S (30 mg/kg), n=5). Previous research suggests that a dose of 5-10 mg/kg/day is sufficient to determine the effect of sitagliptin on the heart independent of its impact on blood glucose (Reimer et al., 2012; T.-M. Lee, Chen, & Chang, 2016). To evaluate the potential cardiac effects of sitagliptin treatment independent of its metabolic effects, we administered a dose of 10 mg/kg to rats based on the literature review. Sitagliptin treatment was initiated after 10 weeks of diabetes (10 mg/kg/day, once daily, 4 weeks) (Figure 1). For this purpose, Januvia® tablets (128.5 mg sitagliptin phosphate monohydrate equivalent to 100 mg sitagliptin) were used to prepare the suspension administered orally. Distilled water was given to the C and D groups. On the day of the experiment, just before euthanasia, body weight and blood glucose levels were recorded.

Papillary muscle experiments

Rats were euthanized under aether anaesthesia and their hearts were rapidly excised. The left ventricular papillary muscle was dissected and mounted in a superfusion organ bath. The muscle was paced at a cycle length of 1700 ms with a 2-ms stimulus pulse at double threshold voltage. The muscle was perfused with Tyrode's solution containing 116 mM NaCl; 5 mM KCl; 2.7 mM CaCl2; 1.1 mM $MgCl_2$; 0.33 mM $NaHPO_4$; 24 mM $NaHCO_3$, and 5 mM glucose, flowing at a rate of 5 ml/min (30°C, 95% O2/ % CO_2, pH= 7.4). Muscle tension was recorded using a mechanoelectrical force transducer (Commat Pharmacology & Physiology Instruments, Ankara, Turkiye). The papillary muscle was allowed to stabilize for 60 min before being progressively stretched. Dose-response curves were generated at 90% of the maximum tension. The response mediated by β_1- and β_2-ARs was assessed using isoprenaline, a non-selective β-AR agonist, across a range of concentrations from 0.1 nM to 30 mM.

Figure 1. Design of the study and experimental timeline.

Langendorff-perfused cardiac experiments

Rats were euthanized under ether anaesthesia. Hearts were rapidly excised in ice-cold Krebs-Henseleit solution (in mmol/L; 120 NaCl, 4.8 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11 glucose; pH 7.4) and then retrogradely perfused at a rate of 10 ml/min (37°C, 95% O_2/ 5% CO_2). The heart rate was maintained at 300 bpm by pacing the right ventricle with a stimulator (Grass Instrument Inc., Quincy, MA, USA). A latex balloon connected to a pressure transducer was inserted into the left ventricle, and the volume of the balloon was adjusted to set the left ventricular end-diastolic pressure to 10 mmHg at the beginning of each experiment. Hearts were perfused with a constant flow for 30 min until they reached a steady state. The β_3-AR-mediated relaxation response was then assessed using the selective agonist CL 316,243, which was added to the Krebs-Henseleit solution at increasing concentrations (0.1 pM-1 µM). Left ventricular developed pressure (LVDP, the difference between LV systolic pressure and end-diastolic pressure) was measured using software (version 3.5.0 for Windows, Biopac Systems Inc., Santa Barbara, CA). Data were recorded online via an analogue-to-digital interface (model MP100; Biopac Systems Inc.).

Western Blotting

The left ventricular tissue was homogenised using RIPA solution (comprising RIPA buffer, sodium orthovanadate and protease inhibitor cocktail). After sonication, the homogenate was agitated for 2 h at +4°C and then centrifuged at 16.000g for 30 min at +4°C. Protein concentration was determined by the bicinchoninic acid (BCA) assay. Protein samples ranging from 10-100 µg were loaded onto a polyacrylamide gel (TGX Fast Cast, 7.5%) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane at 100 V for 2 or 4 h. The membranes were blocked with 5% bovine serum albumin (BSA) in tris-buffered saline containing 0.1% Tween 20 (TBST), followed by overnight incubation with primary antibodies at +4°C. The primary antibodies used included β_3-AR (1/500), endothelial nitric oxide synthase (eNOS) (1/500), phosphorylated eNOS (p-eNOS) (1/1000), sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) (1/2000), and glucose-regulated protein 78 (GRP78) (1/1000). After washing, the membranes were incubated with secondary antibodies for 2 h at +4°C, including anti-chicken (1/3000) and anti-rabbit (1/2000) antibodies. Protein bands were visualised using enhanced chemiluminescence assay and exposed to film. Quantification of protein bands was performed using ImageJ software (NIH, USA), and expression levels were normalised to the housekeeping gene, α-tubulin (1/10.000).

Statistical Analysis

Results are presented as mean ± standard deviation (SD). Statistical significance was assessed using one-way ANOVA, followed by Bonferroni's multiple comparison test to evaluate differences between groups. A p-value < 0.05 was considered statistically significant. All statistical analyses and graph plotting were performed using Prism software (version 9.5.0 GraphPad, La Jolla, CA, USA).

Chemicals

Sitagliptin (Januvia, 100 mg, Merck Sharp & Dohme, Levent, Istanbul), streptozotocin (Sigma-Aldrich, Missouri, USA), isoprenaline (Sigma-Aldrich, Missouri, USA), CL 316,243 (Sigma-Aldrich, Missouri, USA), anti-β_3 antibody (ab59685, Abcam, Cambridge, UK), anti-eNOS antibody (9572S, Cell Signaling Technology, Massachusetts, USA), anti-p-eNOS (ser1177) antibody (9571S, Cell Signaling Technology, Massachusetts, USA), anti-SERCA2a (4388, Cell Signaling Technology, Massachusetts, USA), anti-GRP78 (ab21685, Abcam, Cambridge, UK) anti-α-tubulin antibody (ab4074, Abcam, Cambridge, UK), anti-chicken antibody (29710, AnaSpec, Fremont, USA), anti-rabbit antibody (7074S, Cell Signaling Technology, Massachusetts, USA), RIPA buffer (Sigma-Aldrich, Missouri, USA), BCA kit (Thermo Fischer, Massachusetts, USA), TGX Fast Cast (Bio-Rad, California, USA), ECL kit (Thermo Fischer, Massachusetts, USA), BSA (A7030, Sigma-Aldrich, Missouri, USA), Film (Kodak, New York, USA).

RESULTS

General characteristics of rats

Blood glucose levels were significantly higher in group D after 14 weeks of diabetes; however, sitagliptin treatment did not reduce the blood glucose levels (Table 1). Diabetic rats lost body weight as expected, with body weight remaining lower in the S group (Table 1). Heart weight was comparable between

the groups (Table 1). The ratio of heart weight to body weight was higher in the D group and was significantly improved by sitagliptin treatment (Table 1).

β_1- and β_2-AR-mediated contractile responses in papillary muscles

The dose-dependent contractile response to isoprenaline in the papillary muscle was attenuated in group D and was not recovered by the treatment (Figure 2A). Similarly, the maximal response to the agonist was reduced in group D and did not increase with sitagliptin treatment (E_{max}; C: 153.50±61.19; D: 46.18±24.07; S: 71.92±32.51; %, Figure 2B).

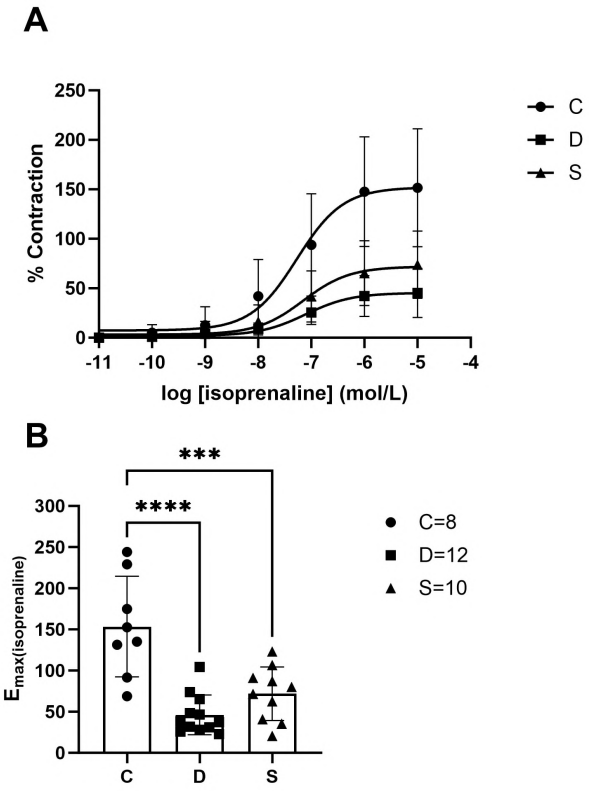

Figure 2. Isoprenaline-induced contractility. **A.** Cumulative concentration response curve. **B.** Maximum response. C, control group; D, diabetic group; S, sitagliptin-treated diabetic group. E_{max}: maximum effect. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. ***, p<0.001, ****, p<0.0001.

β_3-AR-mediated relaxation response in Langendorff heart preparation

β_3-AR-mediated relaxation was determined using the selective agonist CL 316,243, which induced dose-dependent relaxation in Langendorff-perfused rat hearts. The negative inotropic effect was enhanced in group D; however, sitagliptin treatment did not reduce it (Figure 3A). The maximal response was also higher in the D and S groups (E_{max}; C: 91.34±7.66; D: 80.15±11.71; S: 87.37±6.97; %, Figure 3B).

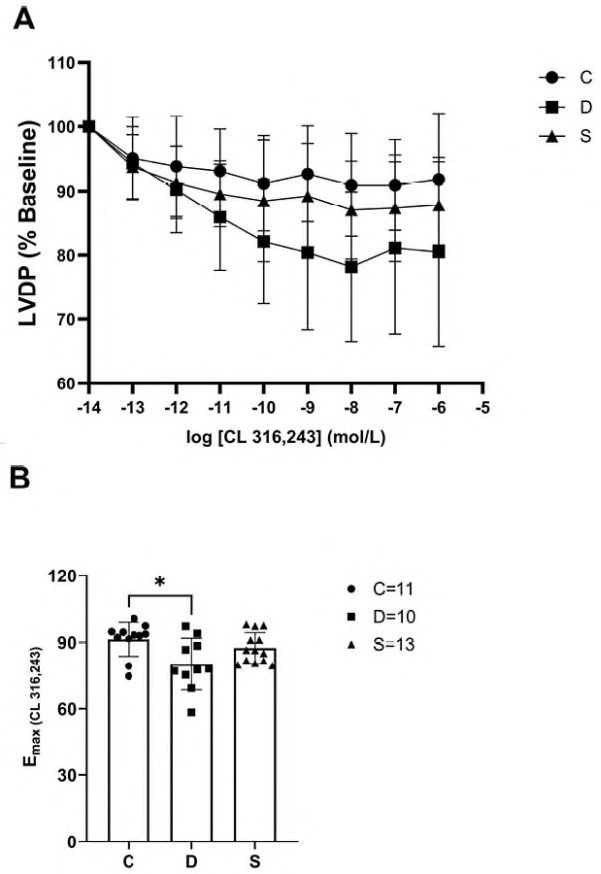

Figure 3. CL 316,243-mediated relaxation. **A.** Cumulative concentration response curve. B. Maximum response. C, control group; D, diabetic group; S, sitagliptin-treated diabetic group. E_{max}: maximum effect. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. *, p<0.05.

Protein expression of SERCA2a and GRP78

SERCA2a was downregulated in the D group but not improved in the S group (C: 100.00±14.20 D: 58.59±25.20; S: 60.24±31.78, Figure 4A). The protein expression of GRP78, a marker of ER stress, was slightly increased in the D group but not significantly altered in the S group (C: 100.00±19.32; D: 140.00±50.42; S: 128.90±22.50, Figure 4B).

Protein expression of β_3-AR, eNOS, and p-eNOS

β_3-AR was upregulated in group D and was significantly decreased by sitagliptin treatment (C: 100.00±37.90; D: 279.60±22.32; S: 180.20±32.16, Figure 5A). eNOS expression, on the other hand, did not differ between groups (eNOS; C:100.00±22.10; D: 89.56±36.83; S: 91.99±38.42; Figure 5B). p-eNOS or the ratio of p-eNOS to eNOS also did not change

Table 1. General characteristics of the rats.

	C (n=14)	**D (n=20)**	**S (n=16)**
BG (mg/dl)	99.93±9.84	502.90±89.38****	459.20±103.40****
	C (n=15)	**D (n=16)**	**S (n=16)**
BW (g/g)	394.70±46.29	295.10±54.88****	318.80±37.49***
HW (g)	1.55±0.33	1.44±0.24	1.35±0.22
HW/BW (g/g)	0.00393±0.00048	0.00496±0.00062****	0.00424±0.00058##

BG, blood glucose; BW, body weight; HW, heart weight. ***, p<0.001, ****, p< 0.0001 compared to C; ##, p<0.01, compared to D. C, control group; D, diabetic group; S, sitagliptin treated diabetic group

significantly despite a slight increase in the D and S groups (p-eNOS; C: 100.00±32.65; D: 154.10±82.70; S: 86.33±28.74; p-eNOS/eNOS; C: 100.00±17.38; D: 174.70±123.50; S: 107.40±59.00; Figure 5C and Figure 5D).

DISCUSSION

The present study indicates that sitagliptin treatment did not improve isoprenaline-stimulated β-AR-mediated cardiac contractility in STZ-induced diabetic rats. In addition, it did not reduce hyperglycemia or prevent body weight loss in the diabetic group. However, the study identified one positive outcome of sitagliptin, which is an improvement in cardiac hypertrophy in diabetes, as evidenced by the heart weight/body weight ratio.

β-ARs play a critical role in cardiac contractile response (Brodde, Michel, & Zerkowski, 1995), and the impact of diabetes on β-AR-mediated contractility has been extensively studied. Despite conflicting results in the literature, reduced β-adrenergic responsiveness is often associated with diabetic heart (Erdogan, Michel, & Arioglu-Inan, 2020). Consistent with these observations, our study revealed attenuated isoprenaline-induced contraction in the papillary muscles of diabetic rats, a response not improved by sitagliptin treatment. This lack of improvement may be linked the persistent hyperglycemia in diabetic rats, as sitagliptin failed to correct this metabolic imbalance. However, this explanation appears unlikely given that studies in non-diabetic models have demonstrated beneficial cardiac effects of sitagliptin (Esposito et al., 2017; Ibrahim et al., 2018). Our findings on blood glucose are unsurprising, as the antihyperglycemic effect of this drug class is primarily driven by insulin secretion stimulation, which is compromised in the STZ diabetic rat model due to pancreatic β-cell destruction. Nevertheless, varying results regarding glycemic control with DPP-IV inhibitors in this model have been reported (Marques et al., 2019; Kizilay, Ersoy, Cerkezkayabekir, & Topcu-Tarladacalisir, 2021).

The beneficial cardiovascular effects of DPP-IV inhibitors have been demonstrated in numerous preclinical studies, including those involving cardiac pathologies unrelated to diabetes (Nakajima et al., 2019; Yamaguchi et al., 2019). Among these investigations, Connelly et al. reported that high-dose sitagliptin treatment did not reduce blood glucose; however, it did improve certain cardiac parameters and adverse remodelling induced by myocardial infarction in STZ-diabetic rats (Connelly et al., 2013). In addition, sitagliptin treatment was found to reduce passive left ventricular stiffness in obese type 2 diabetic mice (Hamdani et al., 2014). In this study, an increase in left ventricular stroke volume was observed, which was attributed to the stimulation of the cardiac cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase (PKG) pathway, rather than glycemic control. In contrast to these reported findings, our study did not observe any beneficial effect of sitagliptin on cardiac contractility.

It has been suggested that cardiac contraction or relaxation in response to β-adrenergic agonists may be influenced by altered SERCA2a activity (Kranias & Hajjar, 2012). Furthermore, the SERCA2a regulator phospholamban (PLN) has been implicated as a key component of the β-adrenergic agonist-induced cardiac response (Kranias & Hajjar, 2012), as demonstrated in PLN-deficient mice (Wolska, Stojanovic, Luo, Kranias, & Solaro, 1996). In the current study, we observed the downregulation of SERCA2a in the diabetic heart, a common occurrence in the STZ-diabetic rat model (Arioglu-Inan, Ozakca, Kayki-Mutlu, Sepici-Dincel, & Altan, 2013). Additionally, GPR78, an ER stress marker, was found to be upregulated in the diabetic heart. The reduced expression of SERCA2a may be associated with increased ER stress, similar to the findings in OLETF diabetic rats (Takada et al., 2012). In our study, sitagliptin could not correct either the downregulation of SERCA2a or the upregulation of GPR78. Thus, decreased SERCA2a expression could have contributed to the impaired contractile response observed in the diabetic heart. Our findings indicating that sitagliptin did not improve β-AR-mediated contractility in diabetic rats may

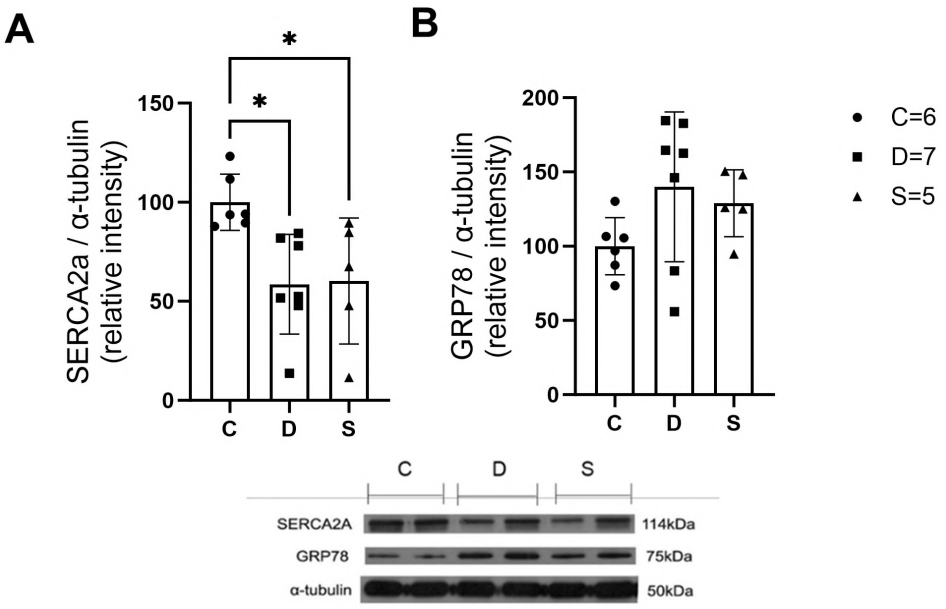

Figure 4. Protein expression levels of SERCA2a and GRP78. **A.** % relative intensity of SERCA2a. **B.** % relative intensity of GRP78. C, control group; D, diabetic group; S, sitagliptin-treated diabetic group. SERCA2a, sarcoplasmic reticulum calcium ATPase 2a; GRP78, glucose-regulated protein 78. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. *, $p<0.05$.

also be attributed to the persistent downregulation of SERCA2a, as the expression of this channel remained low in the treated group compared with the control. Nevertheless, we acknowledge that the expression of SERCA2a may not be the sole determinant of heart contractility.

Our findings regarding SERCA2a or GPR78 are not consistent with the current literature, as most studies have reported positive effects of DPP-IV inhibitors on the expression of SERCA2a and ER stress markers. For instance, sitagliptin treatment improved the downregulation of SERCA2a in ventricular myocyte from spontaneously hypertensive rats (T. Lee et al., 2013). Similarly, Aroor et al. (2013) demonstrated that linagliptin, another DPP-IV inhibitor, increased SERCA2a expression in Zucker-obese rats. In addition, sitagliptin reversed the upregulation of GRP78 and C/EBP homologous protein (CHOP) in the aorta of rats fed a high-fat diet (Cao et al., 2021) . It also reduced the upregulation of GRP78 in the testes of STZ-diabetic rats (Kizilay et al. , 2021). Moreover, sitagliptin treatment corrected the increased mRNA expression of CHOP, another ER stress marker, in the livers of insulin-resistant rats (Ahmed, Ali, Mohamed, Rashed, & Mohamed, 2021).

In our study, we also investigated the β_3-AR-mediated cardiac response. Using the Langendorff heart preparation, we observed that the β_3-AR-mediated negative inotropic effect was augmented in the diabetic heart, consistent with our previous findings (Kayki-Mutlu, Arioglu-Inan, Ozakca, Ozcelikay, & Altan, 2014). Furthermore, we noted an upregulation of β_3-ARs, which is in line with our previous studies of this subtype being elevated in STZ-diabetic rats (Dincer et al. , 2001; Amour et al., 2007). Sitagliptin treatment had no effect on the augmented relaxation response; however, it markedly reduced the upregulation of β_3-ARs. Given that the signalling pathway of cardiac β_3-ARs is proposed to involve nitric oxide (NO)(Gauthier et al., 1998), we examined the expression of eNOS, the enzyme responsible for NO production in cardiac tissue. The expression of eNOS was comparable across all the groups. We also measured the expression of phosphorylated eNOS (p-eNOS), as it has been suggested that diabetes may alter the phosphorylation of this enzyme without affecting the total protein levels (Kayki-Mutlu et al. , 2014). However, the expression of p-eNOS and the ratio of p-eNOS to eNOS were comparable in all groups, aligning with our previous results (Arioglu-Inan et al. , 2013). In support of our results, Hamdani et al. (2014) also reported unaltered eNOS phosphorylation in sitagliptin-treated obese type 2 diabetic mouse hearts. In contrast, Aroor et al. reported that both total and p-eNOS were upregulated in Zucker-obese rats (Aroor et al., 2013). Therefore, further studies are required to investigate the impact of DPP-IV inhibitors on eNOS/p-eNOS expression.

CONCLUSION

Contrary to numerous studies reporting the beneficial cardiac effects of DPP-IV inhibitors, our study found that sitagliptin treatment did not improve β-AR-mediated contractility in STZ-diabetic rat hearts. The lack of metabolic control in our study does not appear to account for this discrepancy, given that cardiac benefits of this drug class have also been reported in non-diabetic models. However, our current dataset is insufficient to

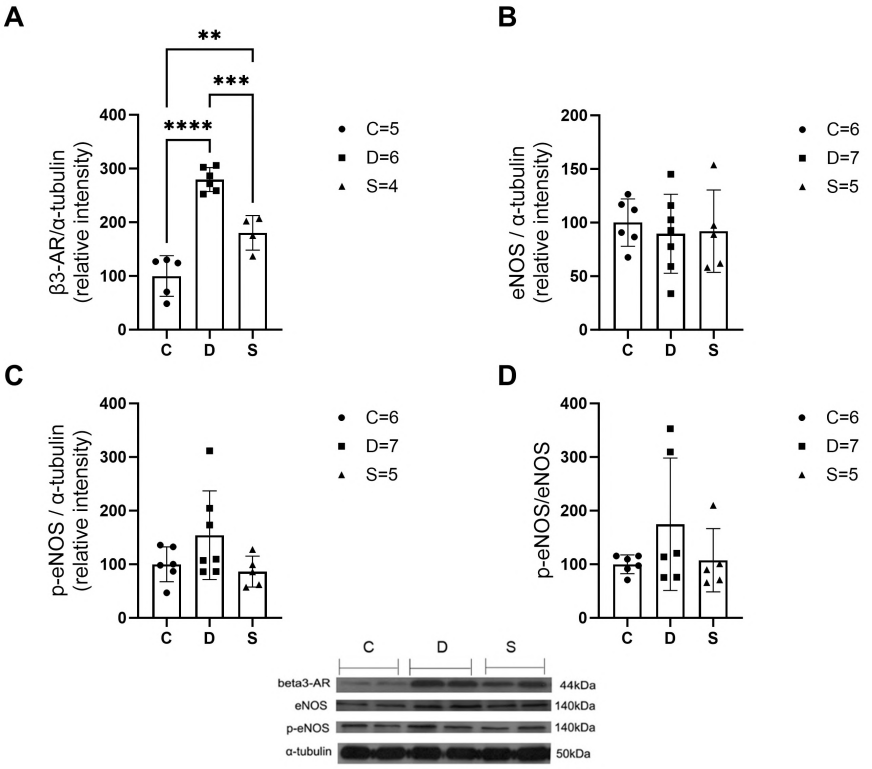

Figure 5. Protein expression levels of β_3-AR, eNOS, p-eNOS, and p-eNOS/eNOS. **A.** % relative intensity of β_3-AR. **B.** % relative intensity of eNOS. C. % relative intensity of p-eNOS. **D.** the ratio of p-eNOS to eNOS. C, control group; D, diabetic group; S, sitagliptin-treated diabetic group. β_3-AR, beta-3 adrenoceptor; eNOS, endothelial nitric oxide synthase; p-eNOS, phosphorylated eNOS. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. **, p<0.01, ***, p<0.001, ****, p<0.0001.

fully elucidate the differences between our findings and those of other research groups. Therefore, future studies are needed to clarify the effects of sitagliptin and other DPP-IV inhibitors on β-AR mediated cardiac responses.

STUDY LIMITATIONS:

This study has several limitations. First, a sitagliptin-treated control group was not included, which precluded us from assessing the potential effects of sitagliptin on the heart of healthy rats. Second, we focused solely on the expression of SERCA2a but not PLN. Exploring alterations in PLN or its phosphorylation could have provided insights into the improved contractile response despite decreased SERCA2a expression. Third, our Western blot experiments did not yield data on β_1-AR protein expression. As a result, we lack information on the components of the β_1- or β_3-AR-mediated signalling pathways in the heart, preventing us from commenting on either β_1-AR-mediated contractility or β_3-AR-mediated relaxation.

Ethics Committee Approval: The study was approved by the local ethical committee of Ankara University (2014-24-161).

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study: C.U.B., E.A.İ., V.M.A.; Data Acquisition: C.U.B., B.R.E., A.E.M., G.K.M., Z.E.Y.D., İ.K., V.M.A., E.A.İ.; Data Analysis/Interpretation: C.U.B., B.R.E., E.A.İ.; Drafting Manuscript: C.U.B., B.R.E., G.KM., E.A.İ., V.M.A.; Critical Revision of Manuscript: C.U.B., B.R.E., A.E.M., G.K.M., Z.E.Y.D., İ.K., V.M.A., E.A.İ.; Final Approval and Accountability: C.U.B., B.R.E., A.E.M., G.K.M., Z.E.Y.D., İ.K., V.M.A., E.A.İ.

Conflict of Interest: The authors have no conflict of interest to declare.

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Original Article

Some histone deacetylase inhibitors protect against dextran sulphate sodium-induced hepatotoxicity in mice

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ABSTRACT

Background and Aims: Ulcerative colitis is an inflammatory bowel disease that affects many people worldwide and has extraintestinal effects. Dextran sulphate sodium (DSS) is a synthetic polysaccharide widely used to model ulcerative colitis in experimental animals. Histone deacetylase (HDAC) inhibitors are molecules that cause changes in gene expression and play a role in many biological events such as inflammatory response formation, cell growth, and differentiation. The aim of this study was to reveal the effects of HDAC inhibitors such as sodium phenylbutyrate (PBA) and suramin on liver morphology, inflammatory mediators, oxidative stress, and antioxidant system in DSS-induced liver injury.

Methods: In this study, 48 male C57BL/6 mice were divided into six groups: control mice; mice administered PBA (150 mg/kg/d, intraperitoneally) or suramin (25 mg/kg/d, intraperitoneally) for 7 days; mice administered 3% DSS orally for 5 days; animals treated with PBA and DSS; and mice treated with suramin and DSS. The effects of PBA and suramin on liver histology were examined microscopically; their impacts on antioxidant parameters and oxidative stress were assessed spectrophotometrically; and their influence on COX-2 and TNF-α expressions was analysed by Western blotting in liver tissues of mice administered DSS.

Results: DSS application resulted in extensive necrosis, increased lipid peroxidation levels, and myeloperoxidase activity, as well as decreased GSH levels and SOD activities in liver tissues. It also increased COX-2 and TNF-α expressions in DSS-induced liver toxicity. PBA or suramin treatment prevented liver injury by mitigating the effects of DSS.

Conclusion: This study showed that PBA and suramin have cytoprotective, anti-inflammatory, and antioxidant effects on DSS-induced hepatotoxicity. Consequently, HDAC inhibitors such as PBA and suramin may be considered effective prophylactic and therapeutic agents against DSS-induced liver injury.

Keywords: Histone Deacetylase Inhibitors, Sodium Phenylbutyrate, Suramin, Dextran Sulphate Sodium, Liver, Mice

INTRODUCTION

Ulcerative colitis is an inflammatory bowel disease (IBD) that begins in the rectum and may spread to the entire colon. It affects not only the gastrointestinal tract but also has extraintestinal effects. Extraintestinal effects commonly seen in IBDs include liver diseases such as nonalcoholic fatty liver disease (NAFLD), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) (Klein et al., 2020; Larsen, Bendtzen & Nielsen, 2010). In addition, drugs used for the treatment of IBD cause liver injury as well as side effects such as abdominal pain, sickness, and pancreatitis (Rogler, 2010). Therefore, new and alternative treatment methods are sought to prevent the disease and increase the quality of life of patients. To elucidate the pathogenesis of colitis and develop effective treatment methods, the dextran sulphate sodium (DSS) induced colitis model is widely preferred (Jurjus, Khoury & Reimund, 2004). DSS, a synthetic polysaccharide, is a polyanionic derivative of dextran produced by esterification with chlorosulfonic acid. Because of its large molecular weight, DSS cannot cross cellular membranes and is poorly absorbed. DSS causes inflammation by disrupting the epithelial cell barrier in the colon (Hu et al., 2017).

Chemical compounds that inhibit histone deacetylase enzymes are known as histone deacetylase inhibitors (HDACi). The mechanism of action of HDAC inhibitors involves the inhibition of histone deacetylation. Because of hyperacetylation, the chromatin structure is loosened, which may enable the initiation of gene transcription. Apart from histone proteins, HDACi also affect pathways related to apoptosis, DNA

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repair, mitotic division, redox pathways, cell cycle progression, and angiogenesis. Therefore, HDACi have important effects on physiological and pathological processes, immune response, apoptosis-autophagy control, and inflammation and are considered therapeutic targets for many diseases, including cancer and inflammatory diseases (Tang, Yan & Zhuang 2013). Histone modifications are believed to contribute to the pathogenesis and progression of various liver diseases, including alcoholic liver disease (ALD), metabolic-associated fatty liver disease (MAFLD), viral hepatitis, autoimmune liver disease, and liver fibrosis or cirrhosis (Cai, Gan, Tang, Wu & Gao, 2021). Due to their roles, reversible histone modifications like acetylation and methylation may represent promising therapeutic targets in these diseases (Arechederra et al., 2021; Claveria-Cabello et al., 2020; Liu et al., 2021). Givinostat, an HDACi, potently alleviated diet-induced hepatic steatosis, inflammation, liver injury, and fibrosis (Huang et al., 2022). Administration of the HDAC inhibitor butyrate in mice with alcoholic liver disease (ALD) alleviates pathological damage and inflammation by suppressing lipopolysaccharide (LPS), tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) levels (Zhang et al., 2024).

Sodium phenylbutyrate (PBA) is an aromatic fatty acid salt consisting of an aromatic ring and butyric acid. PBA inhibits HDAC I and IIA classes, which have various physiological functions such as cell proliferation, cell survival, insulin resistance, and gluconeogenesis (Carducci et al., 2001). PBA is known to have immunomodulatory and anti-inflammatory effects (Park, Lee, Lee, Kim & Kim, 2007; Roy et al., 2012). PBA exhibits antioxidant and hepatoprotective effects in CCl4-induced liver injury, reduces liver enzyme levels in the ischaemia/reperfusion mouse model, and demonstrates antiapoptotic and protective effects on the liver (Lee, Marahatta, Bhandary, Kim & Chae, 2016; Vilatoba et al., 2005).

Suramin is an HDAC inhibitor that can suppress the deacetylase activities of sirtuins (SIRTs) (Li & Alam, 2011). Because of its large molecular structure and charge, it cannot pass through the blood-brain barrier, and its half-life is quite long because of its high binding affinity for serum proteins (Bacchi, 2009; Sahu, Sharma, Singla & Panda, 2017). It is thought that the six sulphonate groups in the structure of suramin are responsible for its superoxide and hydroxyl radical scavenging effects (Sahu et al., 2017). Studies in macrophage-like cell lines and collagen-induced arthritis (CIA) model rats have reported that suramin has anti-inflammatory and antioxidant effects (Firsching, Nickel, Mora & Allolio, 1995; Han et al., 2012). In a liver failure model, it was determined that suramin treatment decreased the mortality rate in mice and suppressed the production of TNF- and IL-6 (Goto et al., 2006). Suramin has been reported to exhibit antioxidant effects on the liver in a collagen-induced arthritis model and to possess hepatoprotective and anti-inflammatory properties in ethanol-induced liver damage (He et al., 2013; Sahu et al., 2017).

The literature review revealed no studies demonstrating the effects of PBA and suramin on liver injury caused by DSS. The aim of this study was to reveal the potential effects of PBA and suramin on liver morphology, oxidative stress, antioxidant system, and inflammatory mediators on DSS-induced hepatotoxicity in mice. In order to better analyse the mechanisms of action of PBA and suramin on liver toxicity, we examined the effects of both histone deacetylase inhibitors comparatively.

MATERIALS AND METHODS

Istanbul University Aziz Sancar Experimental Medicine Research Institute Animal Experiments Local Ethics Committee approved this study with decision letter 07 dated 25.02.2016. In this study, 48 C57BL/6 male mice, 8-10 weeks old, were used. Mice were randomly selected and divided into six groups. Group I, control animals were injected with phosphate-buffered saline (PBS, pH 7.4) as the vehicle. Group II mice were injected with 150 mg/kg PBA intraperitoneally (i.p) once a day for 7 consecutive days. Group III, animals were injected with 25 mg/kg suramin (i.p) once a day for 7 sequential days. Group IV consists of mice administered 3% DSS orally for 5 days. Clinical evaluation of colitis was conducted by determining the Disease Activity Index (DAI) in mice (Cooper, Murthy, Shah & Sedergran, 1993). Mice with a DAI score of ≥3 at the end of the experiment in the DSS-given experimental groups were considered to have ulcerative colitis (Ozal-Coskun, 2018). Group V, animals given both PBA and DSS (in the same dose and time). Group VI, animals that were both injected with suramin and given DSS (in the same dose and time). All injections into mice were performed intraperitoneally at the same time every day at 0.1 mL/day. PBA (Sigma SML0309), suramin (Sigma S2671), and PBS i.p. injections were administered for 7 consecutive days. DSS (MP Biomedicals, MW: 36.000-50,000) application was started on the 3rd day, and DSS was applied for 5 days. On the 8th day of the experiment, all animals were sacrificed under anaesthesia. A schematic of the experimental design is shown in Figure 1.

Histopathological assessment

Samples from liver tissue were fixed in Bouin's fixative and embedded in paraffin. Sections of 5 µm thickness taken from paraffin-embedded tissues were stained with Masson's trichrome and Hematoxylin&Eosin dye and examined with an Olympus BX53F light microscope and photographed with an Olympus DP27-CU camera. In the sections, histopathological criteria such as hypertrophy, vacuolisation, pyknotic nucleus, mononuclear cell infiltration, hyperaemia and necrosis were examined. Each criterion was scored between undamaged (0) and severely damaged (3).

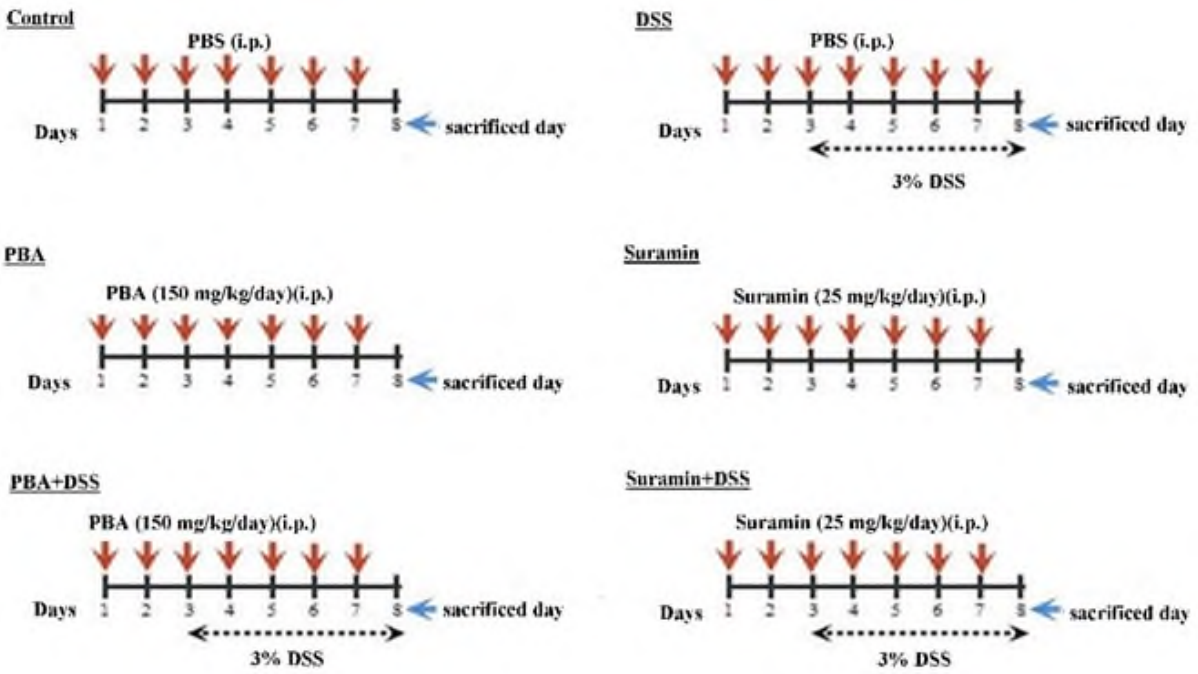

Figure 1. Schematic of the designated experimental groups.

Biochemical analyses

Liver tissue samples were homogenised with 10 mL of 0.9% sodium chloride solution per 1 g of tissue for spectrophotometric analysis. Homogenates were centrifuged at 10.000 g for 15 min (+4°C) and supernatants were used for analysis.

The formation of lipid peroxidation products was assayed by measuring TBARS (thiobarbituric acid reactive substances) levels, which are based on the reaction of malondialdehyde (MDA) with thiobarbituric acid at 532 nm, according to Buege & Aust (1978). The absorbance of the sample was read at 532 nm. The values of TBARS were calculated using an extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} and expressed as nmol/mg protein.

SOD activity was assayed using a standard colorimetric assay in which xanthine oxidase serves as a free radical generator and causes the reduction of nitro blue tetrazolium (NBT). SOD inhibits the reduction of NBT by scavenging the free radicals generated by xanthine oxidase (Aruoma, Halliwell, Hoey & Butler 1989). The reaction was measured spectrophotometrically at OD_{560} nm for 10 min at 25°C. Results were expressed as U/mg protein.

Catalase activity was measured using the method of Aebi (1974). Homogenate was added to the cuvette, and the reaction was started by the addition of freshly prepared H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from the change in absorbance at 240 nm. The activity of catalase was expressed as U/mg protein.

Tissue MPO activity was measured using a modification of the method described by Hillegass et al (Hillegass, Griswold, Brickson & Albrightson-Winslow, 1990). Briefly, tissue samples were homogenised in ice-cold PBS and centrifuged at 13.000 g for 10 min at 4°C. The supernatants were discarded, and the insoluble pellets were rehomogenized in PBS containing 0.5% hexadecyltrimethylammonium bromide (HTAB). After final centrifugation, the supernatants were used to measure MPO. The changes in absorbance at 460 nm were recorded over 5 min. Results were expressed as units of MPO per g of wet tissue.

The level of reduced glutathione (GSH) was measured in liver tissues by the enzymic recycling procedure using 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) and glutathione reductase (DTNB-GSSG reductase recycling assay) as described by Tietze and modified by Anderson (Anderson, 1985). DTNB, distilled water, sample, and stock buffer containing NADPH were mixed in a cuvette and equilibrated to 30°C. To the warmed solution, glutathione reductase was added, and 5-thio-2-nitrobenzoic acid formation was monitored continuously at 412 nm. The GSH content of the aliquot assayed was determined by comparing the rate observed with a standard curve generated using GSH. The results were expressed as nmol of GSH/mg protein.

The protein concentration in the samples was determined using the bicinchoninic acid (BCA) protein assay. The BCA method was performed by optimising the protocol recommended by the manufacturer.

Western blotting

Liver tissue taken for western blot analysis were immediately transferred to liquid nitrogen and stored in a -86°C freezer until the day of analysis. Homogenisation of liver tissue was performed using RIPA buffer containing EDTA and a protease inhibitor. Homogenates were centrifuged at 10.000 g to obtain their supernatants. The amount of protein in the supernatants was determined using the Bradford method (Bradford, 1976). Proteins were separated electrophoretically on a 4-12% Bis-Tris gel (Thermo Fisher Scientific) in MES buffer. Gels were transferred to the nitrocellulose membrane using an iBlot transfer system (Invitrogen, USA). Membranes were blocked with 5% BSA in tris-buffered saline (TBST) for 1 h at room temperature. Primary antibodies were applied to the membranes at +4°C overnight and secondary antibodies for 1 h at room temperature. All antibodies were diluted with 1% BSA (in TBST). The dilution ratio was determined to be 1:2000 for the cyclooxygenase-2 (COX-2) antibody (Novus NB100-689) and 1:1000 for the TNF-α (Abcam ab9739) antibody. β-actin bands were used for normalisation of proteins.

Statistical analysis

All data were evaluated using the SPSS 28.0 statistical programme. The normality test and homogeneity test of variances were applied to the data of all parameters. For the data of all parameters, an independent t-test was used between pairs, following the one-way ANOVA test. The results were given as "mean ± standard error". $p<0.05$ was considered statistically significant.

RESULTS

Histopathological findings

Images of liver sections stained with Masson's trichrome dye and findings regarding liver damage scoring are shown in Figure 2.

Normal histological appearance predominates in the liver sections of the control, PBA, and suramin group mice. Findings such as necrosis, mononuclear cell infiltration, and hyperaemia were not observed in the liver tissue sections of these mice. The finding of widespread severe necrosis in the liver tissue of DSS group mice. In addition, it has been determined that DSS application causes mononuclear cell infiltration in the liver, hyperaemia in blood vessels, and moderate vacuolisation and pyknotic nucleation formation in hepatocytes. When the liver tissue of the DSS+PBA and DSS+suramin group mice are examined, the liver injury caused by DSS is significantly reduced. While necrosis, mononuclear cell infiltration and hyperaemia findings are almost never observed in the liver sections of these two groups, slight vacuolisation was occasionally observed in hepatocytes.

Considering all these parameters, it was determined that the liver damage score in the DSS group increased significantly compared with that in the control group ($p<0.001$). It appears that treatment with PBA or suramin significantly reduces the damage caused by DSS in the liver ($p<0.001$).

Findings of the biochemical analysis

The findings of the biochemistry analysis of all groups are shown in Figure 3.

GSH levels in liver tissue were significantly decreased in the DSS group mice compared with the control group mice ($p<0.001$). There was a significant decrease in GSH levels in the group to which PBA was applied alone compared with the control group ($p<0.001$). PBA treatment caused an increase in low GSH levels caused by DSS in the liver; however, this increase was not statistically significant. In addition, a significant increase in GSH levels was detected in the Suramin group compared with the control group ($p<0.001$). The highest GSH levels among all groups were measured in mice in this group. Suramin treatment of DSS group mice also caused a statistically significant increase in decreased GSH levels ($p<0.001$). GSH levels decreased due to DSS but approached the control group levels after suramin treatment.

When all groups were examined, it was determined that there were higher MDA levels in the liver tissue of the DSS group. In this group, MDA levels were significantly increased compared with the control group ($p<0.01$). In addition, a significant increase in MDA levels was detected in the PBA group compared with the control group ($p<0.05$). It was observed that the administration of PBA to the DSS group did not decrease the increased MDA levels. There was no significant difference in MDA levels between the suramin group and the control group. In addition, it was determined that suramin treatment of DSS group mice caused a significant decrease MDA level ($p<0.001$).

Among the groups, the highest MPO activity in liver tissue was observed in the DSS group. The MPO activity in the DSS group increased significantly compared with the control group mice ($p<0.001$). MPO activity in the PBA group increased significantly compared with the control group ($p<0.01$), whereas there was no significant difference in the suramin group when compared with the control group. Separately administration of both PBA and suramin to DSS group mice significantly reduced the activity of MPO increased with DSS in the liver ($p<0.001$). There was a significant decrease in SOD activity in the liver tissue of the DSS group compared with the control group ($p<0.05$). No significant changes in SOD activity were detected between the PBA and suramin groups compared with the control group. Treatment of PBA ($p<0.01$) and suramin ($p<0.001$) to the DSS group caused a significant increase in SOD activity in the liver homogenates.

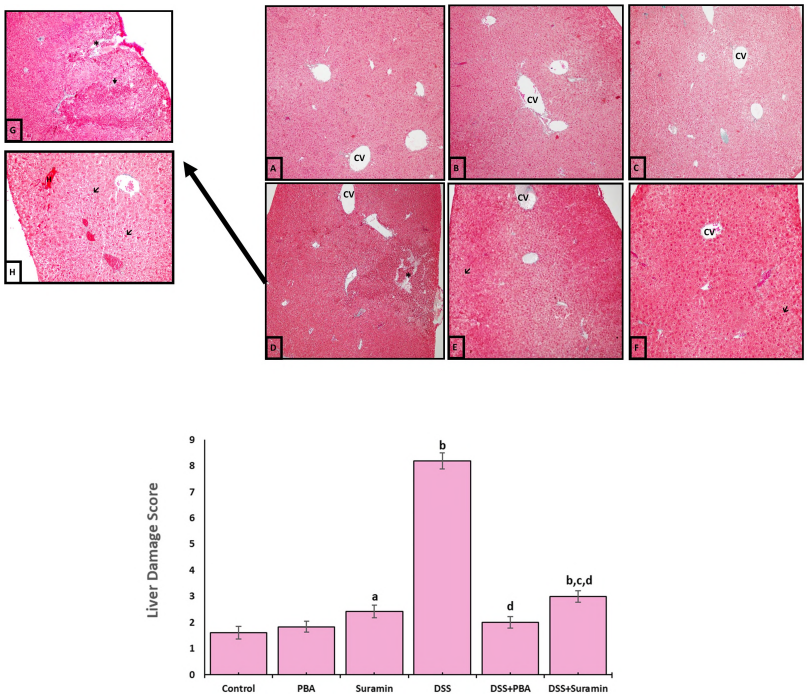

Figure 2. Masson's trichrome staining in the liver of mice in all groups. **A:** Control group, **B:** PBA group, **C:** Suramin group, **D:** DSS group, **E:** DSS + PBA group, **F:** DSS + suramin group. (Original magnification x100). **G:** Widespread necrotic areas on the liver of the DSS-given group, **H:** Severe hyperaemia and vacuolisation observed on the liver section of DSS-given mice. (Original magnification x200). Central vein (CV), Mononuclear cell infiltration (→), necrosis (*), hyperaemia (H), vacuolisation (→). Findings of liver damage score. a: (p<0.05) vs. control group b: (p<0.001) vs. control group c: (p<0.05) vs. PBA group d:(p<0.001) vs. DSS group.

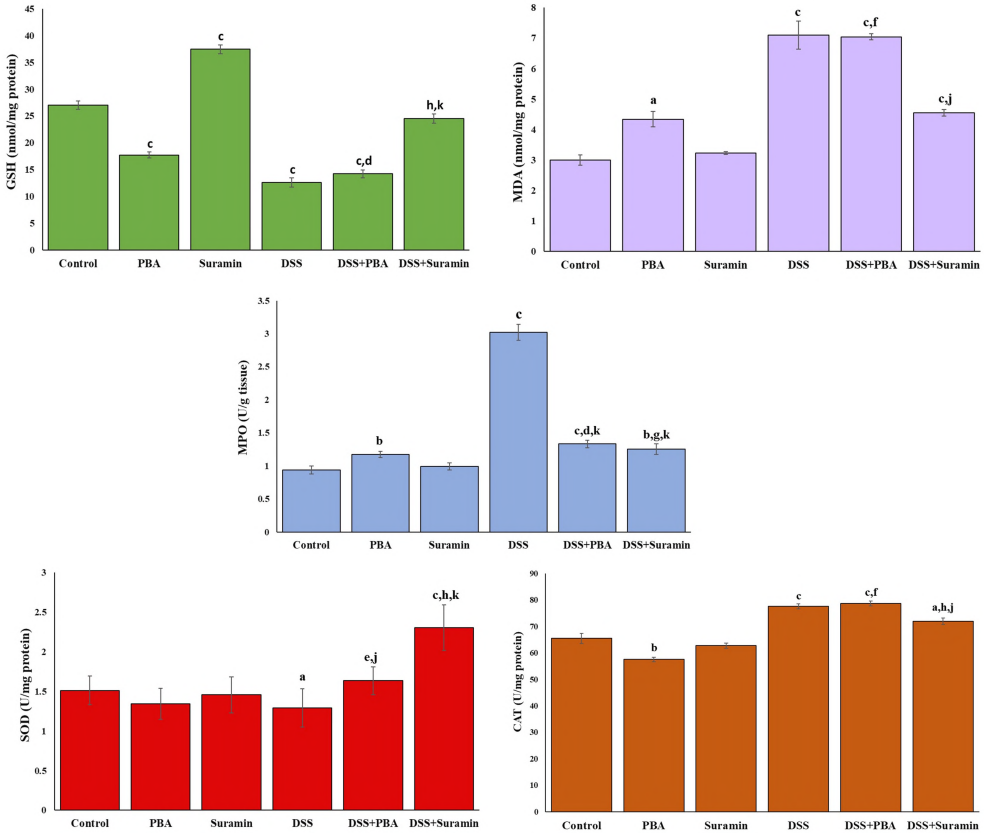

Figure 3. GSH, MDA, and MPO levels and SOD and CAT enzyme activities in liver homogenates according to groups. (Mean ± Standard error). a: (p<0.05) vs. control group; b: (p<0.01) vs. control group; c: (p<0.001) vs. control group; d: (p<0.05) vs. PBA group; e: (p<0.01) vs. PBA group; f: (p<0.001) vs. PBA group; g: (p<0.01) vs. suramin group; h: (p<0.001) vs. Suramin group; i: (p<0.05) vs. DSS group; j: (p<0.01) vs. DSS group; k: (p<0.001) vs. DSS group.

DSS administration did not decrease CAT activity in liver tissue. CAT activity in the PBA group showed a significant decrease compared with that in the control group ($p<0.01$). However, the decrease in the suramin group compared with the control group was not statistically significant. It was determined that PBA treatment of the DSS group caused a non-significant increase in CAT activity compared with the DSS group, but it significantly increased CAT activity compared with the control ($p<0.001$) and PBA ($p<0.001$) groups. Although suramin application to the DSS group caused a significant increase in CAT activity compared with the control ($p<0.05$) and suramin ($p<0.001$) groups, it was determined that there was a decrease in CAT activity in this group compared with the DSS group ($p<0.01$).

Changes in COX-2 and TNF- α protein expression levels

The COX-2, TNF-α and β-actin bands of liver tissue and the COX-2 and TNF-α expression according to the groups are shown in Figure 4.

There was a significant increase in COX-2 and TNF-α expression in the liver tissue of the DSS group compared with the control group ($p<0.001$). It was determined that PBA treatment of the DSS group caused a significant decrease in both COX-2 ($p<0.01$) and TNF-α ($p<0.001$) expression. Suramin treatment also caused a significant decrease in COX-2 ($p<0.001$) and TNF-α ($p<0.001$) expression, which were increased by DSS, similar to PBA.

DISCUSSION

Inflammatory bowel diseases (IBD), such as ulcerative colitis, have extracolonic effects. The most common and serious extracolonic effects of IBD include hepatobiliary abnormalities (Lichtenstein, 2011). It has been found that 5-10% of patients with IBD develop hepatobiliary disorders (Memon, Memon & Memon, 2000). In addition, it has been reported that drugs used for the treatment of IBD can also cause liver damage. (Koller et al., 2017; Mazza et al., 2021). New and effective treatment methods for IBD are sought because of the risk of developing colon cancer, the decreased quality of life in patients, and the serious side effects of the drugs used. Approximately 70% of the liver's blood supply comes from the portal vein, and thus, liver more responsive to the mediators originating from intestine (Adawi, Molin & Jeppsson, 1999).

The pathogenesis of IBD-associated liver injury is typically linked to increased intestinal mucosal permeability. In conditions like ulcerative colitis, the heightened release of inflammatory mediators and toxins in colon tissue triggers inflammation-related signalling pathways upon reaching the liver (Duan et al., 2020). These activated pathways lead to increased production of pro-inflammatory cytokines in the liver, which stimulates hepatocyte damage and inflammation. Primary sclerosing cholangitis (PSC) is typically characterised by fibrosis due to persistent inflammation in the liver, leading to cirrhosis and failure. It is the most common hepatobiliary disease associated with inflammatory bowel diseases (IBDs), with approximately 70-80% of PSC patients also having IBD. (Broomé & Bergquist, 2006). Autoimmune hepatitis (AIH) is characterised by necrosis, severe portal inflammation, and lymphocyte infiltration on liver histology and has been particularly described in patients with ulcerative colitis. (Trivedi & Chapman, 2012).

DSS-induced colitis model is the most widely used animal model to study the pathogenesis and potential therapeutic agents of ulcerative colitis. Various studies have reported histopathological findings such as necrosis, inflammation, and fibrosis in the liver tissues of experimental animals administered DSS to induce colitis (Trivedi & Jena, 2013; Farombi et al., 2016; Li et al., 2021). DSS-induced colitis is characterised by inflammation in the colon and disruption of the mucosal barrier. After 5 days of DSS administration to mice, an increase in the DAI score, inflammation, and oxidative damage in the colon were clearly observed (Ozal-Coskun, 2018). In the current study, DSS-induced hepatotoxicity was examined as an extraintestinal effect of colitis. This study determined that DSS had hepatotoxic effects by causing necrotic damage, which is consistent with findings in the literature. Histone deacetylase inhibitors (HDACi) are known to exhibit anti-inflammatory effects in some inflammatory disease models (Glauben et al., 2006; Sailhamer et al., 2008; Gillespie et al., 2012). PBA is used as a drug in urea metabolism disorders and in the treatment of various diseases. It is an HDACi that has been tested for clinical use (Maestri, Brusilow, Clissold & Bassett, 1996). In our study, it was observed that PBA treatment of mice at a dose of 150 mg/kg for 7 days suppressed the histopathological damage caused by DSS in the liver and showed protective effects. Suramin is an HDACi used for therapeutic purposes against African trypanosoma. In our study, it was determined that suramin, used at a dose of 25 mg/kg for 7 days, reduced the damage to the liver that increased with DSS application, similar to PBA. PBA exhibits anti-inflammatory and anti-apoptotic effects in various liver injury models, protecting the liver against damage and fibrosis. (Wang et al., 2013; Shimizu et al., 2014; Lee, Marahatta, Bhandary, Kim & Chae, 2016). Similarly, studies have shown that suramin has antiapoptotic, anti-inflammatory, antioxidant, and hepatoprotective effects on the liver (Doggrell, 2004; He et al., 2013; Tayel et al., 2014). However, no study has investigated the effects of PBA or suramin on liver injury in DSS-induced ulcerative colitis. In our study, we comparatively examined the antioxidant and anti-inflammatory effects of PBA and suramin on liver injury caused by DSS. Our goal was to understand the protective effects of these agents on liver injury and to explore the potential therapeutic effects of HDAC inhibitors on liver diseases.

MPO is considered an indicator of inflammatory damage and a biomarker of leukocyte infiltration (Zheng, Gao & Wang,

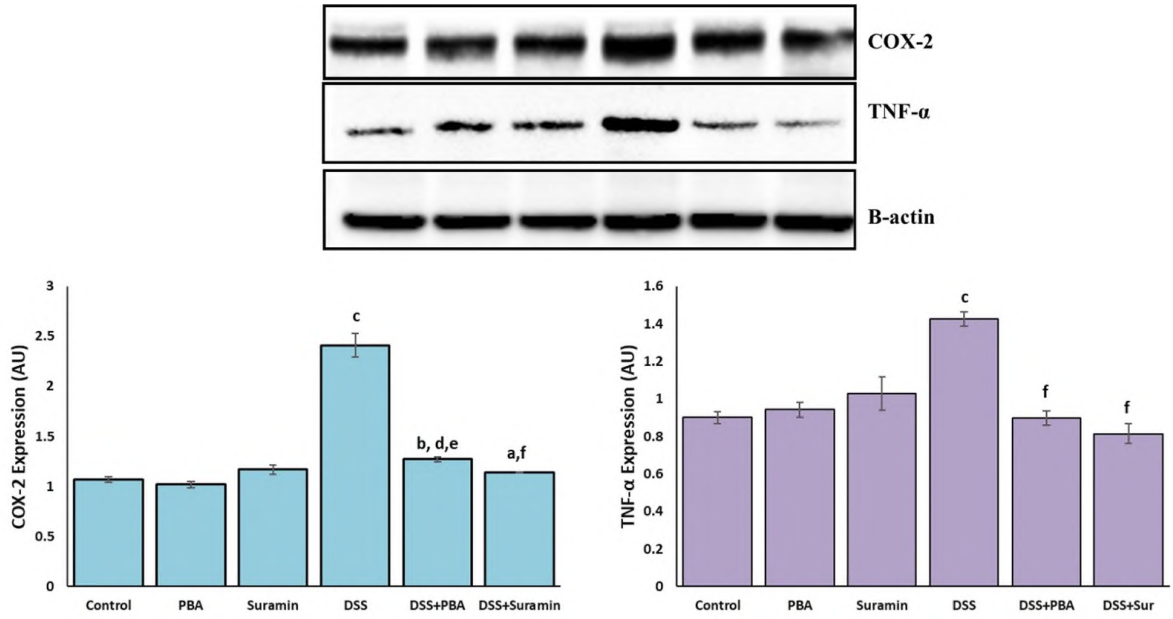

Figure 4. COX-2, TNF-α and β-actin bands of liver tissue and the COX-2 and TNF-α expression level of all groups analysed by western blotting. The data are presented as mean ± standard error (SE). a: ($p<0.05$) vs. control group, b: ($p<0.01$) vs. control group, c: ($p<0.001$) vs. control group, d: ($p<0.001$) vs. PBA group, e: ($p<0.01$) vs. DSS group, f: ($p<0.001$) vs. DSS group.

2000). DSS causes an increase in MPO levels in the colon by stimulating neutrophil infiltration (Sangaraju et al., 2019; Zhao et al., 2022). Oral DSS administration similarly increases MPO levels in the liver tissue. Previous studies have shown that DSS application leads to a decrease in the activities of antioxidant enzymes, including SOD and CAT, in tissues and an increase in the level of MDA, a marker of lipid peroxidation. In the DSS colitis model, it was determined that GSH levels decreased, whereas MPO and MDA activities increased as a result of liver damage. (Trivedi & Jena, 2013). In another study, it was reported that DSS application increased MPO and lipid peroxidation levels and decreased GSH levels and activities of enzymes such as SOD, CAT, and GPx in the liver and colon (Farombi et al., 2016). Several studies have demonstrated that DSS administration leads to a reduction in antioxidant enzyme activities and GSH levels in the liver and an increase in hepatic MPO, LDH, and MDA activities, thereby suppressing the antioxidant system and causing oxidative damage in the liver. (Mouzaoui, Rahim & Djerdjouri, 2012; Rtibi et al., 2016). The increase in MPO levels observed in the liver in our study is an indicator of inflammatory damage caused by leukocyte infiltration in mice treated with DSS. In addition, there was a decrease in SOD levels along with decreased GSH levels and increased MDA levels in the liver tissue in the DSS group compared with the control group. These findings indicate that DSS causes oxidative damage in the liver. PBA has been reported to protect tissue against oxidative damage by reducing the levels of MDA and MPO, which increase as a result of damage in various tissues, and by increasing the levels of antioxidant enzymes (Vilatoba et al., 2005; Daosukho et al., 2007; Jangra, Sriram & Lahkar, 2016). Similarly, suramin has been shown to exhibit antioxidant activity by reducing the levels of MPO and MDA, which increase due to oxidative damage in the liver, and by elevating the levels of SOD (Tayel et al., 2014; Sahu et al., 2017). In our study, it was determined that PBA and suramin application to groups administered DSS decreased MPO levels. Suramin significantly reduced elevated MDA levels in liver tissue, whereas PBA administration did not effectively reduce lipid peroxidation levels. In this study, the fact that PBA and suramin caused a decrease in GSH levels and an increase in SOD enzyme activity with DSS is consistent with the literature. Our study found that DSS application did not decrease CAT levels in the liver tissue. When comparing the effects of the two HDAC inhibitors on the antioxidant system and oxidative stress, suramin was observed to increase GSH levels and SOD enzyme activities in liver tissue more than PBA. In addition, suramin was more effective than PBA in reducing MPO and MDA levels. Considering these findings, we can conclude that HDAC inhibitors such as PBA and suramin protect liver tissue against oxidative damage caused by DSS.

Cytokines are mediators that play a key role in the initiation and development of inflammation and the treatment of inflammatory diseases (Strober & Fuss, 2011). TNF-α is a key pro-inflammatory cytokine in inflammatory bowel diseases, stimulating the release of inflammatory mediators and reactive oxygen species (ROS) (Brown & Mayer, 2007). Mucosal levels of TNF-α are increased in patients with IBD, and anti-TNF-α therapies have been developed with their inhibition and neutralisation (Järnerot, et al., 2005). COX-2 is an enzyme that can stimulate some cytokines, including TNF-α, and is responsible for the formation of prostanoids. It has been reported that

DSS administration increases the levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and COX-2 in liver tissue (Trivedi & Jena, 2013; Farombi et al., 2016). In the acute colitis model induced by 2.5% DSS, the relative mRNA expression levels of inflammatory mediators such as TNF-α, IL-1β, and IL-6 increased in liver tissue (Rohwer, et al., 2023). Various studies have determined that PBA and suramin have anti-inflammatory effects (Novales-Li, 1996; Ono et al., 2014). It has been reported that TNF-α and IL-6 levels, which increase as a result of liver tissue damage, decrease as a result of PBA application (Vilatoba et al., 2005; Qiao, Qian, Wang, Ma & Wang, 2014). Suramin has also been observed to reduce increased levels of cytokines such as TNF-α, IL-1β, and IL-6 in both liver tissue and serum in liver injury models (Goto et al., 2006; He et al., 2013). In our study, it was determined that DSS application caused a significant increase in both COX-2 and TNF-α levels in liver tissue compared with the control group. It was observed that PBA or suramin treatment led to a decrease in COX-2 and TNF-α levels, which increased with DSS. Two different HDACi showed similar effects in suppressing increased COX-2 and TNF-α levels in liver tissue with DSS.

CONCLUSION

As a result, HDAC inhibitors such as PBA and suramin prevent hepatotoxicity by exhibiting cytoprotective, antioxidant, and anti-inflammatory effects on liver injury caused by DSS. Considering these properties, PBA and suramin may be considered effective prophylactic and therapeutic agents against colitis-induced liver injury because they do not cause hepatotoxicity and possess cell-protective effects.

Ethics Committee Approval: İstanbul University Aziz Sancar Experimental Medicine Research Institute Animal Experiments Local Ethics Committee approved this study with decision letter 07 dated 25.02.2016.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study: C.O.C., P.A.; Data Acquisition: C.O.C., N.O., A.U.; Data Analysis/Interpretation: P.A., N.O.; Drafting Manuscript: C.O.C.; Critical Revision of Manuscript: C.O.C., P.A.; Final Approval and Accountability: C.O.C., P.A., N.O., A.U.

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Original Article

Effects of Anatolian propolis on absence seizures and anxiety in rats with genetic absence epilepsy

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ABSTRACT

Background and Aims: Anatolian propolis, which is rich in phenolic compounds, may offer neuroprotective benefits due to its anti-inflammatory and antioxidant properties. This study explored how Anatolian propolis affects the frequency of absence seizures and anxiety levels in rats with genetic absence epilepsy from Strasbourg (GAERS).

Methods: Adult male GAERS were orally administered Anatolian propolis samples at concentrations of 15% (120 mg/kg/day) and 30% (180 mg/kg/day), whereas the control group received an equivalent volume of tap water by oral gavage for 35 days. A 3-h EEG was recorded 9.00 and 12.00 a.m after 35 days of sub-chronic administration. The effects of Anatolian propolis on spike-and-wave discharge (SWD) duration, number, and mean duration of each SWDs were evaluated and compared with the control group. The elevated plus maze test was then performed to measure the anxiety level of GAERS rats. Finally, brains were isolated, and interleukin-1 beta (IL-1β) levels were measured in freshly frozen isolated brains using an ELISA method.

Results: Oral administration of Anatolian propolis (180 mg/kg/day) significantly reduced the number of SWDs and decreased IL-1β levels in the brain tissue of adult GAERS after 35 days of sub-chronic administration ($p<0.05$). Propolis treatment did not alter anxiety levels in terms of time spent in the closed and open arms.

Conclusion: This study represents an initial exploration of the effects of Anatolian propolis on absence seizures in GAERS. Our findings indicate that Anatolian propolis could offer therapeutic advantages by reducing the levels of the brain's pro-inflammatory cytokine IL-1β in GAERS, potentially mitigating absence seizures. However, additional research is necessary to understand the potential mechanisms driving this benefit.

Keywords: Absence epilepsy, Anatolian Propolis, Anxiety, GAERS, IL-1β, Neuroinflammation

INTRODUCTION

Childhood absence epilepsy is a form of genetic generalised epilepsy characterised by bilateral synchronous spike-and-wave discharges (SWDs) at a frequency of 3–4 Hz on EEG, along with behavioural arrest and rhythmic eyelid movements (Crunelli & Leresche, 2002; Hirsch et al., 2022). Approximately 40% of children with absence epilepsy may also have behavioral and cognitive problems (Masur et al., 2013). Currently, the treatment of choice for absence epilepsy alone is ethosuximide (Shorvon, 2011), which is one of the oldest anti-absence drugs that was introduced into clinical practise in 1958 (Zimmerman & Burgemeister, 1958). Gastrointestinal side effects (nausea, vomiting, anorexia, and diarrhea) occur in 4%–29% of patients receiving ethosuximide (Shorvon, 2011). More than 50 years after its induction, ethosuximide still represents the optimal initial empirical monotherapy for absence epilepsy, and no new antiseizure drug has proven significant efficacy against typical absences (Vrielynck, 2013; Brigo, Igwe, & Lattanzi, 2019). Valproic acid and lamotrigine are effective in numerous patients, but they tend to elicit more adverse effects in comparison to ethosuximide (Kessler & McGinnis, 2019). Therefore, there is a need for more effective and better-tolerated treatments for absence epilepsy and related comorbidities (Löscher & Schmidt, 2011).

Propolis is a bee product with antibacterial, antioxidant, anti-inflammatory, and neuroprotective activities(Zulhendri, Perera, & Tandean, 2021). Bees produce it using a combination of beeswax and saliva to protect their hives. In general, propolis contains flavonoids, phenolic compounds, and terpenoids, although its exact chemical composition varies depending on the

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plant species native to the region where it is produced. Propolis exerts anti-inflammatory effects via mechanisms such as the inhibition of cyclooxygenase and prostaglandin synthesis, scavenging of free radicals, immunosuppressive activity, and reduction of inflammatory cytokine levels (Braakhuis, 2019; Zullkiflee, Taha, & Usman, 2022). Anatolian propolis composition differs from that of other propolis samples and has a high concentration of phenolic compounds, such as caffeic acid phenethyl ester (CAPE). CAPE, known for its anti-inflammatory and antioxidant properties, contributes to the neuroprotective potential of Anatolian propolis (Altuntaş, Güzel, & Özçelik, 2023). Preclinical studies testing the effect of bee products on experimental epilepsy models showed that propolis significantly decreased the frequency of seizures and reduced excitability (Kwon et al., 2004; Swamy, Suhaili, Sirajudeen, Mustapha, & Govindasamy, 2014). The effect of propolis on absence seizures has not been evaluated. In this study, for the first time, we aimed to investigate the possible effect of Anatolian propolis on absence seizures, anxiety levels, and brain pro-inflammatory cytokine interleukin-1β (IL-1β) levels in genetic absence epilepsy rats from strasbourg (GAERS), a thoroughly validated and commonly used rat model of genetic generalised epilepsy (Danober, Deransart, Depaulis, Vergnes, & Marescaux, 1998). Adult GAERS rats display SWDs on EEG that closely resemble absence seizures observed in patients with childhood absence epilepsy. Furthermore, their pharmacological response aligns with medications commonly used in clinical practice. Additionally, they exhibit pronounced anxiety- and depression like behaviours (Jones et al., 2008), which are commonly observed comorbidities in patients with epilepsy (Ott et al., 2003). Considering these characteristics, this model is an appropriate epilepsy model to investigate the effects of Anatolian propolis on spontaneous SWDs and anxiety levels.

MATERIALS AND METHODS

Drugs and chemicals

Anatolian propolis 15% and 30% were obtained as a gift from SBS Scientific Bio Solutions Co., Türkiye. BEE'O UP commercially available propolis preparations in standardized doses containing 15% or 30% pure Anatolian propolis extract were used. Extract analyses were already performed at the SBS Scientific Bio Solutions R&D centre, as shown in Table 1.

Animals and Experimental design

Male GAERS rats (n=22) weighing 250–300 g and aged 4-5 months were obtained from Mehmet Ali Aydınlar University, Animal Research and Application Centre (ACU-DEHAM).

All animals were maintained under standard laboratory conditions, with a 12/12 h light/dark cycle, 21±2°C, 45%–65% relative humidity, and free access to food and water. All procedures were carried out in the Experimental Animal Care and Research Unit of Istanbul University Faculty of Pharmacy (EDEHAB), according to approval by the Animal Ethics Committee of Istanbul University (2022/06) conforming with the EU Directive 2010/63/EU for animal experiments of the Istanbul University Local Ethics Committee of Animal Experiments (HADYEK).

Anatolian propolis at concentrations of 15% and 30% (BEE'O UP 15% Soluble Propolis Drops and BEE'O UP 30% Propolis Drops, SBS Scientific Bio Solutions Inc.) were administered orally for 35 days (Table 1). Adult GAERS rats were orally administered Anatolian propolis at a concentration of 15%, at a dose of 120 mg/kg (n=8), and at a concentration of 30% and at a dose of 180 mg/kg (n=6), based on previous studies that demonstrated the efficacy and safety of these concentrations in various experimental models (Oršolić & Bašić, 2003; Zingue et al., 2017; Gocmez et al., 2019; El Adaouia Taleb, Djebli, Chenini, Sahin, & Kolayli, 2020; Guler, Bilir, Kocak, Atas, & Samanci, 2022). The control group (n=8) received the same volume of tap water (Figure 1). EEG recordings were performed to evaluate the effect of Anatolian propolis on absence seizures. Subsequently, the rats underwent the elevated plus maze test to determine their anxiety levels. At the end of the protocol, the brains were isolated for pro-inflammatory cytokine; IL-1β measurements.

Stereotaxic surgery

EEG recording electrodes were implanted into the animals using stereotaxic surgery under ketamine/xylazine anaesthesia (100 mg/kg; 10 mg/kg; i.p.). Before surgery, carprofen (5 mg/kg) was administered subcutaneously for analgesia. The animals' levels of consciousness and anaesthesia depth were evaluated by pinching their hind paws. Cortical screw electrodes were implanted bilaterally into the frontal-parietal cortices for EEG recording. A reference cortical screw electrode was placed on the cerebellum. The EEG screw electrodes were soldered to the microconnectors, which were then fixed to the skull bone with cold acrylic cement. After the surgical procedure, the animals were placed in individual cages and allowed to recover for 1 week.

EEG recordings and analysis

After the recovery period, the animals were placed in plexiglass cages, and 3 h EEG recordings were recorded between 09:00 and 12:00 a.m. (ADI Instruments, Power Lab). EEG signals were amplified using a BioAmp ML 136 amplifier and filtered at bandpass settings of 1–40 Hz. These signals were then recorded and analyzed utilizing the Chart v.7 program (PowerLab8S ADI Instruments, Oxfordshire, UK). The cumulative SWD duration, number of SWDs, and mean duration of SWDs were evaluated after 35 days of oral Anatolian propolis administration.

Table 1. LC-MS/MS analysis of the phenolic substances in 15% and 30% Propolis drop soluble in water.

Phenolic compounds	15 % Propolis	30 % Propolis	Unit
p-Hydroxybenzoic acid	161,4	196,2	mg/L
Epicatechin	225,7	431,5	mg/L
Caffeic acid	858,7	1613,2	mg/L
p-Coumaric acid	392,6	669,4	mg/L
Ferulic acid	743,1	1938,7	mg/L
Resveratrol	32,5	63,9	mg/L
Luteolin	20,1	58,9	mg/L
Quercetin	123,6	249,4	mg/L
t-Cinnamic acid	58,2	186,3	mg/L
Apigenin	343	686,3	mg/L
Hesperetin	284,6	563,8	mg/L
Rhamnetin	374,2	592,8	mg/L
Chrysin	4416	8404,7	mg/L
Pinocembrin	790,3	1714	mg/L
Caffeic acid phenethyl ester (CAPE)	8152,5	11365	mg/L

LC-MS/MS: Liquid chromatography with tandem mass spectrometry.
Adapted from https://www.beeo.com.tr/analizlerimiz#fancybox-grup-84)

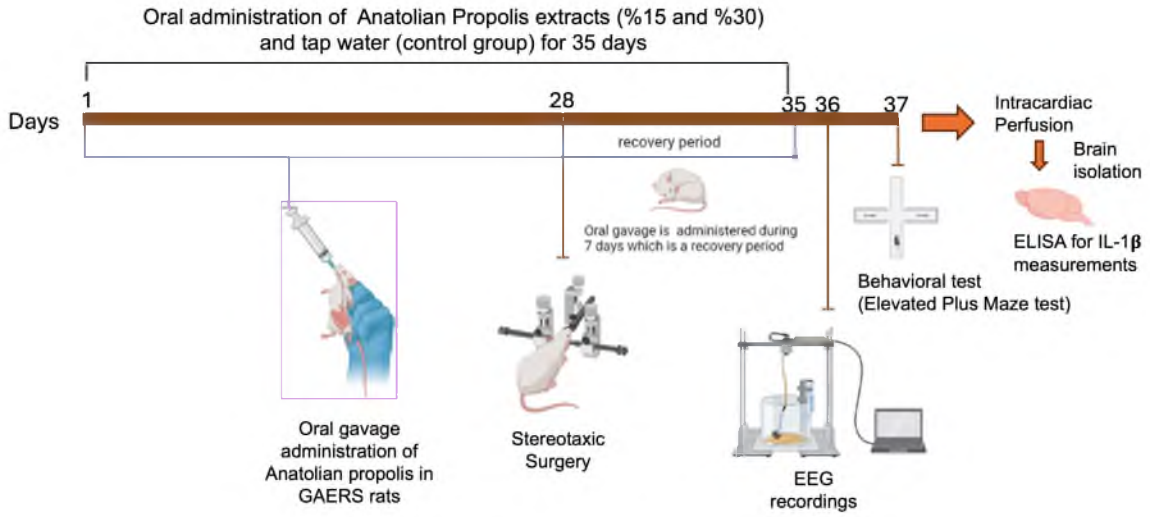

Figure 1. Experimental timeline. Adult GAERS rats were orally administered Anatolian propolis at a concentration of 15%, at a dose of 120 mg/kg (n=8), and at a concentration of 30% at a dose of 180 mg/kg (n=6). The control group (n=8) received the same volume of tap water. On the 28th day after the administration started, rats were implanted with EEG electrodes stereotaxically. After 7 days of recovery on the 36th day, EEG recordings were performed to evaluate the effect of Anatolian propolis on absence seizures. Subsequently, on day 37, rats underwent the elevated plus maze behavioral test to determine their anxiety levels. At the end of the protocol, rats were perfused and then brains were isolated for pro-inflammatory cytokine IL-1β measurements with ELISA metod.

Elevated plus maze test

The anxiety levels of the GAERS rats were measured using the elevated plus maze test. Adult male GAERS rats were placed on a platform that was 50 cm above the ground and consisted of two open arms (50 cm×10 cm), two closed arms (50 cm×10 cm×50 cm), and a central area (10cm×10cm) that connected the four arms. The rats were placed in the central area facing an open arm. Measurements of arm exploration (including duration and frequency of entries on open and closed arms and time spent in open and closed arms) were recorded and scored manually by a blind observer over 5 min (300 sec) (Hu et al., 2017; Mehta, Parashar, & Udayabanu, 2017; Słupski, Trocha, & Rutkowska, 2017). Using these measures, animal anxiety was estimated as an experimental outcome.

Measurement of proinflammatory cytokine (IL-1β) levels in brain tissue

At the end of the behavioural experiments, rats were deeply anaesthetised with ketamine/xylazine anaesthesia (100 mg/kg; 10 mg/kg; i.p.) and transcardially perfused with 0.1 M phosphate buffered saline (PBS). The brains of the rats were isolated and stored at -80 °C until the IL-1β measurements. For IL-1β measurements, brains were weighed and then homogenised in PBS (tissue weight (g): PBS (mL) volume = 1:9) with Omni Bead Ruptor®. The homogenates were then centrifuged for 10 mins 5000 x g at 4 °C to obtain the supernatant. IL-1β measurement in brain supernatant samples was performed using the ELISA method. To determine the dilution ratio that corresponds to the range of optical density (OD) values of the standards provided in the kit, a test was performed with different dilutions of one sample from each group. Based on the appropriate dilution ratio determined from the test, all samples were diluted with the sample diluent provided in the kit and processed according to kit instructions. All samples, standards, and blanks were studied in duplicate, and the average of two values was used for calculations. Diagnostic Automation, Inc.'s DA'R800 spectrometer and KC Junior software were used to determine the optical density values.

Statistical analysis

All data are given as the mean ± standard error of the mean (SEM). In all experiments, "n" represents the number of GAERS rats. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used for the statistical analysis of the effect of propolis on SWDs (cumulative duration, number, and mean duration), IL-1β levels by ELISA, and anxiety scores. Statistical analysis was performed using Graphpad Prism version 9.0.1 program. *p<0.05 was considered statistically significant.

RESULTS

The effect of Anatolian propolis on SWDs

Cumulative SWD duration in the 15% (n=8) and 30% propolis (n=6) groups did not differ from that in the control (n=8) group (p>0.05; Figure 2A). Anatolian propolis administration had a significant treatment effect on the number of SWDs (F (2, 21) = 3.922; P=0.039; Figure 2B). The number of SWDs in the 30% Anatolian propolis group (147.8 ± 15.80) was significantly lower than that in the control (206.4 ± 14.08) group, as revealed by Tukey's multiple comparison test (P=0.039, Figure 2B). No significant change was observed in the mean duration of individual SWDs when comparing the propolis groups with the control group (p>0.05, Figure 2C).

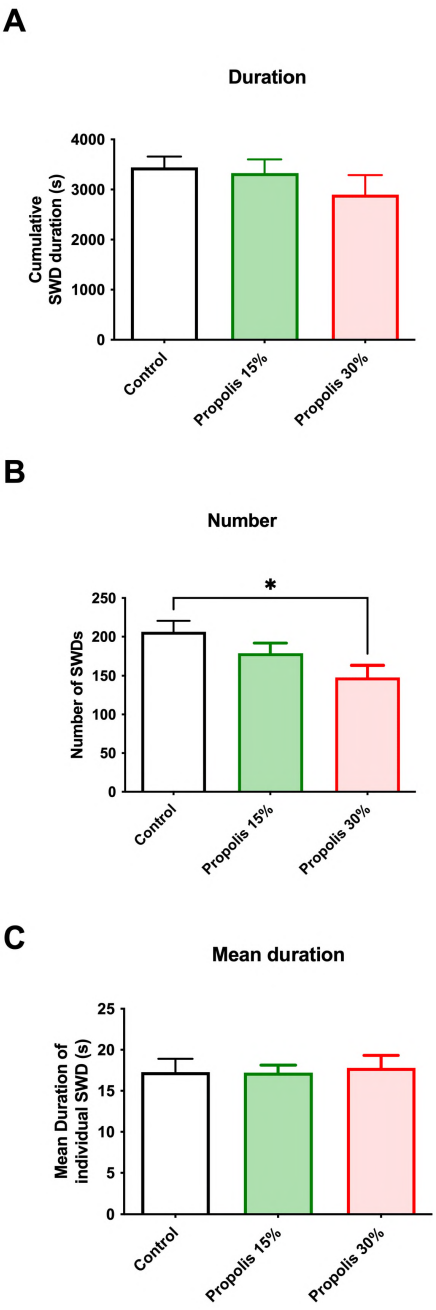

Figure 2. The effect of Anatolian propolis on absence seizures (SWDs). Effect of treatment with Anatolian propolis (15% and 30%) on cumulative SWD duration (A), number of SWDs (B), mean duration of individual SWD (C). *p<0.05, One-way analysis of variance, Tukey post-test. Data were expressed as mean±SEM.

Effect of Anatolian propolis on IL-1β levels

To evaluate whether Anatolian propolis administration affected neuroinflammation in GAERS, we measured IL-1β levels in brain tissue using ELISA. Anatolian propolis administration had a significant treatment effect on IL-1β levels (F (2, 21) = 3.341; P=0.048; Figure 3). Anatolian propolis at 30% concentration significantly reduced IL-1β levels (184.3 ± 15.15)

compared with the control (265.1 ± 33.04) group (P=0.034; Figure 3), as revealed by Tukey's multiple comparison test. Anatolian propolis at a 15% concentration also reduced IL-1β levels, but this change did not achieve statistical significance (p>0.05; Figure 3).

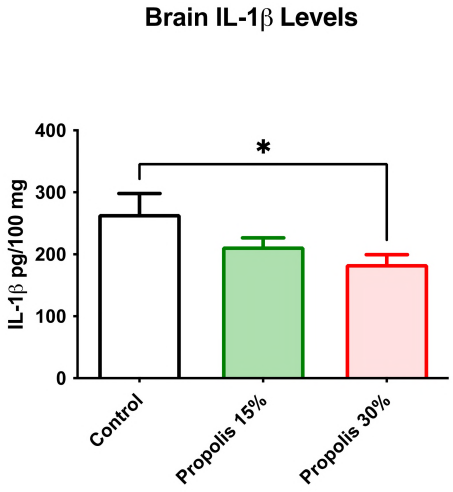

Figure 3. The effect of Anatolian propolis on IL-1β levels. Effect of treatment with Anatolian propolis (%15 and %30) on brain IL-1β Levels in GAERS, * p<0.05, One-way analysis of variance, Tukey post-test. Data were expressed as mean±SEM.

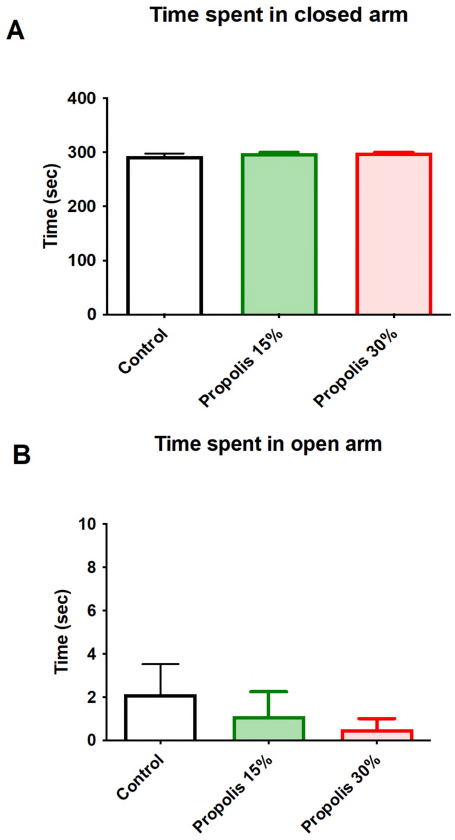

Figure 4. The effect of Anatolian propolis on anxiety levels. Effect of treatment with Anatolian propolis (%15 and %30) on time spent in closed (A) and open (B) arms. One-way analysis of variance, Tukey post-test. Data were expressed as mean±SEM.

Effect of Anatolian propolis on anxiety levels

After propolis administration, we compared the effect of propolis on the time spent in the open and closed arms to estimate the anxiety level of GAERS rats. No significant change was observed in the time spent in the closed and open arms in the propolis groups compared with the control (Figure 4A-B, p>0.05).

DISCUSSION

In the present study, oral administration of 30% Anatolian propolis (180 mg/kg/day) reduced the number of SWDs by decreasing brain pro-inflammatory cytokine; IL-1β levels in GAERS after 35 days of sub-chronic administration. However, propolis treatment did not change the anxiety levels in terms of the time spent in the closed and open arms. This is the first study to demonstrate the effect of Anatolian propolis on SWDs and brain IL-1β levels in GAERS.

Limited data have demonstrated that propolis significantly attenuates seizures in temporal lobe epilepsy rat models (Kwon et al., 2004). This effect has been attributed to the neuroprotective and anti-inflammatory effects of propolis (Kwon et al., 2004; Mannaa, El-Shamy, El-Shaikh, & El-Kassaby, 2011; Kulkarni, Vaidya, Narula, & Sharma, 2021; Zulhendri et al., 2021). Our results also confirmed these findings, suggesting that the effect of propolis on absence seizures in the GAERS model may be associated with its anti-inflammatory properties, potentially mediated by a decrease in the pro-inflammatory cytokine IL-1β.

Considering that the exact substance in propolis that is responsible for this effect has not yet been identified, CAPE is a possible candidate (Kulkarni et al., 2021). Anatolian propolis composition differs from other propolis samples and boasts an exceptionally rich content of phenolic constituents, notably CAPE, which is renowned for its neuroprotective potential due to its anti-inflammatory and antioxidant properties (Altuntaş et al., 2023). The anti-inflammatory and antioxidant properties of Anatolian propolis containing high levels of CAPE might play a significant role in attenuating absence seizures in GAERS. Further studies investigating the specific role of CAPE in absence seizures might provide better insight into the involvement of neuroinflammation in absence epilepsy.

The increase in the expression of the pro-inflammatory cytokine IL-1β was shown to be linked to the onset of absence seizures in GAERS. In rat models of genetic absence epilepsy, there was an observed tendency for the expression of IL-1β to increase before the onset of seizures. (Akin et al., 2011). Our data showing the reduced IL-1β levels in Anatolian propolis-

treated GAERS demonstrate that blocking IL-1β biosynthesis as a specific anti-inflammatory approach that may be helpful for managing absence epilepsy. Research further supports this by showing that in the GAERS model, a specific anti-inflammatory approach suppressed the progression of absence seizures and comorbid depressive-like symptoms. This approach involved blocking the IL-6 signaling pathway using tocilizumab, a monoclonal antibody targeting the IL-6 receptor (Leo et al., 2020). In line with previous studies, levels of IL-6 and IL-8 in the CSF fluid were found to be linked with childhood absence epilepsy in humans (Billiau et al., 2007), and valproic acid treatment lowers plasma levels of IL-6 in children with tonic-clonic generalised seizures (Steinborn et al., 2014). Addressing pro-inflammatory cytokines and chemokine could offer a promising avenue for developing targeted anti-epileptogenic therapies aimed at managing non-convulsive epilepsy and related neuropsychiatric comorbidities.

There are studies showing the anxiolytic effects of propolis (Reis et al., 2014; Da Silveira et al., 2016) in experimental animals. In the present study, however, anxiety levels did not differ between the propolis administration and tap water-treated control groups. This finding could be attributed to variations in the experimental setup for measuring anxiety levels or the duration of propolis treatment. Longer treatment protocols with propolis extract or CAPE may reveal promising effects on anxiety levels in GAERS.

As confirmed by our findings, previous studies have largely demonstrated the beneficial effects of bee products, such as propolis, on various health conditions, including their antioxidant, anti-inflammatory, and neuroprotective properties. However, emerging evidence shows that not all bee products exert beneficial effects. For instance, Kuru et al. (2014) explored the effect of toxic honey, specifically grayanotoxin-containing honey, in genetic absence epilepsy rat model (Kuru et al., 2014). Bees that feed on the nectar of certain Rhododendron species produce this type of honey, also known as "mad honey." The intracerebroventricular administration of toxic honey led to generalised seizures in both GAERS and non-epileptic Wistar rats. This finding is particularly intriguing because it highlights the dualistic nature of bee products, in which certain types can intensify rather than alleviate seizure activity. Our study underscores the importance of considering the specific type and source of bee products when evaluating their neurological effects. It also emphasises the need for rigorous chemical characterisation and standardisation of bee products used in experimental settings. Thus, although the therapeutic potential of bee products is promising, it is crucial to recognise and account for the variability in their chemical composition and biological effects.

CONCLUSION

Overall, Anatolian propolis (30%) treatment reduced pro-inflammatory cytokine (IL-1β) levels in GAERS but did not modify anxiety levels. The potential effects of Anatolian propolis in absence seizure and anxiety have not been evaluated in GAERS, and thus, this study constitutes original findings on this promising relationship with the anti-inflammatory effect of Anatolian propolis and absence seizure in epilepsy. On the other hand, addressing some limitations in the current study may provide a better understanding of the anti-inflammatory effects of Anatolian propolis. We suggest that measurement of plasma IL-1β levels along with brain sample measurements may be more supportive of the correlation between the systemic and local anti-inflammatory effects of Anatolian propolis treatment. Further investigation to understand the neuroprotective effect of Anatolian propolis in relation with absence seizure will be an intriguing research target in favour of epilepsy and its associated neuropsychiatric comorbidities.

Ethics Committee Approval: All procedures were carried out in the Experimental Animal Care and Research Unit of Istanbul University Faculty of Pharmacy (EDEHAB), according to approval by the Animal Ethics Committee of Istanbul University (2022/06) conforming with the EU Directive 2010/63/EU for animal experiments of the Istanbul University Local Ethics Committee of Animal Experiments (HADYEK).

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Author Contributions: Conception/Design of Study: B.Y., N.Ç.; Data Acquisition: B.Y., Ş.A.P., E.C .; Data Analysis/Interpretation: B.Y., Ş.A.P., E.C., D.K.D., N.Ç.; Drafting Manuscript: B.Y., E.C.; Critical Revision of Manuscript: Ş.A.P., D.K.D., N.Ç.; Final Approval and Accountability: B.Y., Ş.A.P., E.C., D.K.D., N.Ç.

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Original Article

High Performance Liquid Chromatography-Tandem Mass Spectrometric determination of carcinogen nitrosamine impurities from pharmaceuticals and DNA binding confirmation aided by molecular docking application

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ABSTRACT

Background and Aims: Nitrosamines are amine compounds attached to a nitroso group. The reaction of amines and nitrosating agents forms nitrosamines. Nitrosamine impurities are classified as Class 1 based on the carcinogenicity and mutagenicity data by ICH M7 (R1). The recent discovery of nitrosamines in some pharmaceutical products has caused concern. Nitrosamines are carcinogenic, so it is necessary to determine the possible nitrosamines in pharmaceutical products.

Methods: An Inertsil ODS-3, (4.6x250 mm, 5 µm) column was used for separation. A triple quadrupole mass detector with electrospray Ionisation (ESI) was used for detection. Multiple reaction monitoring (MRM) was used for quantification. The transition ions are 75.1 > 43.3 for NDMA and 103.0 > 75.1 for NDEA. The calibration curves consist of 5 concentration levels, including NDMA and NDEA (5, 10, 50, 100, 150 ng/mL). The mean r^2 value was 0.997 for NDMA and 0.999 for NDEA. For NDMA and NDEA, LOD: 2 ng/mL, LOQ: 5 ng/mL.

Results: Comprehensive in silico and in vitro results indicate that the method has good accuracy and precision.

Conclusion: DNA binding interactions of the molecules NDMA and NDEA, were investigated through the molecular docking and molecular dynamics methods. Molecular Docking simulations showed that these small organic molecules have high-affinity scores and strongly bind to the minor groove of the hDNA via strong hydrogen bonds.

Keywords: DNA binding, Molecular docking, MD, Mass spectrometry, Nitrosamine, NDMA, NDEA

INTRODUCTION

Nitrosamines are amine compounds attached to a nitroso group (R1N(-R2)-N=O). Nitrosamines are formed by the reaction of amines and nitrosating agents under acidic conditions (Figure 1). Nitrosamines are amine derivatives that are generally volatile and chemically stable (Control of nitrosamine impurities in human drugs, 2021).

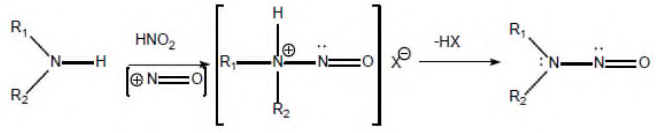

Figure 1. The nitrosamine formation reaction

N-nitroso compounds, which began to be used as solvents in the industry in the 1930s, were later observed to have hepatotoxic effects. Then, due to studies on experimental animals, it was understood that these compounds were not only hepatotoxic but also potent carcinogens (Barnes & Magee, 1954; Magee & Barnes, 1956). In the 1960s, after it was reported that NDMA (N-nitrosodimethylamine) was formed in meat that used nitrite as a preservative, research was started on environmental samples. (Nawrocki & Andrzejewski, 2011; Preussmann, 1984).

The unexpected detection of nitrosamine impurities has highlighted the need to determine their presence in any pharmaceuticals (such as metformin, ranitidine, etc.). In July 2018, the FDA (Food and Drug Administration) and EMA (European Medicines Agency) announced that NDMA and NDEA (N-Nitrosodiethylamine) are found in the drugs, known as "sar-

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tans", used to treat patients with hypertension. In September 2019, Unacceptable levels of NDMA were found in some common heartburn products (ranitidine, commonly known as Zantac, and nizatidine, commonly known as Axid). In December 2019, some countries reported the presence of NDMA in metformin, which is used for treating type 2 diabetes. In May 2020, tests proved that certain metformin lots contained NDMA above the acceptable intake limit. The FDA and EMA evaluated the processes used in API synthesis and found that common manufacturing processes can reveal other nitrosamine impurities besides NDMA (Nitrosamine impurities in human medicinal products, 2020).

Nitrosamine impurities are classified as Class 1 based on carcinogenicity and mutagenicity data by ICH M7 (R1) (M7(R1) assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk, 2018). Nitrosamines are classified as probable human carcinogens by the IARC (International Agency for Research on Cancer) (Ponting, Dobo, Kenyon, & Kalgutkar, 2022). These Nitrosamine impurities affect the genetic material through the DNA adduct. Since the damage caused to DNA by low numbers of nitrosamines threatens human health, it is necessary to determine very low numbers of possible nitrosamines in drug products.

Nitrosamine impurities can be incorporated into drug products mainly through processing, direct administration, cross-contamination, or degradation. The manufacture of drug products includes raw materials, intermediates, reagents, chemicals, and solvents. During these stages, if these impurities are formed, they can be incorporated into the drug products. Secondary, tertiary, and quaternary amines and nitrosating agents such as sodium nitrite are considered to be precursors for the formation of nitrosamine impurities. Recovered solvents and catalysts can pose a risk of nitrosamine formation. These solvents or catalysts can cause the formation of nitrosamine impurities as they are treated with sodium nitrite or nitric acid to remove the residual azide. Contaminated starting material or raw material supplied by the vendor may introduce nitrosamine impurities into the drug product. Cross-contamination in production processes can lead to contamination of nitrosamine impurities. The use of certain packaging materials for the finished product may form nitrosamine impurities. According to one hypothesis, a packaging material containing nitrocellulose can react with amines in the printing ink to form nitrosamine impurities in the drug product. Trace numbers of these impurities may form due to the degradation of the solvents or other materials used in the synthesis of drug substances. Similarly, by-products formed in the drug synthesis process can be transferred to drug substances as nitrosamine impurities. Solvents such as dimethylformamide, dimethylacetamide, or diethylacetamide can form NDMA and NDEA impurities. Experts suggest that the NDMA impurity in valsartan may have come from sodium nitrite, which is used to remove the residual sodium azide (NaN_3) reagent. Under acidic conditions, the nitrite ion forms nitrous acid, which can then react with traces of dimethylamine, a degradation product of the dimethylformamide (DMF) solvent, to form nitrosamine (Figure 2) (Sedlo, Kolonić, & Tomić, 2021; Shaikh, Gosar, & Sayyed, 2020).

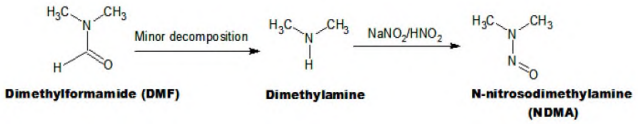

Figure 2. Formation of N-nitrosodimethylamine

The FDA calculated the limit for NDMA and NDEA impurities as 96.0 ng/day and 26.5 ng/day, respectively (Control of nitrosamine impurities in human drugs, 2021).

Some HPLC methods have been reported for nitrosamine impurities. The determination of nitrosamines in food products using the HPLC-UV method with liquid-phase extraction was reported (Li et al., 2018). The determination of nitrosamine in meat using the HPLC method was reported (Al-Kaseem, Al-Assaf, & Karabeet, 2014). An C18 (4.6 × 150 mm, 5 µm) column was used for chromatographic separation. The compounds were detected at a 231 nm wavelength using a spectrophotometric detector. Masada et al. determined the LOD and LOQ for NDMA in valsartan as 8.5 ng/mL and 28.5 ng/mL, respectively (Masada et al., 2019).

Some LC-MS/MS methods have been reported for nitrosamine impurities. The characterisation of nitrosamines in water using the LC-MS/MS method was reported by Zhao et al. (Zhao, Boyd, Hrudey, & Li, 2006). The detection limits were 0.1-10.6 ng/L. Ngongang et al. (Ngongang, Duy, & Sauvé, 2015) reported that nitrosamines can be analysed using LC-MS on a Q-Exactive instrument. The detection limits ranged from 0.4 to 12 ng/L. Scherf-Clavel et al. (Scherf-Clavel et al., 2019) quantified trace amounts of NDMA and NDEA impurities in valsartan samples using the LC-MS method. NDMA was formed during the chlorination of ranitidine as reported by Roux et al. (Roux, Gallard, Croué, Papot, & Deborde, 2012). The detection limit for NDMA formation by ozonation of ranitidine was reported at 4 ng/mL by Lv et al. (Lv, Wang, & Li, 2017).

Atmospheric Pressure Chemical Ionisation (APCI) is a popular complementary soft ionisation technique to Electrospray Ionisation (ESI). LC-MS/MS studies of nitrosamines in the literature have mostly used the APCI ion source. This choice is primarily due to its ability to reduce noise in complex and varied matrices, minimise matrix effects, and analyse very low concentrations. However, the APCI ion source is not as widely used as the ESI ion source, which is more common in LC-MS/MS. In this study, low detection limits were achieved using the ESI ion source. However, by eliminating matrix effects through sam-

ple preparation techniques, the ESI source can also be used efficiently (Kul & Sagirli, 2023).

The molecular docking approach can be used to model the interaction between a small molecule and DNA and a protein at the atomic level, which allows us to characterise the behaviour of small molecules in the binding site of the target (McConkey, Sobolev, & Edelman, 2002). Essentially, molecular docking aims to predict the ligand-receptor complex structure using computation methods. Genotoxicity and carcinogenicity data on nitrosamines are sparse (Thresher et al., 2020). It is thought that some DNAs will guide nitrosamine interaction studies (Li & Hecht, 2022).

The developed and validated method offers an accurate and reliable method for pharmaceutical products, with a calibration curve prepared only by the pre-extraction method and placebo.

MATERIALS AND METHODS

Chemicals and solutions

NDMA and NDEA were purchased from a commercial vendor (Sigma-Aldrich, Germany) and were used as the standard. Acetonitrile and formic acid were purchased from a commercial vendor (Merck KGaA, Darmstadt, Germany) and were used as the solvent and the mobile phase. Deionised water was obtained from a Milli-Q ultrapure water system (Millipore, Barnstead). All reagents and solvents used were of the best analytical grades available.

Chromatographic conditions

The Agilent Technologies liquid chromatography system and 6460 Triple Quad detector system were used in this study. A Inertsil ODS-3, (4.6x250mm, 5µm) column maintained at 40°C was used for separation. The Liquid chromatography mobile phase consisted of acetonitrile:1% formic acid in deionised water (70:30% v/v). The flow rate was 0.600 mL/min. The injection volume was 25.0 µL and the runtime was 10.0 min.

Mass spectrometry conditions

A triple quadrupole mass detector with electrospray Ionisation (ESI) was used for detection. Multiple reaction monitoring (MRM) was used for quantification. The transition ions were 75.1 > 43.3 for NDMA and 103.0 > 75.1 for NDEA, with a dwell time of 100 ms. Nitrogen gas was used as the collision gas. The optimised conditions were 45 psi for nebuliser pressure, 350°C for the gas temperature, 5 L/min for the gas flow, and 3500 V for the capillary. The fragment voltages were 35 V for NDMA and 52 V for NDEA. The collision energies were 14 V for NDMA and 6 V for NDEA.

Preparation of the stock and working solutions

The certified standards were 5 mg/mL for NDMA and 950 mg/mL for NDEA. Intermediate stock solutions for NDMA and NDEA were prepared by diluting the certified standards with acetonitrile. The calibration curve consists of 5 calibration standards. Quality control (QC) samples consist of 3 standards. Calibration standards and QC samples containing both analytes were prepared by diluting the intermediate stock solutions of NDMA and NDEA with acetonitrile to obtain the following analyte concentrations. 5, 10, 50, 100, and 150 ng/mL were prepared for the calibration standards and 5, 75, and 150 ng/mL for the QC samples.

Method validation

The method was validated according to ICH Q2 (R1) on method validation (Guideline, 2005). The evaluated parameters were system suitability, specificity, carryover, accuracy, precision, linearity, LOD, and LOQ.

System suitability

System suitability tests are required for the analytical methods. The overall purpose of system suitability testing is to monitor chromatographic results to ensure chromatographic compatibility and stable system performance. System suitability tests are specific tests that contribute to analytical methods that give accurate and precise results. The system suitability acceptance limits are given according to the FDA guidelines.

Samples preparation

Effervescent, capsule, and tablet drug products were prepared by extracting with acetonitrile. A calibration curve was prepared for each drug product by adding standard impurities to its placebo. After the vortex and centrifugation processes, the samples were filtered into vials and analysed using the validated method.

Matrix Effect

The matrix effect used in the mass spectrometric analysis should be examined. The matrix may increase or decrease the ion intensity, which must be determined. The matrix effect was calculated from the peak areas of the LOQ prepared in the solvent and the LOQ prepared in the relevant sample placebo. The calculated matrix effect is absent when ME = 0%. Ion suppression occurs when ME > 0%, and ion intensity increases when ME < 0%.

Geometry Optimization of the Ligands

Before the simulation studies, the geometries of the ligands, NDMA and NDEA, were optimized using the Gaussian 09 programme (Frisch et al., 2009) with density functional theory (DFT)/B3LYP functional (Becke, 1993) and utilising the 6-31G(d,p) basis set. This process led to the formation of the most stable molecular structures of NDMA and NDEA with 1BNA, intended for further computational and simulation-based research, as depicted in Figures 3 and 4. For molecular docking and molecular dynamics computations, as well as post-processing of the output files, Gauss View 6.0 and Avogadro 1.95 software programmes (Dennington, Keith, & Millam, 2009) were employed to prepare the input files.

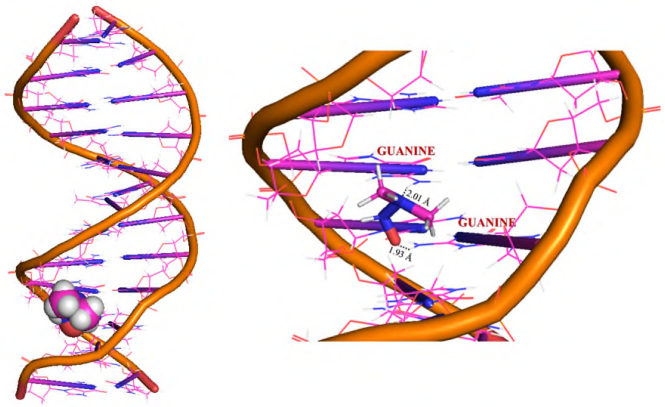

Figure 3. Snapshot pictures taken from the MD simulation, illustrating the stable equilibrium pose of the ligand NDMA in the proximity of the hDNA

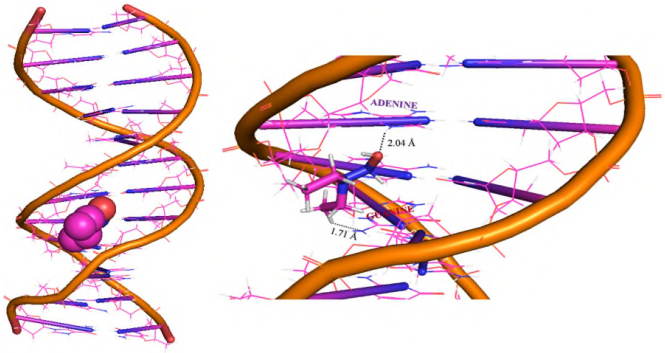

Figure 4. Snapshot pictures taken from the MD simulation, illustrating the stable equilibrium pose of the ligand NDEA in the proximity of the hDNA.

Molecular Docking Procedure

The molecular docking simulations were carried out using the AutoDock Vina 1.1.2 software program (Gaillard, 2018). A total of 400 poses were generated, with 100 poses for each simulation. As a representative of the double helix hDNA structure, 1BNA (PDB NDB ID: BDL001), which is a dodecamer of 5'-D(*CP*GP*CP*GP*AP*AP*TP*TP*CP*GP*CP*G)-3', was retrieved from the PDB database. The grid box dimensions were set to 44 × 44 × 88 $Å^3$ for blind docking. The simulations involved the NDMA and NDEA ligands and their interactions with the receptor structure of 1BNA. The simulations demonstrated the interactions and the drug's binding to the receptor. The docking scores obtained in kcal/mol represent the Gibbs free binding energy. Within all the simulations, the most accurate docking poses and favourable binding energies, identified among the best-clustered data, were selected as the initial molecule design structures and input files for the subsequent molecular dynamics (MD) simulations.

Molecular Dynamics (MD) Simulations

The input files and their corresponding chemical structures for the MD simulations were selected from the docking poses with the most favourable binding energies (Cheraghi et al., 2023; Şenel et al., 2022; Şenel et al., 2020) as shown in the scientific literature. Schrödinger's Maestro Desmond Programme (Desmond, 2017) was utilised for running the molecular dynamics (MD) simulations, each spanning 50 ns with 5000 poses at 10 ps intervals. To ensure accuracy, each MD simulation was repeated three times with varying different seed numbers, confirming the correctness of the simulation parameters and the structures of the complexes formed by the NDMA and NDEA with 1BNA. Throughout the MD simulations, the dynamic characteristics of the ligand-receptor complexes were evaluated longitudinally. A grid box measuring 110 × 110 × 110 $Å^3$ with a 0.5Å spacing was utilised to define the simulation area, allowing for comprehensive coverage during the simulation. TIP3P-type water molecules were incorporated within the grid box, and 0.15 M NaCl ions were introduced to maintain system neutrality. The simulation conditions were set to NPT at 310 K using the temperature coupling equations of Nose-Hoover (Evans & Holian, 1985), alongside a constant pressure of 1.01 bar achieved through Martyna Tobias-Klein pressure coupling (Martyna, Tobias, & Klein, 1994). The system was not constrained, and the default fitting for the OPLS 3.0 standards provided the initial velocity values for the forefield calculations. Hydrogen bonds that formed during the interaction of ligands with the 1BNA structure were investigated. The NDMA- and NDEA-bound DNA complexes were (Figure 3-4) to illustrate the interaction and binding site of the ligands on 1BNA.

RESULTS

Selectivity

The selectivity of the method against the matrix components was evaluated against a blank solvent. No peak at the retention time of the analytes was observed in the solvent chromatogram (Figure 5).

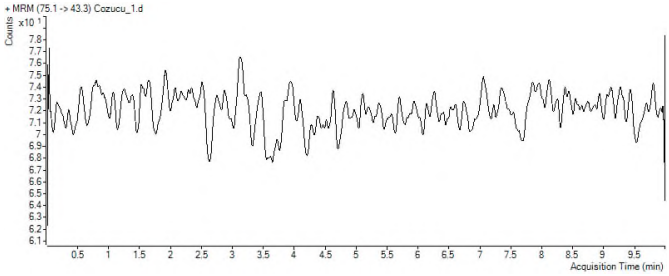

Figure 5. Blank solvent chromatogram

Carryover

During validation, the carryover was determined by the solvent injected between the calibration standards and the QC samples. There was no carryover in the injected solvent chromatogram after injecting the highest concentration level standard solution (Figure 6).

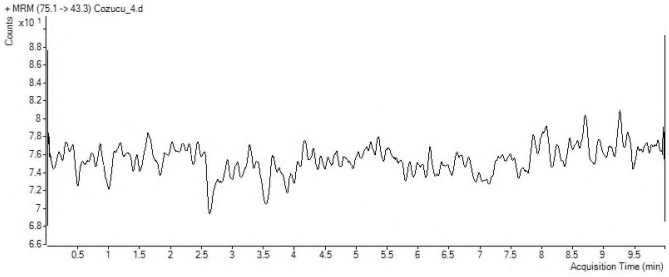

Figure 6. Blank solvent chromatogram after the highest concentration standard solution

Linearity

The linearity of the detector response at decreasing concentrations in the analytical dilutions was investigated. The calibration curves were prepared for the quantification of the samples. The calibration curves consist of 5 concentration levels including NDMA and NDEA (5, 10, 50, 100, 150 ng/mL). The correlation coefficients of the calibration curves are in the acceptable range of 0.99 for both NDMA and NDEA. The accuracy and precision results of the calibration curve concentration levels are given in Table 1-2.

Limit of detection (LOD) and limit of quantitation (LOQ)

The analyte concentrations with a signal-to-noise ratio of ≥ 3.0 and ≥ 10.0 are determined as the LOD and LOQ, respectively. The LOQ is also the initial concentration level of the calibration curve. In the LOD study, it was determined as S/N:3.24 for NDMA and S/N:13.77 for NDEA. In the LOQ study, it was determined that S/N:17.60 for NDMA and S/N:24.79 for NDEA (Figure 7).

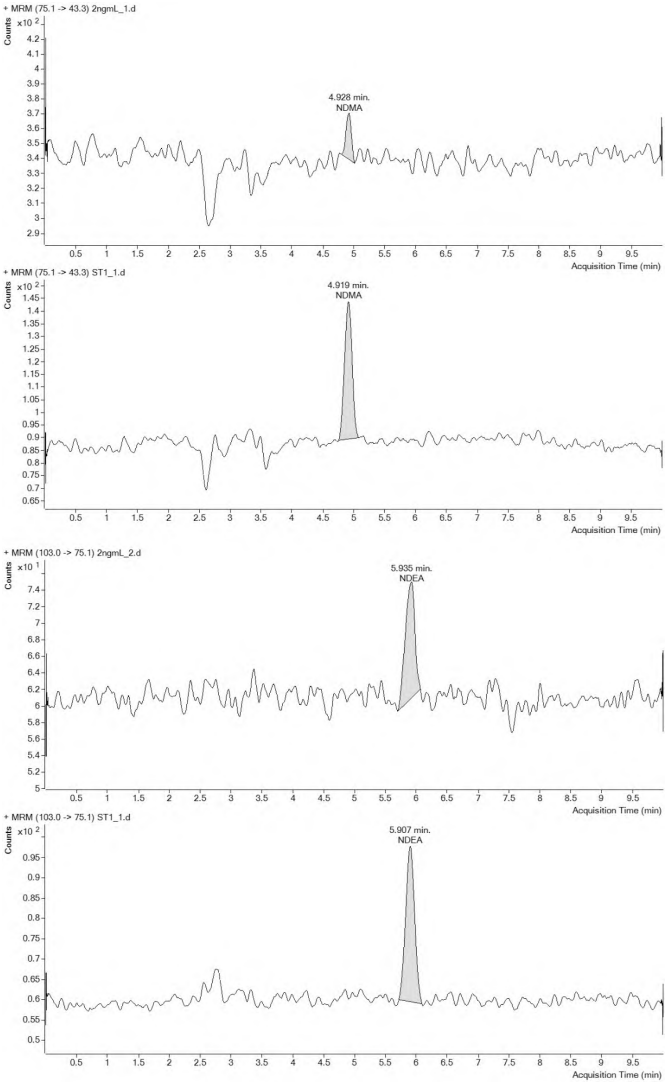

Figure 7. Chromatograms of LOD and LOQ for NDMA and NDEA

Accuracy and precision

The accuracy and the precision were calculated by analysis of low, medium, and high concentration levels of QC samples. The QC samples had 5, 75, and 150 ng/mL concentration levels. The percentage of the coefficient of variation (%CV) and the percentage of recovery were calculated for the presentation of the accuracy and the precision, respectively. A summary of the accuracy and precision is presented in Table 3-4. The results show that the method has good accuracy and precision. The intra-day and inter-day accuracy and precision values for NDMA range from 100.36 to 111.73 and from 1.68 to 5.75, respectively. For NDEA, these values range from 96.24 to 101.81 and from 0.34 to 8.77, respectively.

Table 1. Accuracy and precision results of the calibration standards for NDMA

Calibration standards (ng/mL)	Mean	Recovery %	Standard Deviation (SD)	Coefficient of variation %CV	n
5	4.84	96.73	0.42	8.59	6
10	9.98	99.78	0.68	6.86	6
50	50.29	100.59	2.92	5.81	6
100	99.73	99.73	6.09	6.11	6
150	150.07	100.05	7.74	5.16	6

Table 2. Accuracy and precision results of the calibration standards for NDEA

Calibration standards (ng/mL)	Mean	Recovery %	Standard Deviation (SD)	Coefficient of variation %CV	n
5	4.93	98.65	0.40	8.15	6
10	9.84	98.42	0.73	7.39	6
50	51.11	102.22	1.46	2.85	6
100	99.00	99.00	3.28	3.31	6
150	150.31	100.20	2.69	1.79	6

System suitability

System suitability was calculated as the theoretical plate number (N) > 2000, capacity factor (k') >, resolution (Rs) > 2, and tailing factor (T) ≤ 2 for NDMA and NDEA. Detailed parameters are given in Table 5.

Matrix Effect

The matrix effect was calculated by spiking the matrix (placebo) of a capsule drug product containing the active substance Nizatidine. The matrix effect results were 12.5% for NDMA and 11.6% for NDEA.

Molecular Docking and Molecular Dynamics Results

NDMA is a strong drug impurity and suppresses the DNA via gene silencing, as shown in Figures 3 and 8. It selectively binds onto the minor groove location and chooses solely and strictly Guanine nucleotides with a strong 9.9 kcal/mol inhibition score, Δ(ΔG) for 1BNA. It binds to two Guanine nucleotides simultaneously by forming H-bonds via its amine nitrogen and hydroxyl oxygen at distances 2.01 Å, and 1.93 Å, respectively. The drug impurity positions itself towards the minor groove region of the 1BNA and it is stabilised via the H-bonds. The suppression of 1BNA causes an alteration in the strong, rigid phosphodiester helical structure resembling a bent, distorted shape.

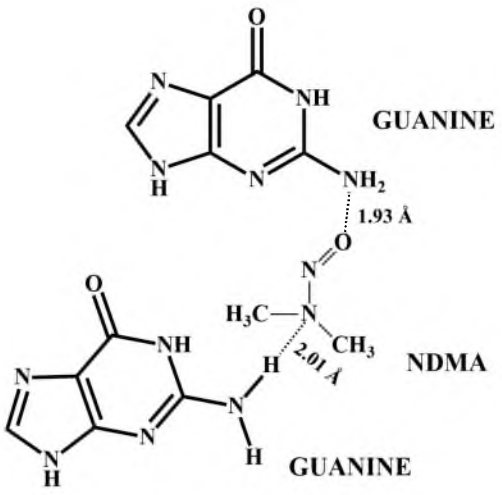

Figure 8. 2D pose of the hydrogen bonding of NDMA's functional groups to Guanine nucleic bases

NDEA is also capable of suppressing the DNA via gene

Table 3. Accuracy and precision results of the QC samples for NDMA

	Intra-day					Inter-day				
QC samples (ng/mL)	Mean	Recovery %	SD	%CV	n	Mean	Recovery %	SD	%CV	n
5	5.59	111.73	0.29	5.22	6	5.42	108.35	0.31	5.75	18
75	78.16	104.21	1.43	1.83	6	78.88	105.17	1.32	1.68	18
150	150.54	100.36	4.06	2.70	6	151.11	100.74	4.31	2.85	18

Table 4. Accuracy and precision results of the QC samples for NDEA

	Intra-day					Inter-day				
QC samples (ng/mL)	Mean	Recovery %	SD	%CV	n	Mean	Recovery %	SD	%CV	n
5	5.08	101.61	0.34	6.66	6	5.05	101.02	0.44	8.77	18
75	76.33	101.78	1.48	1.93	6	76.36	101.81	1.80	2.36	18
150	144.36	96.24	1.47	1.02	6	145.30	96.86	2.03	1.40	18

Table 5. System suitability results and acceptability limits

Parameter	Value for NDMA	Value for NDEA	Limit (FDA guideline)
Retention time (min)	4.912	5.886	-
Peak Width (W)	0.114	0.141	-
Tailing (T)	1	1.1	$T \leq 2$
Theoretical Plates (N)	9259	2220	$N > 2000$
Resolution (Rs)	48.1	23.6	$Rs > 2$
Capacity Factor (k)	490.2	587.6	$k' > 2$

silencing with nucleotide regioselectivity by coordinating itself into the minor groove domain of the hDNA in Figures 4 and 9. Its regioselectivity is equally the same for Guanine and Adenine nucleotides. The inhibition score was a bit higher than NDMA where $\Delta(\Delta G)$ value was found to be -10.3 kcal/mol for 1BNA.

Since 1BNA mimics the human DNA template in the scientific literature, its utilisation under the physiological pH of 7.4 proves us here that these two drug impurities, NDMA and NDEA, cause a DNA suppression, DNA toxicity, with the minor groove type of mode of binding and significantly efficient inhibition scores via strong Hydrogen bonds. In Figure 10, the RMSD data can be observed vindicating the MD results of Figures 3 to 4, where both NDMA and NDEA reach a much more stabilised complex structure, oscillations are much lower compared to hDNA (1-BNA dodecamer) itself. This is the evidence of the great suppressive/inhibitive nature of NDMA and NDEA towards human DNA, it forms a quite strong Hydrogen bonding after 10 ns passes in the simulation and keeps the oscillations of all the atoms of the complex under 0.5 Å.

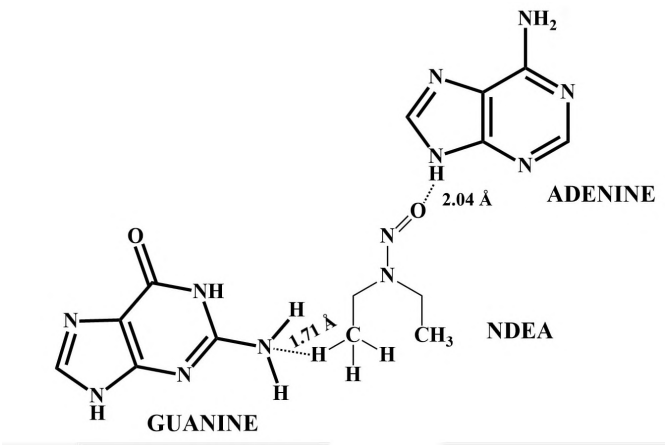

Figure 9. 2D pose of the hydrogen bonding of NDEA's functional groups to Adenine and Guanine nucleic bases

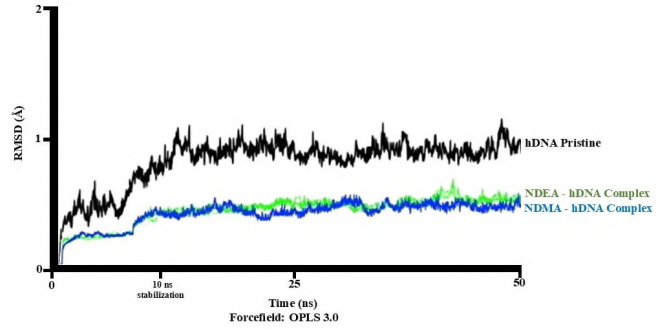

Figure 10. The root mean square deviation (RMSD) plot of the MD result is illustrated. The NDEA-hDNA complex is in green, the NDMA-hDNA complex is in blue, and the hDNA (1-BNA dodecamer) is in black colour

DISCUSSION

Nitrosamine impurities need to be limited to acceptable levels in drug products because of their highly mutagenic and carcinogenic properties. Regulatory authorities such as the FDA and EMA have published several notices to guide drug manufacturers in controlling and analysing these impurities to ensure they remain at acceptable levels. LC-MS/MS and GC-MS/MS techniques are the analytical methods used for the determination of nitrosamine impurities. A potential source of nitrosamine impıirities can enter drug substances, raw materials, reagents, catalysts, and solvents.

The non-bonded interactions are stronger due to the larger size of NDEA than NDMA. One of the hydrogen of the ethyl group approaches Guanine at a distance of 1.71 Å to form a vdW-type interaction, which is absent in NDMA. The typical H-bond occurs hydroxy oxygen of the ligand and Adenine at a distance of 2.04 Å (Figure 4). The binding mode of the drug impurity NDEA is the same as that of NDMA, but the nucleotide preference of oxygen changes to Adenine and the ligand is coordinated onto the minor groove region of the 1BNA.

CONCLUSION

LC-MS/MS is the most reliable technique for determining nitrosamine impurities in drug products. The developed method can detect NDMA and NDEA at trace levels, and it is both fast and simple. In the matrix effect studies, the extraction method yielded low matrix effect results, indicating that very low nitrosamine concentrations can also be effectively detected using the ESI ion source instead of the APCI ion source.

Since 1BNA mimics the human DNA template in the scientific literature, its utilisation under the physiological conditions as a target for the drug impurities, NDMA and NDEA enabled us to show that these small organic molecules have high-affinity scores and strongly bind to the minor groove of the hDNA via strong hydrogen bonds. It is highly probable that they may cause DNA suppression and damage DNA integrity.

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Original Article

Efficacy of aztreonam/avibactam against *Stenotrophomonas maltophilia* alone and in combination with tigecycline

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ABSTRACT

Background and Aims: *Stenotrophomonas maltophilia* is a multidrug-resistant opportunistic pathogen that threatens human and public health because of its widespread intrinsic and acquired antibiotic resistance. These bacteria become resistant to aztreonam by degrading it using the beta-lactamases. The aim of this study was to evaluate the effect of aztreonam/avibactam on aztreonam resistance in clinical strains of *S. maltophilia* and to assess the synergistic potential of aztreonam in combination with tigecycline.

Methods: Minimum Inhibitory Concentrations (MICs) of aztreonam, aztreonam/avibactam, tigecycline, and doxycycline were determined using broth microdilution in sixty-six *S. maltophilia* isolates. Additionally, six isolates with the most common MICs of aztreonam/avibactam against the strains, 2 µg/ml and 4 µg/ml were selected from among the sixty-six tested strains, and the effectiveness of the combination of aztreonam/avibactam with tigecycline was determined using both the checkerboard test and the time-dependent killing method.

Results: Aztreonam/avibactam restored aztreonam activity in 96.9% of resistant *S. maltophilia* isolates. Half of the isolates were susceptible to tigecycline, whereas all were susceptible to doxycycline. The combination of aztreonam/avibactam with tigecycline was found to have an additive effect against all isolates in the checkerboard experiment in which the activity of aztreonam/avibactam in combination with tigecycline was investigated against six isolates. In the time-dependent killing experiment, the combination exerted a synergistic effect against two isolates.

Conclusion: Aztreonam/avibactam appears to be an important alternative for reversing aztreonam resistance in *S. maltophilia*. Additionally, tetracyclines, such as tigecycline and doxycycline, are highly effective against these bacteria. To confirm these promising findings, further *in vitro*, *in vivo*, and clinical studies are required.

Keywords: *Stenotrophomonas maltophilia*, Aztreonam/avibactam, Tigecycline, Combination

INTRODUCTION

Stenotrophomonas maltophilia is a multidrug-resistant Gram-negative pathogen that causes life-threatening infections, especially in immunocompromised and intensive care patients (Liu, Xiang, & Zhang, 2024; Mojica et al., 2022). Natural resistance and multidrug resistance, which occur through the transfer of antibiotic resistance genes via gene transfer mechanisms such as plasmids, cause the treatment options for Gram-negative pathogens to gradually decrease and place an important focus on health studies related to the discovery of new antibiotics. *S. maltophilia* is particularly notable for causing respiratory tract infections, but it can also be encountered as the causative agent of bacteremia, skin and soft tissue infections, osteomyelitis, meningitis, endocarditis, and urinary tract infections (Brooke, 2021). *S. maltophilia* respiratory tract infections can have a mortality rate of almost 50%. In the case of septic shock, this rate can increase (Hafiz et al., 2022). *S. maltophilia* is naturally resistant to antibiotics such as carbapenems and aminoglycosides, which are critical in infections with multidrug-resistant Gram-negative pathogens. Resistance to antibiotics occurs especially in the presence of genes encoding efflux pumps and enzymes that degrade antibiotics (Gil-Gil, Martínez, & Blanco, 2020). Additionally, acquired resistance is common due to the large number of antibiotic resistance genes and gene mutations. The empirical antibiotic treatment approach preferred for other Gram-negative pathogen infections may not be applicable for *S. maltophilia*. In order to prevent unnecessary antibiotic use and

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not contribute to antibiotic resistance, the treatment regimen should be evaluated after reviewing factors such as bacterial culture results, the patient's general condition, and risk factors (Tamma et al., 2023). It has been observed that a history of previous broad-spectrum antibiotic use increases the risk of *S. maltophilia* infections in hospitalised patients (Hafiz et al., 2022; Brooke, 2021).

Efflux pump-mediated resistance is observed against trimethoprim/sulfamethoxazole, which is considered the first step in the treatment of *S. maltophilia*. Similarly, resistance to levofloxacin and minocycline is also transmitted through the presence of various resistance genes and can be transferred between bacteria (Mojica, Bonomo, & Van Duin, 2023; Tamma et al., 2023). A meta-analysis reported that *S. maltophilia* resistance to levofloxacin, trimethoprim/sulfamethoxazole, and minocycline can be observed worldwide (Dadashi et al., 2023).

Serine β-lactamases and metallo β-lactamases, which cleave the β-lactam rings necessary for the antimicrobial activity, inactivate the beta-lactam antibiotics. In order to overcome this critical challenge, β-lactamase inhibitors that inhibit β-lactamases have been synthesized and combined with β-lactam antibiotics. L1 metallo β-lactamase and L2 serine β-lactamase are responsible for the β-lactam resistance observed in *S. maltophilia*. L1 β-lactamases hydrolyse penicillins, cephalosporins, and carbapenems, while L2 serine β-lactamases are responsible for the hydrolysis of aztreonam and extended-spectrum cephalosporins. L2 serine β-lactamases can be inactivated by the β-lactamase inhibitors clavulanic acid and avibactam (Mojita et al., 2022). With the approval of avibactam, a new serine β-lactamase inhibitor, in 2016, its combination with ceftazidime was approved and introduce to increase the effectiveness of ceftazidime (Tyers & Wright, 2019). The combination of aztreonam, a monobactam, and avibactam has been the subject of research in recent years and has been found to be an effective combination (Sader, Carvalhaes, Arends, Castanheira, & Mendes, 2021; Sader, Castanheira, Kimbrough, Kantro, & Mendes, 2023; Cornely et al., 2020). In 2024, aztreonam/avibactam was approved by the European Medicines Agency (EMA) for the treatment of Gram-negative bacterial infections, such as complicated intra-abdominal and urinary tract infections and hospital-acquired pneumonia. Furthermore, EUCAST has published aztreonam/avibactam clinical cut-off values for Enterobacterales (EUCAST, 2024b). In light of new studies, in cases of resistance to other antibiotics, the combination of aztreonam and ceftazidime/avibactam should be used, as recommended in the 2023 report by the Infectious Diseases Society of America (IDSA) (Tamma et al., 2023).

The most important problem with antibiotic susceptibility testing for *S. maltophilia* is that breakpoints are available for only a few antibiotics because of insufficient in vitro and clinical data (CLSI, 2023). In addition, given antibiotic resistance, there appears to be insufficient data to assess the effectiveness of alternative antibiotics. The IDSA recommends that combination therapy be preferred for *S. maltophilia* infections to increase the chance of treatment success. The use of minocycline in combination with other treatment regimens is recommended to accelerate clinical improvement. Although breakpoints for minocycline have been established by the CLSI, no data on tigecycline are available. Although minocycline is preferred because it has some advantages, such as better tolerance to it than tigecycline, it is also known that tigecycline is an important alternative (Tamma et al., 2023; CLSI, 2023). The aim of this study was to evaluate the efficacy of new drugs/combinations, primarily aztreonam/avibactam, for which CLSI breakpoints have not yet been determined. These drugs may be alternatives in the treatment of *S. maltophilia* infections.

MATERIALS AND METHODS

Bacteria and antibiotics

Sixty-six *S. maltophilia* strains included in the study were isolated from clinical samples collected from Istanbul University, Faculty of Medicine, Istanbul, Turkey between 2005 and 2009. *Pseudomonas aeruginosa* ATCC 27853 was used as a quality control strain to verify the accuracy of the experiments. Tryptic soy agar (TSA-BD DIFCO™) and cation-adjusted Muller–Hinton Broth (MHB-BD BBL™) were used for the growth of bacteria. In this study, aztreonam (Sigma–Aldrich), avibactam (Sigma–Aldrich), doxycycline (Sigma–Aldrich), and tigecycline (Pfizer Inc.) were tested for their effectiveness against these strains. Antibiotics were prepared in accordance with the CLSI recommendations and stored at - 20°C for a maximum of 6 months. For this purpose, aztreonam was dissolved in saturated sodium bicarbonate solution and diluted with sterile distilled water. All other antibiotics were dissolved in sterile distilled water. The aztreonam/avibactam combination was studied in accordance with the CLSI recommendations, with the avibactam concentration fixed at 4 µg/ml in all experiments (CLSI, 2023).

Broth microdilution

Minimum Inhibitory Concentrations (MICs) of aztreonam, tigecycline, doxycycline, and aztreonam/avibactam against the isolates were determined using the broth microdilution method recommended by the CLSI (CLSI, 2006). Bacterial suspensions at a final concentration of 5x105 were added to two-fold serial dilutions of antibiotics (128-0.06 µg/ml) prepared in 96-well U-bottom microplates. After incubating the microplate at 37°C for 24 hours, MICs were determined as the lowest antibiotic concentration at which no growth was observed. In the aztreonam/avibactam combination, the final concentration of avibactam was fixed at 4 µg/ml in all wells. Experiments were repeated three times.

Checkerboard Assay

A checkerboard assay was performed to determine the effects of the aztreonam/avibactam combination with tigecycline on *S. maltophilia* isolates (Eliopoulos & Moellering, 1996). For this purpose, six isolates with the most common MICs of aztreonam/avibactam against the strains, 2 µg/ml and 4 µg/ml were selected from among the 66 tested strains, and the experiments were continued with these isolates. After the MIC values of aztreonam/avibactam and tigecycline were determined separately, two-fold serial dilutions of the antibiotics between 8xMIC and MIC/8 were prepared, and each of these concentrations was added to the microplate to match each other. For this purpose, aztreonam/avibactam was added to the horizontal plane of the microplate, and tigecycline was added to the vertical plane of the microplate at increasing concentrations. Then, bacterial suspensions were added to the microplate to a final concentration of $5x10^5$ and incubated at 37°C for 24 h. The next day, 30 µl of resazurin solution (0.1 mg/ml) was added to all wells, and the mixture was kept for 3 h. Wells with no bacterial growth were identified, and the lowest concentrations of the two antibiotics in these wells were determined. The fractional inhibitory concentration (FIC) index was determined by considering the concentrations. The FIC value for each antibiotic was determined by dividing the lowest antimicrobial concentration in wells with no bacterial growth by the MIC value of that antibiotic alone against the same isolate. The FIC index was obtained by summing the FIC values of both antibiotics. According to the FIC index results, combinations were evaluated as synergistic (values ≤ 0.5), additive (values 0.5-4) or antagonist (values ≥ 4.0) (Odds, 2003).

Time-Kill Assay

A time-kill assay was used to determine the time-dependent effects of the combination of aztreonam/avibactam with tigecycline on six *S. maltophilia* isolates (NCCLS, 2002). After the antibiotics were prepared to have final concentrations corresponding to their MICs, they were added either alone or in combination to bacterial inocula at a final concentration of 10^6 CFU/ml. Samples were taken from these suspensions at 0, 2, 4, 6, and 24 h, dilutions were made, and the plates were then incubated at 37°C for 24 h. The number of bacterial colonies formed the next day was counted and determined. The obtained data were used to create time-kill curves, with time on the x-axis and bacterial counts expressed logarithmically on the y-axis. The results were then evaluated as synergistic, additive, or antagonistic based on the National Committee for Clinical Laboratory Standards (NCCLS) criteria (NCCLS, 2002). Bactericidal activity was defined as a decrease of ≥3log10 cfu/mL in the number of viable bacteria in the initial inoculum. Antibiotic combinations were evaluated by comparing the effects of each combination with those of the individual antibiotics. Synergy and antagonism were determined by changes in colony numbers. A ≥ 2log10 decrease was considered to indicate synergy and a ≥ 2log10 increase for antagonism. If no 2log10 change was observed, the effect of the combination was considered additive.

Statistical analysis

The graphs were generated using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed on the time-kill data using two-way analysis of variance followed by Tukey's post-hoc test, with p-values of < 0.05 considered statistically significant.

RESULTS

Antibiotic susceptibility test results

The MICs (µg/ml) of the antibiotics against sixty-six *S. maltophilia* isolates are provided in Supplementary 1. MIC_{50} and MIC_{90} values (µg/ml), representing the lowest MICs that inhibited 50% and 90% of the isolates, respectively, are presented in Table 1.. As the breakpoints, the susceptibility breakpoint for aztreonam published by CLSI for *P. aeruginosa*, which is ≤8 µg/ml, was applied (CLSI, 2023); for aztreonam-avibactam, the provisional PK/PD susceptibility breakpoint of ≤8/4 µg/ml was used (Singh et al., 2015). According to the results, all isolates except one (n:65) were determined to be aztreonam-resistant. When aztreonam is used together with avibactam, MIC values decreased significantly. All strains, except two, were evaluated as susceptible to aztreonam/avibactam. Although the MIC50 and MIC90 values for aztreonam were found to be >128 µg/ml, these values for aztreonam/avibactam were 4/4 and 8/4 µg/ml, respectively. All isolates tested were susceptible to doxycycline according to the breakpoints for *Enterobacterales* (CLSI, 2023). The tigecycline susceptibility breakpoint, ≤2 µg/ml, determined by the US Food and Drug Administration (FDA) (2023) for *Enterobacterales*, was applied. As a result, 9 of 66 isolates were found to be resistant to tigecycline, 24 were intermediately susceptible, and 33 were susceptible to tigecycline.

Table 1. MIC_{50} and MIC_{90} values of antibiotics

Antibiotics	*MIC_{50} (µg/ml)*	*MIC_{90} (µg/ml)*
Doxycycline	1	2
Tigecycline	2	8
Aztreonam	>128	>128
Aztreonam/Avibactam	4/4	8/4

MIC breakpoints (µg/ml): Dox: S≤4 I=8 R≥16; Tig: S≤2 I=4 R≥8; Azt: S≤8 I=16 R≥32; Azt/Avi: S≤8 I=16 R≥32

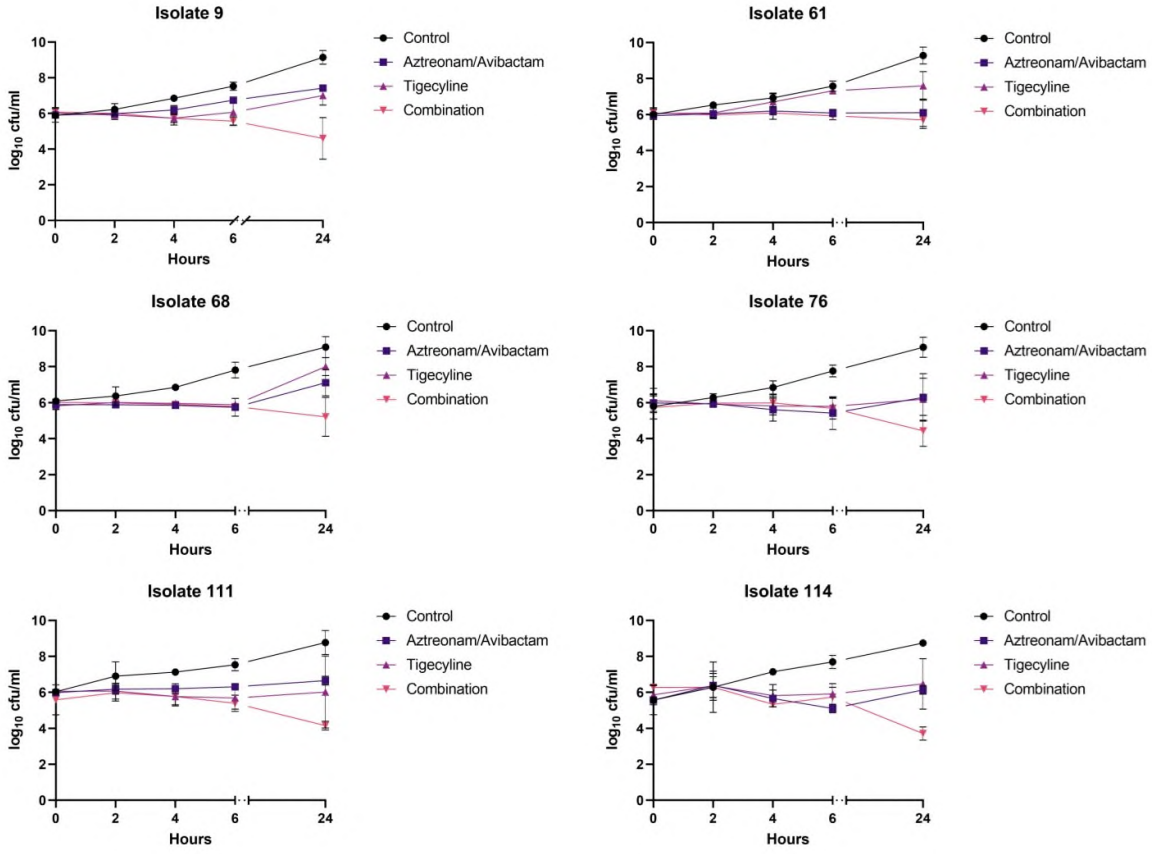

Figure 1. Time-kill curves of antibiotics and combination

Checkerboard assay results

To determine the effect of the combination of aztreonam/avibactam with tigecycline, three of the six selected isolates had MIC values of 4/4 µg/ml for aztreonam/avibactam, and the other three had 2/4 µg/ml. The isolates used in the combination experiments are described in Table 2.

Table 2. MIC values of isolates used in combinations

Isolate number	*Antibiotics*	
	Aztreonam/avibactam	**Tigecycline**
	MICs (µg/ml)	
9	2/4	4
61	2/4	2
68	4/4	4
76	4/4	4
111	2/4	4
114	4/4	4

The combination experiments were repeated three times, and the results are shown in Table 3. According to the FIC index values, the combination of aztreonam/avibactam and tigecycline had additive effects on all six isolates tested. Synergism or antagonism was not observed.

Table 3. FIC index values of the aztreonam/avibactam and tigecycline combinations

Isolate number	*FIC index of aztreonam/avibactam and tigecycline combination therapy*
9	0.75
61	0.625
68	0.75
76	0.625
111	0.625
114	0.75

Time-kill results

Time-dependent killing results showed that aztreonam/avibactam and tigecycline alone or in combination did not cause a 3log reduction in initial bacterial counts. These antibiotics inhibited bacterial growth for most of the

tested isolates at all-time intervals compared with the control group. The effectiveness of the combination was observed at the end of 24-h, and statistical significance was determined between the control and combination groups for all tested isolates ($p < 0.05$). For isolates 9 and 114, the combination of aztreonam/avibactam and tigecycline was found to have at least a 2-log reduction in bacterial counts at the end of the 24th hour compared with the most effective antibiotic alone; therefore, the combination was evaluated as synergistic. For isolates 68, 76, and 111, the combination caused a 1 log decrease in the number of bacteria at the end of the 24th hour compared with the most effective antibiotic alone. No significant effect was observed for isolate 61. The effects of the combination were evaluated as additives for isolates 61, 68, 76, and 111.

DISCUSSION

S. maltophilia is a Gram-negative pathogen that poses a health risk due to its widespread antibiotic resistance and severe mortality and morbidity in immunocompromised patients. Treatment options for *S. maltophilia* are quite limited, and besides clinical data, information on clinical breakpoints for antibiotic efficacy as defined by reference sources, such as CLSI and EUCAST, is also limited (CLSI, 2023; EUCAST, 2024a). The three antibiotics recommended by the CLSI for first-line therapy are trimethoprim/sulfamethoxazole, levofloxacin, and minocycline. However, studies conducted worldwide have shown that clinical *S. maltophilia* isolates may exhibit resistance to these three antibiotics (Dadashi et al., 2023). In addition to these three antibiotics, IDSA's 2023 guide also recommends antibiotics such as aztreonam, tigecycline, ceftazidime/avibactam (Tamma et al., 2023). Results of a study examining 486 patients treated for bloodstream infections caused by *S. maltophilia* showed that levofloxacin was the most commonly used antibiotic. Trimethoprim/sulfamethoxazole was largely preferred as definitive treatment after empirical treatment. The study results also showed that aztreonam, tigecycline, and doxycycline were not widely preferred (Cai, Tillotson, Benjumea, Callahan, & Echols, 2020). Additionally, studies have revealed the existence of tigecycline resistance. Among the 450 *S. maltophilia* strains isolated between 2012 and 2015, tigecycline resistance was 22.22% and doxycycline resistance was 18.67% (Zhao et al., 2018). In our study, half of the 66 *S. maltophilia* strains isolated from respiratory tract samples from patients with cystic fibrosis were found to be susceptible to tigecycline. All isolates were found to be susceptible to doxycycline. These results indicate that in addition to minocycline, other tetracyclines is an important treatment option. Studies evaluating the efficacy of doxycycline against *S. maltophilia* are limited. In a clinical study comparing tetracyclines (minocycline and doxycycline) with trimethoprim/sulfamethoxazole, similar clinical (28.6% vs. 25.4%) and microbiological success rates (55.6% vs. 66.4%) were determined for these two antibiotic groups (Alhayani, Philpott, Liao, Gentene, & Mueller, 2024). Tigecycline has been tested in many studies for *S. maltophilia*, and resistance rates have been reported to be between 11.8% and 28.1% (Banar et al., 2023; Biagi et al., 2020a; Su et al., 2023; Wang, Yu, Hsu, & Wu, 2020). It was determined that minocycline and tigecycline had bacteriostatic effects on *S. maltophilia* isolates carrying the dihydropteroate synthase (*sul*) gene (Zhao et al., 2022). Similarly, the results of the time-dependent killing assay conducted with 6 strains in our study revealed that tigecycline had a bacteriostatic effect on these strains. It is thought that these strains may carry the sul gene, and genetic studies are required to confirm this. According to the results of a study conducted by Gülmez et al. (2010), the most effective antibiotics against 25 tested *S. maltophilia* isolates were trimethoprim/sulfamethoxazole, tigecycline, and doxycycline. In the results of this study, resistance percentages were determined as 4% and 0% for tigecycline and doxycycline, respectively. In our study, the resistance percentages were the same for doxycycline but higher for tigecycline (13.6%) (Gülmez, Cakar, Şener, Hasçelik, & Karakaya, 2010).

The efficacy of aztreonam/avibactam against *Enterobacterales* has been demonstrated in numerous studies, and broth microdilution breakpoints have been published by EUCAST (EUCAST, 2024b). Results from a large surveillance study involving 63 countries determined that aztreonam/avibactam was 99.4% potent against metallo β-lactamase positive *Enterobacterales* isolates (Rossolini, Arhin, & Kantecki, 2024). The combination of ceftazidime/avibactam with aztreonam has been recommended in the guidelines published by IDSA, as its effectiveness has been proven in the treatment of *S. maltophilia* (Tamma et al., 2023; de Almeida Torres, Junior, Lopes, Zeigler, & Uip, 2023; Ranieri et al., 2023; Emeraud et al., 2019). Avibactam enhances the in vitro activities of both ceftazidime and aztreonam when combined with them in *S. maltophilia* isolates (Lin et al., 2020).

Aztreonam resistance is common among *S. maltophilia* isolates (Anđelković et al., 2019). Avibactam has been reported to competitively and reversibly inhibit *S. maltophilia* L2 β-lactamases, thus restoring the susceptibility of this bacteria to aztreonam (Mojica et al., 2017). Other β-lactamase inhibitors, when combined with aztreonam, restored activity to a lesser extent than avibactam. Following avibactam (98%), relebactam (71%), clavulanate (61%) and vaborbactam (15%) were determined as effective β-lactamases, respectively (Biagi et al., 2020b). Similarly, another large-scale study examining 1,839 *S. maltophilia* isolates from different geographic regions and infection types showed that aztreonam/avibactam was effective against 97.8% of the isolates. The same study determined that the sensitivity of the isolates to tigecycline was 85% (Sader et al., 2020). Our study demonstrated that aztreonam resistance was eliminated by aztreonam/avibactam in *S. maltophilia* strains isolated from Türkiye. Additionally, 86.4% of the iso-

lates were found to be susceptible or intermediate to tigecycline in our study.

Antibiotic combinations are frequently preferred in situations that require rapid and effective treatment to achieve broad-spectrum effects, reduce the risk of resistance development, decrease the efficacy of existing resistance, target heterogeneous bacterial populations by combining different antibiotics, and enhance clinical efficacy in cases in which in vitro synergism is observed (Roemhild, Bollenbach, & Andersson, 2022). However, it is possible that antibiotics may interact with each other, resulting in synergism or antagonism. Synergism between antibiotics used in combination is preferable for treatment, but antagonism can make treatment unsuccessful. Therefore, when making antibiotic combinations, this possibility should be considered, and appropriate studies should be conducted. Combination studies on aztreonam/avibactam have been limited to testing combinations of ceftazidime/avibactam and aztreonam. Hence, it is important to test the efficacy of aztreonam/avibactam along with another highly effective antibiotic with a different mechanism of action, such as tigecycline. A previous study determined that combinations of tigecycline with cefoperazone-sulbactam and levofloxacin had a synergistic effect (Karamanlıoğlu & Dizbay, 2019). Although many methods are used in the literature to test the effectiveness of combinations, the most frequently used are the checkerboard assay and the time-kill method. Both of these methods have advantages and disadvantages. While the checkerboard method is a more static method that examines the effects of antibiotics on the growth of bacteria, the time-kill method is a dynamic method based on the principle that antibiotics kill bacteria in a time-dependent manner (White, Burgess, Manduru, & Bosso, 1996). This fundamental difference between them may have caused some inconsistencies in the results of the two tests. In our study, although the checkerboard assay results indicated that the combination was additive in all isolates, the time-kill experiment results indicated that the combination of tigecycline and aztreonam/avibactam, a β-lactam-β-lactamase inhibitor, showed synergism for two isolates. A meta-analysis showed that synergisms detected by the time-kill method were greater than those detected using the checkerboard method (Zusman et al., 2013). Similarly, previous studies have reported that the time-kill method is more sensitive in detecting synergism (Visalli, Jacobs, & Appelbaum, 1998; Rizvi, Ahmed, Khan, Shukla, & Malik, 2013). In parallel with these findings, synergism was observed in two isolates using the time-dependent killing method in our study.

CONCLUSION

The results obtained from this study, in parallel with the literature, showed that aztreonam/avibactam restored aztreonam activity in *S. maltophilia* isolates that were isolated from Türkiye and resistant to aztreonam. In addition, the effectiveness of tigecycline and doxycycline against *S. maltophilia* isolates indicated that they could be important alternatives for treating these infections, and further studies are therefore necessary. According to the results of the time-kill experiment performed to determine the efficacy of the combination of aztreonam/avibactam with tigecycline, the observation of synergism in the two isolates was interpreted as promising for the combined use of these antibiotics. However, clinical studies are needed to confirm the results.

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Original Article

Assessment of antioxidant and neuroprotective activity of plants from the Lamiaceae family

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ABSTRACT

Background and Aims: Plants from the Lamiaceae family are shown to have pharmacological activities, including anti-inflammatory, antidiabetic, antiasthmatic, hypolipidemic, antibacterial. This research measured the antioxidant and neuroprotective capacity of methanol extracts from the *Marrubium vulgare* L., *Phlomis armeniaca* Willd., *Thymus haussknechtii* Velen. and, *Thymus kotschyanus* Boiss. & Hohen plants in cellular and cell-free systems.

Methods: The neuroprotective potential of *Marrubium vulgare, Phlomis armeniaca, Thymus haussknechtii*, and *T. kotschyanus* were determined against H_2O_2 toxicity in SH-SY5Y, the human neuroblastoma cell line. The antioxidant capacity of methanol extracts was examined by radical scavenging assays using static exempt chemical groups, 2.2'-diphenyl-1-picrylhydrazyl-hydrate (DPPH) and 2.2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Folin-Ciocalteau and Aluminum chloride assays were used to measure phenolic and flavonoid contents of the extracts.

Results: Following these experiments, the effect of three different concentrations of extracts (1, 10 and 100 µg/mL) on cell viability was assessed by a WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium)]. The methanol extract of *Marrubium vulgare* was found to have the highest antiradical activity against DPPH (76.439±0.42%). The *Thymus kotschyanus* (10µg/mL) protected cells from H_2O_2-induced toxicity in all the extracts ($p<0.05$).

Conclusion: In our report, we suggest that *Thymus kotschyanus* have potential neuroprotective activity because of the existence of polyphenolic compounds, flavonoids, and phenolic acids.

Keywords: Antioxidant activity, Lamiaceae, Neuroprotective, Oxidative stress

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurological disorder with a prevalence of 5% among individuals over 65 years old, increasing to 30% among those over 85 years old. It is the most common form of dementia, as well as the most prevalent neurodegenerative disorder, and dramatically affects cognitive and behavioral skills. One of the contributing factors in AD's progression is the presence of oxidative stress, a disturbance in the balance between oxidants and antioxidants, in favor of the oxidants (Ververis et al., 2020). Antioxidants are the key players combating oxidative stress in diseases. Several studies have shown the high antioxidant capacity of plant species which are efficient in cell development, adjusting membrane potential, or inhibiting lipid peroxidation (El Houri, & Rosado, 2019; Silva et al., 2019; Poznyak et al., 2020). These studies correlated with the secondary metabolites of plants such as lipo- and water-soluble nutrients and polyphenols (Ginsburg, Kohen & Koren, 2011). The drugs obtained from medicinal plant materials from members of the family Lamiaceae have a neurotropic effect, enhance the affinity of gamma-aminobutyric acid (GABA) for GABA-receptors in the subcortical formations, primarily in the reticular formation, weakening its stimulating effect on the cerebral cortex. Flavonoids, triterpenic acids (ursolic and oleanolic), phenylpropanoids (rosmarinic and caffeic acids), terpenoids and aromatic compounds which are components of the essential oil (linalool, linalyl acetate, thymol, carvacrol, etc.), alkaloids, alkaloid-like compounds and iridoids, are often studied in this context (Zvezdina et al., 2020).

The Lamiaceae (Labiatae) family is represented by 258 gen-

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era and around 7886 species in the world (Abdelhalima & Hanrahan, 2021). The family has 48 genera and 782 taxa (603 species, 179 subspecies and varieties), 346 taxa (271 species, 75 subspecies and varieties) of which 44% are endemic in Turkiye (Celep, & Dirmenci, 2017). Many plant taxa of this family are used as culinary herbs, sources of aroma and in traditional medicine (Selvi, Polat, Çakılcıoğlu, & Celep, 2022). Members of the Lamiaceae family gained importance in areas such as medicine, food, cosmetics, and perfumery (Ozturk, 2015).

In Turkiye the genus *Marrubium* L. is represented by 34 taxa of which 17 are endemic with an endemism rate of 50% (Deshmukh, Reddy, & Shende, 2022). Recent studies demonstrated that *M. vulgare* offered different *in vivo* and *in vitro* pharmacological activities, including antihypertensive, anti-inflammatory, antidiabetic, vasodilator, antiasthmatic, hypolipidemic, antibacterial, and antifungal (Aćimović et al., 2020; Gavaric et al., 2022; Akbulut, Kose, Demirci, & Baykan, 2023). More than 54 secondary metabolites were identified from *M. vulgare*. The secondary metabolites of diterpenes, sesquiterpenes, flavonoids, and phenylpropanoids were isolated from various parts of *M. vulgare* (Aćimović et al., 2020). Marrubiin, marrubiinic acid, and marrubenol were the most abundant diterpenes that displayed pain-relieving and edema-relieving activities. Arenarioside, acteoside, forsythoside B, and ballotetroside are phenylpropanoids that have strong antitumor and anti-inflammatory activities (Lodhi, Vadnere, Sharma, & Usman, 2017).

Phlomis armeniaca Willd., known as 'boz şavlak' in Turkiye, is a medicinal plant of Lamiaceae family. The aerial parts of this plant are used as tea in traditional medicine for cold and gastrointestinal problems, including digestion disorders, ulcers, and stomachache (Uysal, Gunes, Sarikurkcu, Celik, Durak, & Uren, 2016). A few studies with the aerial parts of *P. armeniaca* demonstrated that the plant has iridoids, phenylethanoid glycosides, lignans, phenylpropanoids, monoterpenes, and diterpenoids as major compounds. The antinociceptive, antiulcerogenic, anti-inflammatory, antiallergenic, anticancer, and antimicrobial activities are reported for some species of *Phlomis* (Aybey, 2020; Sarıkurkcu & Zeljkovic, 2020; Tarhan, Urek, Öner, Nakipoglu, 2022; Kunter, et al., 2023).

Thymus L. in the Lamiaceae family is represented by 39 species and 59 taxa in Turkiye and the proportion of endemism for this genus is greater than 50%. *Thymus haussknechtii* is another endemic plant in Turkiye and in conventional remedies, this species is utilized for its antibacterial, antifungal, antihelminthic, antispasmodic, sedative, antioxidant, and diaphoretic activities (Ozturk, 2015; Yigitkan et al., 2022).

In this study, the primary goal was to determine the neuroprotective potential of *Marrubium vulgare, Phlomis armeniaca, Thymus haussknechtii* and *T. kotschyanus* against H_2O_2-induced apoptosis. Additionally, radical scavenging assays were performed to confirm their antioxidant activities.

MATERIALS AND METHODS

Chemicals

Folin Ciocalteu reagent and methanol were purchased from Merck (Germany). Sodium hydroxide, 2.2'-diphenyl-1-picrylhydrazyl, α-tocopherol, aluminum chloride, and gallic acid were obtained from Sigma Chemical (Sigma-Aldrich Gmbh, Germany). SH-SY5Y, the human neuroblastoma cell line, was purchased from the American Type Culture Collection (ATCC, Cat. No. CRL-2266). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were provided by Life Technologies (Grand Island, NY). The WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium)] was acquired from Takara Bio USA (Mountain View, CA, USA). An analytical grade of other synthetic compounds and solvents were used.

Plant materials and extraction

The aerial parts of *M. vulgare, P. armeniaca, T. haussknechtii* and *T. kotschyanus* (Lamiaceae) were collected in June 2018 from Elazig, Turkiye. The identification of plants was made by Ugur Cakilcioglu, from Tunceli University. The voucher specimens (herbarium numbers: 1635 for *M. vulgare*, 1636 for *P. armeniaca*, 1637 for *T. haussknechtii*, and 1638 for *T. kotschyanus*) were saved in the herbarium of the Faculty of Pharmacy, Ege University, Izmir, Turkiye. *M. vulgare, P. armeniaca, T. haussknechtii* and *T. kotschyanus* were entitled as MV, PA, TH, and TK, separately. The collected plant materials were air-dried at room temperature. The powdered samples (40g) were extracted with 400mL methanol using a Soxhlet type extractor at 60°C for six hours. After the solvent was filtered by Whatman No. 1 filter paper, extracts were dissipated by a rotating evaporator under low pressure. Extracts were kept at + 4°C in the dark until use. The percentage yield of methanol extracts of MV, PA, TH and TK were found as 8.25%, 11.60%, 7.42%, and 8.84%, respectively.

Cell culture studies

The cells were suspended in DMEM with the addition of 10% FBS, 1% penicillin, and streptomycin. For the WST-1 assay, cells were transferred to 96-well plates at a density of 2×10^3 cells/well. Stock solutions of the extracts were prepared in sterile DMSO (dimethyl sulfoxide). The final concentration of DMSO was kept lower than 0.1% in cells.

Cell viability assay

The 96-well plates of seeded cells were incubated for 24 hours. Following the 24 hour incubation period, the cells were treated with three concentrations of extracts (1, 10, and 100µg/mL) for two hours. Then, cells were cleaned twice with sterile

phosphate-buffered saline (PBS) and treated with 250mM H_2O_2 in sterile Ca^{2+} and Mg^{2+} -added PBS for 60 minutes. The H_2O_2 in the well was replaced with a warm medium after one hour and incubated for an additional 17 hours. Cell proliferation after 18 hours was determined by the WST-1 assay as described previously (Loubidi et al., 2016).

Antioxidant activity assays

DPPH radical scavenging assay

The DPPH (2.2'-diphenyl-1-picrylhydrazyl) radical scavenging activity of methanol extracts was assessed by the method described by Fukumoto and Mazza (Fukumoto & Mazza, 2000). 1000µL of 1mg/mL methanol extracts were appended to 4mL of 0.004% DPPH in methanol. The absorbance was assessed following 30 minutes at 517nm. The percentage inhibition of free radicals was calculated as follows:

$$\%Inhibition = [(A_c - A_s)/A_b] \times 100$$

(A_c: the absorbance of the control, As: the absorbance of the sample).

ABTS radical scavenging assay

The ABTS solution was arranged and diluted with ethanol until it gave a 0.750 absorbance in 734nm by the ABTS assay (Re et al., 1999). A 0.1mL of concentrates and 10µL α-tocopherol were appended to 1mL ABTS+ solution, and an absorbance change was seen in 734nm during the six minutes. The α-tocopherol was utilized as a standard solution. The ABTS % inhibition was determined as follows:

$$ABTS\%inhibition = (Abs_1 - Abs_2)/Abs_1 \times 100.$$

(Abs_1: the initial absorbance, Abs_2 : the absorbance at 6 min).

Determination of total phenol and flavonoid contents

As indicated by the method for Folin-Ciocalteu, the total phenol content of 0.1mL concentrates were added to 2.8mL deionized water. A 2mL 2% sodium carbonate and 0.1mL of 0.1 N Folin–Ciocalteu reagent was included in this solution. After standing for 30 minutes at room temperature, the absorbance of the solution was assessed at 750nm on an UV/Vis spectrophotometer (Unicam 8625). A standard solution of gallic acid solution was utilized (Chang, Yang, Wen, & Chern, 2002). Subsequently, the results were shown as mg gallic acid equivalents.

The aluminum chloride assay was utilized to determine the total flavonoid content (Woisky & Salatino, 1998). According to this method, 1.5mL of ethanol, 0.1mL of 10% aluminum chloride, and 2.8mL of purified water were added to the 0.5mL extracts. The mixture solution stayed at room temperature for 30 minutes, and the absorbance was assessed at 415nm on the UV/Vis spectrophotometer. As a standard solution of quercetin was utilized.

Statistical Analysis

The antioxidant capacity experiments were executed in triplicate. The data was presented as ± S.D. The comparison between groups was executed by a one-way analysis of variance (ANOVA). The P values of less than 0.05 were considered significant.

RESULTS

The antioxidant activities and measurements of the total phenolic and flavonoid contents of *M. vulgare, P. armeniaca, T. haussknectii*, and *T. kotschyanus* are shown in Table 1. The most active plant found for its antioxidant capacity and measure of total phenolic and flavonoids with activity assays was *T. kotschyanus*. For defensive impact against oxidative stress on a neuroblastoma cell line (SH-SY5Y) the most effective plant was the *T. kotschyanus.*

The total phenolic content in the taxa extended from 53.02 to 408.31mg gallic acid equivalent/100g, and the complete flavonoid content went from 41.98 to 138.62 to mg quercetin equivalent/100g, being most noteworthy for *T. kotschyanus* and least for *M. vulgare*. The most noteworthy DPPH radical scavenging activity (76.439%) was resolved for *M. vulgare*; the most noteworthy ABTS radical scavenging activity (54.867%) values were resolved for *T. kotschyanus*, while *T. haussknectii* and *M. vulgare* displayed the most reduced DPPH (28.417%) and ABTS (3.438%) values.

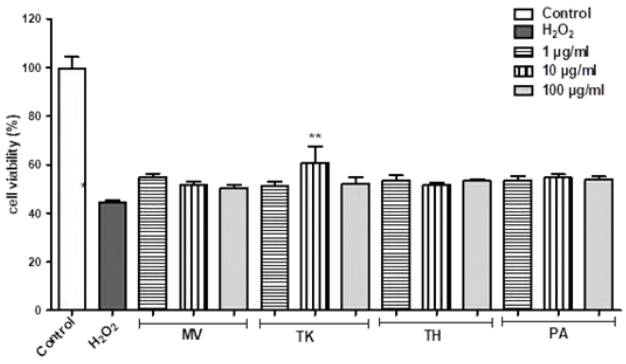

Figure 1. Cell viability analysis following methanol extract treatments are shown against H_2O_2-induced toxicity. The cells were pre-treated with various concentrations (1, 10 and 100µg/mL) of methanol extracts of MV (*Marrubium vulgare),* TK (*Thymus kotschyanus*), TH (*Thymus haussknectii*) and PA (*Phlomis armeniaca*) for two hours and exposed to H_2O_2 for one hour. Cell viability was measured by the WST-1 assay. All values are means ± SDs (n = 5),*p≤0.05 vs. untreated cells, **p≤0.05 vs. H_2O_2-treated cells.

Table 1. Antioxidant activities, total phenolic and flavonoid contents of *M. vulgare, P. armeniaca, T. haussknechtii* and *T. kotschyanus*

Plant name	DPPH (inhibition %)	ABTS (inhibition %)	TPC (mgGAE/100 g)[a]	TFC (mgQE/100 g)[b]
Marrubium vulgare	76.439±1.05[c]	3.438±4.02	53.02±2.66	41.98±3.84
Phlomis armeniaca	52.487±1.28	36.204±1.28	38.49±1.86	28.06±1.96
Thymus haussknectii	28.417±3.84	16.328±2.84	28.36±0.58	19.42±2.08
Thymus kotschyanus	36.246±2.56	54.867±4.06	408.31±1.08	138.62±4.06

[a] Total phenolic content expressed as gallic acid equivalents (mg gallic acid equivalent /100 g extract); [b] total flavonoid content expressed as quercetin equivalents (mg quercetin equivalent /100 g extract);
[c] results are mean ± SD of three replicate analysis; TEAC (trolox equivalent antioxidant capacity).

The investigation of cell viability following methanol extract treatments against H_2O_2-induced toxicity on neuroblastoma cell line appear in Figure 1. The expected protective effects of extracts were assessed in the H_2O_2-induced oxidative stress model. The adjustments in cell viability were resolved after exposure to determine the impact of concentrations on cell proliferation rate. Treatment with concentrations for 24 hours showed roughly a similar proliferation rate with control cells but it was not decided as cytotoxic and did not adjust the expansion of cells. The SH-SY5Y cells were pre-treated with extracts changing from 1 to 100µg/mL for two hours, after treatment with 250µM H_2O_2 for one hour. WST-1 measurements were used for to decide the cell viability. Figure 1 demonstrates that every chosen concentration figured out how to expand cell viability against H_2O_2. TK at 10µg/mL significantly decreased the H_2O_2-induced neuronal death, as it appeared with the expansion of cell viability ($p < 0.05$). The cell viability in the H_2O_2 group was discovered to be 44.21% ± 1.80 ($p < 0.05$), and the TK at 10µg/mL displayed at 36.40% neuroprotection against H_2O_2-induced toxicity.

DISCUSSION

A positive correlation between the contents of total flavonoid and total phenols and antioxidant activities of the plants was observed. Typical phenols that own antioxidant activity are recognized as flavonoids and phenolic acids (Meyre-Silva & Cechinel-Filho, 2010; Silva et al., 2019). The TPC of the extracts of *M. vulgare* and *T. haussknectii* were resolved as 19.08 and 320.96 mg/g gallic acid equivalents. The TFC of the concentrates of *M. vulgare* and *T. haussknectii* were acquired as 7.08 and 86.02 mg/g quercetin equivalents, respectively. Numerous flavonoids were isolated from *M. vulgare* and T. haussknectii. There were a few studies on antioxidant activities of*M. vulgare, P. armeniaca, T. haussknectii* and *T. kotschyanus* (Amri et al., 2017; Tohidi, Rahimmalek, & Arzani, 2017; Boroomand, Sadat-Hosseini, Moghbeli, & Farajpour, 2018). Furthermore, there were studies about defensive effects on cell cultures for these plants (Brahmi et al., 2015; Dibas, Yaghi, Mansi, Mhaidat, & Al-Abrounie, 2017). The utilized cell culture systems were not quite the same as in this work. Consequently, we needed to compare our outcomes with previous investigations.

Lamiaceae species are rich in phenolic acids contribute to neuroprotective activity (Dastmalchi, Dorman, Viorela, & Hiltunen, 2007). Polar extracts of *Thymus* species are used in the food, cosmetics, and pharmaceutical industry due to their protective activities (Afonso, Pereira, Neto, Silva, & Cardoso, 2017). Dietary antioxidant intake, such as for terpenes, and phenolic compounds, act as free radical scavenging molecules (Uttara, Singh, Zamboni, & Mahajan, 2009). In previous studies, volatile terpenoids and polyphenolic compounds were isolated from the *Thymus* species. Polyphenolic compounds from *Thymus* plants, predominantly flavonoids and phenolic acids, were published. The most common structure among flavonoids is found to be flavones (luteolin, apigenin and scutellarin) and flavanones (eriodictyol and naringin). The phenolic acids isolated from different *Thymus* species are caffeic acid and rosmarinic acid (Jordan, Martinez, Martinez, Monino, & Sotomayor, 2009). Oxidative damage caused by free radical interaction with neural cells leads to degeneration, while exogenous and endogenous antioxidants such as polyphenols, and flavonoids could retard cell death (Hassan, Ibrahim, Yusuf, Ahmad, & Ahmad, 2021). The neuroprotective activity of luteolin was observed against hydrogen peroxide-induced toxicity in primary neuronal cells.

CONCLUSION

Flavonoids apigenin and luteolin were found to exhibit neuroprotective effects against KCl-induced-Ca2+ overload and oxidative stress, beyond acting as acetylcholinesterase inhibitors (Cavallaro, Baier, Murray, Estevez-Braun, & Murray, 2018). We claimed the neuroprotection of *Thymus kotschyanus* was because of the existence of polyphenolic compounds, flavonoids, and phenolic acids. New research is required for the isolation of components responsible for biological activities and to clarify their structures.

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Original Article

GS/MS analysis and the antioxidant and antimicrobial properties of *Salvia potentillifolia* (Boiss. & Heldr.) ex Bentham

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ABSTRACT

Background and Aims: Sage is traditionally used as an herbal tea in Türkiye. In this study, the phenolic composition and biological potential of *Salvia potentillifolia* (Boiss. et Heldr.) ex Bentham (Lamiaceae) were determined.

Methods: The essential oil constituents of *S. potentillifolia* were determined using gas chromatography/mass spectrometry (GC/MS). The in vitro antioxidant properties of the methanol extract of *S. potentillifolia* were tested spectrophotometrically using phosphomolybdenum assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, hydrogen peroxide (H_2O_2) scavenging, β-carotene bleaching inhibition, Fe^{2+} chelating, reducing power, ferric ions reducing activity (FRAP), and cupric ion reducing activity (CUPRAC) assays. The total phenolic and flavonoid contents of the methanol extract were determined. The antimicrobial effects of the methanol extract and essential oil were detected against 12 bacteria, 1 yeast, and 2 aflatoxigenic fungi strains using agar-well diffusion and the micro-well dilution methods.

Results: Fifty compounds were detected in the essential oil. The major oil component was eucalyptol. It was followed by carvacrol, β-pinene, borneol, camphor, α-terpineol, 4-terpineol, bornyl acetate, and caryophyllene. The methanol extract of *S. potentillifolia* had effective DPPH radical scavenging, Fe^{3+} reducing, and Cu^{2+} reducing activities while exerting weak H_2O_2 scavenging and Fe^{2+} chelating activities. The methanol extract had weak antibacterial activity, whereas the essential oil had moderate antibacterial activity. The methanol extract had no antifungal potency against the tested aflatoxigenic fungi.

Conclusion: The methanol extract of *S. potentillifolia* is a natural antioxidant resource, whereas the essential oil may be a natural antibacterial agent. It is believed that the results of this study will contribute to the recently increasing research on the use of natural antioxidant and antimicrobial compounds as an alternative to synthetic compounds in various industrial fields, such as food, pharmacy, and medicine.

Keywords: Antimicrobial, Antioxidant, Essential oil, *Salvia potentillifolia*

INTRODUCTION

The genus *Salvia* is the largest member of the Lamiaceae family (Kivrak, Göktürk, Kivrak, Kaya, & Karababa, 2019). There are 115 *Salvia* (sage) taxa growing in Türkiye, 63 taxa of which are endemic (54.7%) (Celep & Doğan, 2023). Some members of the *Salvia* genus are used in the pharmaceutical, cosmetic, perfume, and pharmaceutical industries (Kelen & Tepe, 2008; Kivrak et al., 2009; Kivrak, Göktürk, Kivrak, Kaya, & Karababa, 2019). It has been sold commercially as a spice to flavour meats (Kivrak et al., 2009; Sepahvand et al., 2014).

It has been used for the treatment of diseases, including epilepsy, colds, bronchitis, and tuberculosis (Kivrak et al., 2019), wounds, insomnia, skin infections, headache, cerebral ischemia, memory disorders (Sepahvand et al., 2014), stomach ache, headache, wounds, skin infections, colds (Kivrak et al., 2009), diarrhea, gonorrhea, hemorrhoids and eye diseases (Kelen & Tepe, 2008).

It has been used for medical purposes since ancient times and has different traditional uses (Gürdal, Yeşil, Akalın, & Tan, 2019). Many Salvia species have antimicrobial, antioxidant, antiviral, antitumoral, antidiabetic, antifungal, hypoglycaemic, and anticarcinogenic effects (Aghaei Jeshvaghani, Rahimmalek, Talebi, & Goli, 2015; Kivrak et al., 2019). Their secondary metabolites exhibit many pharmacological activities, such as antiplatelet, antiproliferative, and anticancer ef-

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fects, larvicidal and antimicrobial activities, antioxidant, acetyl- and butyrylcholinesterase inhibition, and antinociceptive and anxiolytic effects (Hao et al., 2015).

Salvia plants have various therapeutic secondary metabolites, such as terpenes, phenolics (Hao et al., 2015; Kelen & Tepe, 2008), diterpenoids, sesquiterpenoids, and phenolic acids (Al-Qudah, Al-Jaber, Abu ZArga, & Abu Orabi, 2014). Endemic *S. potentillifolia* called as "Adacayı" or "Salba" in Muğla, Türkiye and has been used as a folkloric drug, rarely as a tea (Kivrak et al., 2009). It is used to treat cold and flu in Türkiye (Gürdal et al., 2019).

The composition and antimicrobial properties of essential oil of *S. potentillifolia* were previously reported by Köse, Öngüt, & Yanıkoğlu (2013). The antioxidant and antimicrobial properties of essential oil and ethanol extracts obtained from *S. potentillifolia* collected in Burdur were previously reported (Kivrak et al., 2009). The same researchers recorded the phenolic compositions of *S. potentillifolia* extracts collected in Burdur by UPLC-ESI-MS/MS (Kivrak et al., 2019). Additionally, their antioxidant activities had been previously determined (Kivrak et al., 2019; Özek, 2017). However, as far as our literature survey was able, the antioxidant activity of the methanol extract of *S. potentillifolia* had not previously been determined by hydrogen peroxide scavenging, FRAP, and CUPRAC assays. In addition, the antimicrobial activity of the methanol extract was not previously determined. Therefore, this research aimed to identify the polyphenol compounds of *S. potentillifolia* essential oil and to determine the antioxidant and antimicrobial activity of the methanol extract.

MATERIALS AND METHODS

Plant

The aerial parts of *S. potentillifolia* were collected from Antalya (Elmalı-Sedir Research Forest Entrance), the southern Anatolia region of Türkiye in July 2015 (36°35'31"K-29°58'22"D, 1240 m) and stored at the Herbarium of the Biology Department at Erciyes University (Voucher no.: Aksoy 2522).

Extraction

The plant was dried at room temperature and pulverized into powder. The ground material was extracted using a Soxhlet-type extractor with methanol. The extract was filtered through filter paper and then evaporated at 40 °C. The yield of the methanol extract was calculated and stored at 4 °C (Albayrak, Aksoy, Sagdic, & Hamzaoglu, 2010).

Essential oil

The plant was hydrodistilled for 3 h using a Clevenger-type distillation apparatus. The obtained essential oil was dried in anhydrous sodium sulphate and stored at 4 °C until use (Albayrak & Aksoy, 2019).

Estimation of total phenol and flavonoid contents

Folin-Ciocalteu and $AlCl_3$ colorimetric assays were performed to examine the total phenolic and flavonoid content of the methanol extract as detailed in our previous work (Albayrak et al., 2010). The results of total phenolic and flavonoid contents are expressed as milligrams of gallic acid equivalents (GAE) and quercetin equivalents (QE)/g extract, respectively.

Analysis of essential oil content

The essential oil composition was detected by gas chromatography/mass spectrometry/quadrupole detection analysis using a Shimadzu QP 5050 system, as detailed in our previous work. The composition (%) was computed from the GC peak areas without any correction factors (Albayrak & Aksoy, 2019).

Antioxidant activity

The antioxidant activity of the methanol extract of *S. potentillifolia* was spectrophotometrically evaluated using several methods, including phosphomolybdenum, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, H_2O_2 scavenging, β-carotene bleaching inhibition, Fe^{2+} chelation, reducing power, ferric reducing antioxidant power (FRAP), and cupric ion reducing activity (CUPRAC) assays. Total antioxidant activity was presented as mg of ascorbic acid equivalents (AAE) /g extract. The ability of the extracts to scavenge DPPH was examined as a percentage of inhibition. IC_{50} (concentration necessary to scavenge 50% DPPH) value was calculated. BHT (Butylated hydroxytoluene) was used as a reference (Albayrak & Aksoy, 2019).

The ability of the extract to prevent bleaching of β-carotene was studied (Cao et al., 2009), and the results are presented as percentage inhibition. The percentages of H_2O_2 scavenging by the extract and gallic acid, BHT and BHA (Butylated hydroxylanisole) standards were calculated. IC_{50} value was determined. The FRAP results for the extract and L- ascorbic acid were expressed as mmol/L of Fe^{2+}. The reducing activity was compared with that of BHT, and the results are presented as absorbance values at 700 nm. Increasing the absorbance of the solution indicates a higher reduction potential. The chelating ability was compared with ethylene diamine tetra acetic acid (EDTA). The inhibition of ferrozine-Fe^{2+} complex formation was determined (Albayrak & Aksoy, 2019).

Determination of antimicrobial activity

Aeromonas hydrophila ATCC 7965, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Klebsiella pneumo-*

niae ATCC 13883, *Listeria monocytogenes* 1/2B, *Proteus mirabilis* ATCC 25933, Methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA), *Streptococcus pneumoniae* ATCC 10015, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* NRRLE 4463, *Yersinia enterocolitica* ATCC 1501, *Candida albicans* 10231, *Aspergillus parasiticus* DSM 5771, and *Aspergillus flavus* NRRL 3357 were used as test organisms.

Antimicrobial activity test was performed in accordance with the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2020). Agar-well diffusion and the micro-well dilution method, which were described in our earlier study, was carried out for the extract at 30 mg/mL (Albayrak & Aksoy, 2019).) For the essential oil, a disc diffusion assay, which was described in our previous study, was used (Albayrak & Aksoy, 2019). The growth inhibition zones were recorded in millimeters. Tetracycline (10 mg/mL), natamycin (30 mg/mL), ampicillin (AMP, 10 µg/disk), kanamycin (K, 30 µg/disk), and penicillin (P, 10 µg/disk) were selected as standards.

To determine the minimum inhibitory concentration (MIC) values, the extract and essential oil were prepared at 30 mg/mL and 2000 µg/mL in 10% dimethylsulfoxide (DMSO). Then, two-fold dilutions were made in the medium. The minimum inhibitory concentration (MIC) was recorded as the lowest sample concentration that prevented visible growth after the incubation period. In the MIC method, the concentrations exhibiting complete absence of visual growth were determined, and 0.1 mL of each culture broth was transferred on to the agar plates. Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were recorded as the lowest concentration at which zero or only one colony was observed on the agar surface.

RESULTS AND DISCUSSION

The hydrodistillation of *S. potentillifolia* yielded a light yellowish oil with a yield of 0.15% (v/w). The phenolic constituents of the essential oil of *S. potentillifolia* were determined by GC/MS. Fifty compounds representing 100.00% of the total oil were determined (Table 1). The major compound was eucalyptol (%23.01). It was followed by carvacrol (6.45%), β-pinene (6.01%), borneol (5.48%), camphor (4.69%), α-terpineol (4.04%), 4-terpineol (3.93%), bornyl acetate (3.92%), caryophyllene (3.92%), caryophyllene oxide (3.84%), α-pinene (3.81%), linalool (3.37%), cymol (2.58%), γ-terpinene (2.55%), α-terpinyl acetate (1.79%), β-myrcene (1.78%), myrtenyl acetate (1.70%), α-humulene (1.60%), *δ*-cadinene (1.44%), germacrene (1.32%), humulene oxide (1.12%) and β-bourbonene (1.01%).

Several authors have reported on the essential oil compositions of Salvia species (Hatipoglu et al., 2016; Wang et al., 2023). Apart from some chemical variations, eucalyptol, camphor, borneol and α, β-pinene are usually main components of *Salvia* essential oils. The essential oil constituents of *S. potentillifolia* (Burdur, Türkiye) was previously analyzed and α, β-pinenes were found as major compounds (Kivrak et al., 2009). Our findings are similar to those obtained for *S. officinalis* (Hayouni et al., 2008; Longaray Delamare, Moschen-Pistorello, Artico, Atti-Serafini, & Echeverrigaray, 2007) and *S. libanotica* (Farhat et al., 2001) oil containing 1,8-cineole, camphor, borneol and α, β-pinene as main compounds. Similarly, 1,8-cineole was found as the main component of *S. aucheri* var. *aucheri* (30.5%), *S. aramienesis* (46.0%) (Kelen & Tepe, 2008), and *S. hydrangea* EO (2.1%) (Kotan et al., 2008). The major components of *S. officinalis* essential oils were determined as α-thujone, camphor, and borneol (Russo et al., 2013). According to these results, there was a significant variation in the content and yield of essential oil among the studied *Salvia* species. The reasons for these variations may be the differentiation of species, geographical, climatic, seasonal, and experimental conditions (Herraiz-Peñalver et al., 2010).

The percent yield of the methanol extract of *S. potentillifolia* was 17.20% (w/w). The total phenolic and flavonoid contents of the methanol extract were 52.20 ± 0.6 mg GAE/g and 9.90 ± 0.0 mg QE/g extract, respectively. It has been previously recorded the total phenolic and total flavonoid contents of the methanol, hexane, ethylacetate, and water extracts obtained from *S. potentillifolia* as range from 49.2 to 62.4 µg pyrocatechol equivalents (PEs)/mg and from 35.1 to 292.2 µg quercetin equivalents (QEs) /mg, respectively. The total phenolic content of the methanol extract of *S. potentillifolia* was found as 168.5 µg QEs/mg (Kivrak et al., 2019). This value is much higher than the value obtained for the methanol extract of *S. potentillifolia* evaluated here. The total phenolic and total flavonoid contents of the methanol extracts of different Salvia species were found as 38-326 mg gallic acid/g and 91-253 mg (+)-catechin/g, respectively (Asadi et al., 2010). The total phenolic and total flavonoid contents of the methanol extract of *S. spinosa* were 377.6 mg GAE/g and 134.8 mg QE/g, respectively (Bahadori et al., 2015). The total phenolic content of the methanol extract of *S. eremophila* was found as 101.25 µg GAE/mg (Ebrahimabadi, Mazoochi, Kashi, Djafari-Bidgoli, & Batooli, 2010). The total phenolic contents were found as 67.67-72.02 mg GAE/g for *S. argentea* extracts and 112.93-161.37 mg GAE/g for *S. officinalis* extracts (Farhat et al., 2013).

To determine of antioxidant effects of the extracts should be used many methods which have several mechanisms (Aruoma, 2003). Thus, several biochemical methods were carried out to evaluate properties of the extract in this work: phosphomolybdenum, DPPH, FRAP, CUPRAC, β-Carotene bleaching, hydrogen peroxide scavenging, and chelating activity methods.

The total antioxidant activity of the methanol extract was determined to be 301.78 ± 0.6 mg AAE/g extract. In the

Table 1. Compositions of essential oil from *S. potentillifolia*

Compounds	RT[b]	%	Compounds	RT[b]	%
α-thujene[a]	5.694	0.25[c]	Bornyl acetate	24.392	3.92
α-pinene	5.942	3.81	(-)-trans-Pinocarvyl acetate	25.123	0.18
Camphene	6.466	0.66	Carvacrol	26.092	6.45
Sabinene	7.254	0.57	Myrtenyl acetate	26.951	1.70
β-Pinene	7.460	6.01	α-Terpinyl acetate	28.504	1.79
β -Myrcene	7.884	1.78	α-Copaene	30.224	0.39
α- Terpinene	9.037	0.62	β - Bourbonene	30.698	1.01
Cymol	9.404	2.58	β - Elemene	31.209	0.08
Eucalyptol (1,8-cineole)	**9.787**	**23.01**	Caryophyllene	32.967	3.92
γ-Terpinene	10.995	2.55	α-Humulene	35.204	1.60
α -Terpinolene	12.369	0.44	β - Farnesene	35.442	0.54
Linalool	13.296	3.37	β - Cadina-1(6),4-diene	36.336	0.18
Nonanal	13.440	0.24	Germacrene	36.839	1.32
α- Thujone	14.071	0.26	β-Cubebene	37.457	0.20
α- Campholenal	14.574	0.28	β -Selinene	37.874	0.33
trans-Pinocarveol	15.460	0.84	γ-Cadinene	38.900	0.20
Camphor	15.707	4.69	sesquisabinene hydrate	39.107	0.52
p-Mentha-1,5-dien-8-ol	16.046	0.09	δ-Cadinene	39.304	1.44
Pinocamphone	16.495	0.25	Elemol	41.192	0.37
Pinocarvone	16.592	0.51	Caryophyllene oxide	42.922	3.84
Borneol	17.397	5.48	Guaiol	44.033	0.16
4-Terpineol	17.921	3.93	Humulene oxide	44.549	1.12
Cymen-8-ol <para->	18.432	0.23	α -Longipinene	45.768	0.43
Myrtenal	18.626	0.67	α- Muurolol	46.640	0.39
α -Terpineol	18.915	4.04	β-Eudesmol	47.280	0.76
			Total		**100**

[a] Compounds listed in order of elution from the FFAP MS column.
[b] Retention time (as minutes).
[c] The percentage composition was computed from the GC peak areas. Bold type indicates major components.

β- carotene bleaching method, the β- carotene is prevented from losing its orange colour in the presence of antioxidants by quenching the linoleate-free radicals formed in the solution (Jayaprakasha, Singh, & Sakariah, 2001). The inhibition values of the methanol extract, butylated hydroxytoluene, and butylated hydroxyanisole at 1 mg/mL were 31.48%, 84.26%, and 94.33%, respectively. The extract showed weaker inhibitor activity than BHT and BHA.

The methanol extract exhibited concentration-dependent DPPH radical scavenging activity (Figure 1). The percentage inhibition of the extract was 15.69%, 37.13%, 69.24%, 91.18, and 92.02% at 0.1, 0.25, 0.5, 1, and 2 mg/mL concentrations, respectively. At 1 and 2 mg/mL, the extract exhibited a high inhibitory effect similar to that of BHT (91.47% and 92.15% at 1and 2 µg/mL, respectively). The IC_{50} was calculated as 11.63 µg/mL. The BHT level was 3.35 µg/mL. Low IC_{50} and high DPPH scavenging percentages indicate high antioxidant activity.

There are many studies on the DPPH scavenging potential of different *Salvia* species. The ethanol extract of *S. potentillifolia* collected from Burdur, Türkiye showed high DPPH inhibitor (IC_{50}= 69.4 µg/mL) and β-carotene bleaching inhibitor (75.4 % at 80 µg/mL) activity (Kivrak et al., 2009). The same researchers reported that ethyl acetate extracts of *S. halophila* showed DPPH scavenging activity with IC_{50}= 248.4 µg/mL and β-carotene bleaching inhibitor effect (IC_{50} = 26.1 µg/mL, respectively (Kivrak et al., 2019).

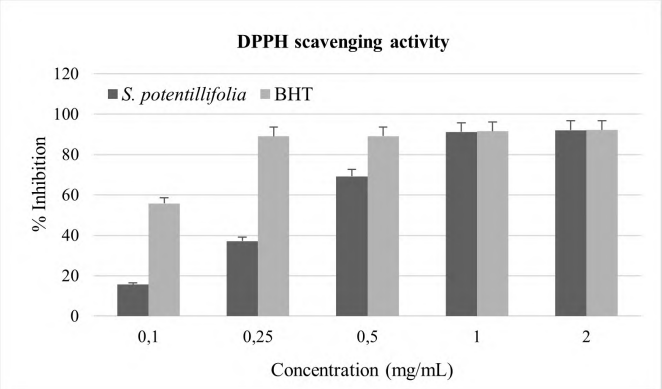

Figure 1. DPPH scavenging activity of *S. potentillifolia* methanol extract

The methanol extract of *S. eremophila* was reported to be an antiradical (IC_{50} = 35.19 µg/mL) and β-carotene bleaching inhibitor (72.42%) agent (Ebrahimabadi et al., 2010). The methanol extract of *S. brachyantha* exerted antioxidant, DPPH scavenging effect (IC_{50}= 46.72 µg/mL) and β-carotene inhibitor effect (69.45%) (Esmaeili & Sonboli, 2010). When the results are compared, it is seen that the methanol extract of *S. potentillifolia* had higher DPPH inhibitor activity (IC_{50} = 11.63 µg/mL) and the lower β-carotene bleaching inhibitor activity than the methanol extracts of *S. eremophila* and *S. brachyantha*. In another study, IC_{50} values of the methanol extracts obtained from *S. virgata, S. nemorosa, S. officinalis, S. sclarea, S. per-*

sica, S. reuterana, and *S. cereal* were in the range of 198 to 1810 µg/mL (Aghaei Jeshvaghani et al., 2015). IC_{50} value of the methanol extract of *S. potentillifolia* was lower than that of these *Salvia* species and thus had higher DPPH scavenger activity. It has been reported that the methanol extract of *S. spinosa* displayed high DPPH scavenging potency with IC_{50} =116.4 µg/mL (Bahadori et al., 2015). However, this IC_{50} was higher than that of *S. potentillifolia*.

Cu^{2+} reducing potentials of the extract and trolox are presented as absorbance values in Fig. 2. Cu^{2+} reducing activity of the extract was concentration-dependent. As shown in Figure 2, *S. potentillifolia* extract had strong CUPRAC activity. The absorbance values of the methanol extract at 450 nm ranged from 0.017 to 3.07 at 0.6-3 mg/mL. The absorbance value of the methanol extract (2.87) was the higher than trolox (2.85) at 1 mg/mL.

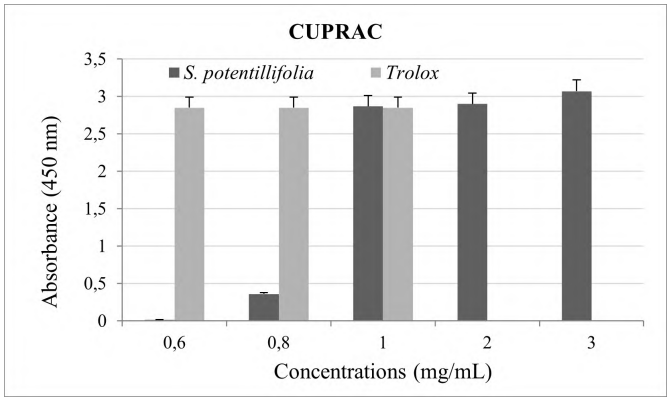

Figure 2. Cu^{2+} reducing activity of *S. potentillifolia* methanol extact

The result indicated that the methanol extract of *S. potentillifolia* had moderate reducing power at 2.28 mM Fe (II)/L compared with L-ascorbic acid (4.52 mM Fe (II)/L) in the FRAP assay. The FRAP values of the methanol extracts obtained from different *Salvia* species were in the range of 81.56 to 197.33 mM Fe(II)/mg (Farhat et al., 2013). Based on this study, it can be concluded that the total phenolic content, composition, and antioxidant capacity of *Salvia* samples collected from different regions may vary.

The methanol extract of *S. potentillifolia*, BHT, BHA, and gallic acid exhibited 34.09%, 76.97%, 64.73%, and 137.61% hydrogen peroxide scavenging activity, at 50 µg/mL, respectively (Figure 3). IC_{50} values were 78.63, 31.09, 23.16 and 17.62 µg/mL, respectively. According to the results, the methanol extract has a weak scavenging potential. The hydrogen peroxide scavenging effect of the methanol extract and standards increased in the order of the methanol extract< BHT< BHA< gallic acid. Similarly, Zhao, Xiang, Ye, Yuan, & Guo, (2006) determined that the extract of *S. miltiorrhiza* has high DPPH scavenging activity but low hydrogen peroxide scavenging activity.

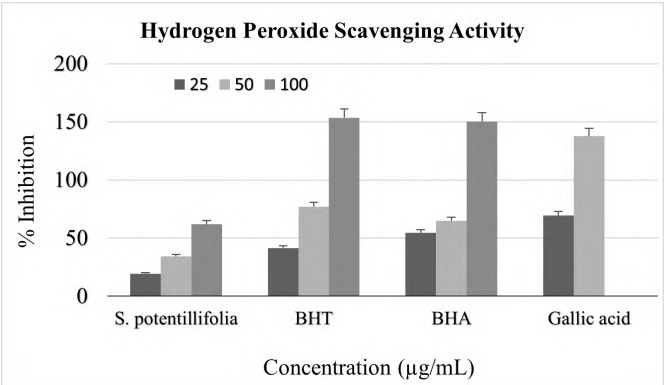

Figure 3. H_2O_2 scavenging effect of *S. potentillifolia* methanol extract

As shown in Figure 4, *S. potentillifolia* extract showed strong reducing power compared with BHT. The reducing activity of *S. potentillifolia* extract and BHT increased with increasing concentrations. *S. potentillifolia* extract has a higher powerful reducing ability than BHT at concentrations of 2.50, 5.0, and 10.0 mg/mL concentrations. The results demonstrated that *S. potentillifolia* extract has high electron-donor properties and thus can terminate very harmful radical chain reactions. The ethanol extract of *S. potentillifolia* has been reported to have high reducing power in a previous study (Kivrak et al, 2009).

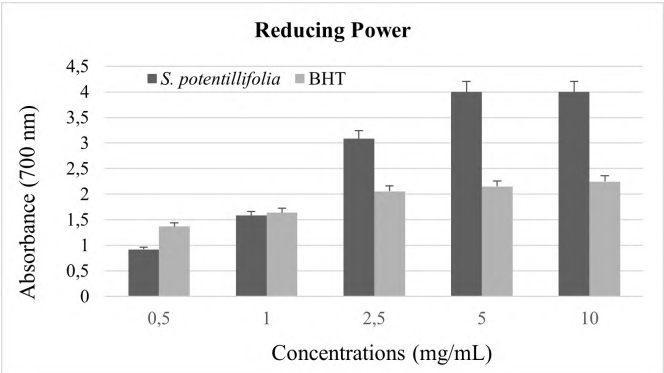

Figure 4. Reducing power of *S. potentillifolia* methanol extract

S. potentillifolia extract showed weak ferrous ion (Fe^{2+}) chelating activity. *S. potentillifolia* extract exhibited 16.25 ± 0.3% chelation of ferrous ions at 5 mg/mL. The value for EDTA was found to be 99.45 ± 0.0% at the same concentration. Similarly, the metal chelation activities of methanolic extracts obtained from five different *Salvia* species were reported to be between 37.35% and 76.25% (Kursat et al., 2023). Phenolic compounds are potential free radical terminators, scavengers, metal chelators, and inhibitor of lipoxygenase (Asadi et al., 2010). Because of differences in experimental methods , standards, and collection locations, it is difficult to compare different species (Aghaei Jeshvaghani et al., 2015).

Table 2. Antimicrobial activities of the methanol extract and essential oil of *S. potentillifolia* (mm, inhibition zones)

Bacteria	Extract (30 µg/mL)			Essential oil			Tetracycline			Ampicillin	Kanamycin	Penicillin
	mm	MIC (µg/mL)	MBC (µg/mL)	mm	MIC (µg/mL)	MBC (µg/mL)	mm	MIC (µg/mL)	MBC (µg/mL)	mm	mm	mm
A. hydrophila	7.0	6.25	12.5	9.0	0.5	1.0	27	<3.9	125	32	19	37
Y. enterocolitica	-	-	-	-	-	-	23	<3.9	125	-	10	-
S. typhimurium	-	-	-	-	-	-	15	62.5	125	11	11	10
L. monocytogenes	-	-	-	10	0.5	0.5	29	<3.9	<3.9	-	-	15
E. coli	-	-	-	-	-	-	24	<3.9	125	8	11	7
K. pneumoniae	10	0.78	0.78	27	0.25	0.5	48	15.6	15.6	23	7	33
S. aureus (MRSA)	-	-	-	-	-	-	25	<3.9	125	-	-	-
P. mirabilis	-	-	-	-	-	-	19	31.5	125	7	14	12
B. cereus	10	6.25	12.5	9.0	0.25	1.0	27	<3.9	<3.9	-	15	11
S. pneumoniae	13	6.25	6.25	9.0	0.5	0.5	24	7.8	7.8	-	-	13
S. enteritidis	-	-	-	-	-	-	25	<3.9	62.5	9	10	10
Yeast							**Natamycin**	**MIC (µg/mL)**	**MFC (µg/mL)**			
C. albicans	-	-	-	-	-	-	23	<3.9	62.5	-	-	-
Moulds												
A. flavus	-	-	-	7	0.06	1.0	17	7.8	>250	-	-	-
A. parasiticus	-	-	-	-	-	-	15	15.6	>250	-	-	-

-: not detected

The antioxidant properties of *Salvia* genus and *S. potentillifolia* were determined using many assays recorded in many reports. However, as far as our literature survey could ascertain, the FRAP and CUPRAC potencies, H_2O_2 scavenging, iron reducing power, and Fe^{2+} chelating potency of *S. potentillifolia* have not been previously reported.

Table 2 gives summary of the findings of the antimicrobial properties of the methanol extract and essential oil of *S. potentillifolia* against 12 bacteria, one yeast and two moulds. The methanol extract of *S. potentillifolia* exhibited weak antibacterial activity (Table 2). The extract had an effect only against *A. hydrophila, K. pneumoniae, B. cereus,* and *S. pneumoniae* with inhibition zones and MIC values in the range of 7.0-13 mm and 0.78-12.5 mg/mL, respectively. No activity against *C. albicans, A. flavus*, and *A. parasiticus* was exerted by the methanol extract. Essential oil had moderate antibacterial activity against all tested bacteria, along with 0.25-1.0 mg/mL MIC and MBC. The essential oil had no inhibitory effect against *Y. enterocolitica, S. typhimurium, E. coli*, methicillin-resistant *S. aureus, P. mirabilis*, and *S. enteritidis*, whereas it showed antibacterial activity against *A. hydrophila* (9.0 mm, inhibition zone), *L. monocytogenes* (10.0 mm), *K. pneumoniae* (27 mm), *B. cereus* (9.0 mm), and *S. pneumoniae* (9.0 mm). *B. cereus* and *K. pneumoniae* were the most sensitive (MIC=0.25 mg/mL) to the essential oil. Essential oil showed weak antifungal activity against aflatoxigenic *A. flavus* (7.00 mm, MIC = 0.06 mg/mL and MFC = 1.0 mg/mL). Essential oil was more effective against *L. monocytogenes, K. pneumoniae*, and *S. pneumoniae* than ampicillin and kanamycin.

The antimicrobial potency of the essential oils of *S. potentillifolia* and its ethanol extract was previously investigated. Contrary to our results, the essential oil and ethanol extract of *S. potentillifolia* was reported to have antibacterial effects against *S. enteritidis, E. coli, Y. enterocolitica, S. aureus* (MIC= 26.5-67.5 µg/mL) and anticandidal activity against *C. albicans* (MIC= 18.5-27.5 µg/mL). When the results are compared, it can be said that ethanol extract of *S. potentillifolia* is more effective than its methanol extract against K. pneumoniae and B. cereus (Kivrak et al., 2009). In a previous study, it was reported that *S. potentillifolia* has anticandidal activity (Celik, Ergin, Arslan, & Kartal, 2010). S. hydrangea essential oil exhibits considerable antifungal activity and a wide spectrum of antibacterial activity (Kotan et al., 2008). Similar results were obtained by Delamare (2007), who showed that the essential oils of *S. officinalis* and *S. triloba* exhibited inhibitory effect on *B. cereus* and *A. hydrophila* (Longaray Delamare et al., 2007). Contrary to our results, the methanol extract and essential oil of *S. spinosa* had effects against *E. coli, S. aureus*, and *C. albicans,* but were not active against *K. pneumoniae* (Ebrahimabadi et al., 2010). The essential oil of *S. sclareoides* strongly prevented *K. pneumoniae, S. aureus,* and *L. monocytogenes*, except for *P. aeruginosa* and *C. albicans* (Sepahvand et al., 2014). Thus, the compositions and biological activities of the essential oils of different *Salvia* species may change (Hayouni et al., 2008). Furthermore, as far as our literature survey could be determined, there were any findings regarding the antimicrobial activity of the methanol extract of *S. potentillifolia*. The phenolic content of essential oils may change due to many factors.

CONCLUSION

The main content of the essential oil of *S. potentillifolia* was eucalyptol (%23.01) determined by gas chromatography–mass spectrometry. The methanol extract of *S. potentillifolia* was found to have strong antioxidant potential. The present results also showed that the methanol extract and essential oil of *S. potentillifolia* exhibited from weak to moderate antimicrobial activity. Based on the obtained results, it can be evaluated as a natural source in the pharmaceutical and food industries. Therefore, further *in vivo* studies on antioxidant activity and action mechanisms are required. The results of this study support its therapeutic use in folk medicine.

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Original Article

Ex vivo anticoagulant effect of *Zingiber officinale* in whole blood samples

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ABSTRACT

Background and Aims: *In vitro* and *in vivo* studies have shown that *Zingiber officinale* (*Z. officinale*, Ginger) may have an anticoagulant effect. Although there are studies on the anticoagulant effect, the results are inconclusive. Our study investigated the anticoagulant effect of *Z. officinale* on the whole blood sample *ex vivo*.

Methods: The inner and shell parts of *Z. officinale* were extracted with methanol, ethanol, and water. 0.1 mg/mL of different volumes (100, 150 and 200 µL) of *Z. officinale* extracts were added to the blood of a healthy volunteer *ex vivo*. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured in the coagulation analyser. Measurements were performed twice, before and after the ginger treatment. International Normalised Ratio (INR) values were calculated using the following mathematical formula: INR = Patient PT/Control PT. The IBM SPSS 25.0 software was used for statistical analyses.

Results: A notable prolongation in PT, aPTT, and INR was detected after the addition of *Z. officinale* extract to blood samples ($p<0.001$). As the volume of *Z. officinale* extract added to the blood sample increased, coagulation parameters were observed to display a corresponding increase ($p<0.001$).

Conclusion: *Z. officinale* was associated with prolonged PT, aPTT, and INR *ex vivo*. *In vivo* studies are needed to demonstrate the mechanism of the anticoagulant effect.

Keywords: Activated partial thromboplastin time, Anticoagulant herbs, Ginger, Prothrombin time, *Zingiber officinale*

INTRODUCTION

Blood vessels, platelets, coagulation factors, and the fibrinolytic system make up the haemostasis system, which helps control bleeding. The vascular constriction phase followed by platelet adhesion. The extracellular matrix facilitates platelet adhesion and aggregation through the secretion of cytokines and inflammatory markers. This process ensures the formation of the platelet plug. Platelets release cytoplasmic granules after adhesion. The cytoplasmic granules contain platelet-activating factors such as adenosine diphosphate (ADP), thromboxane A2 (TXA2), and serotonin. There are two pathways in the coagulation process: extrinsic and intrinsic. Coagulation factors participate in these pathways. Prothrombin is degraded to during preoperative examinations, and in patients with bleeding (Winter, Flax, & Harris, 2017).

Thrombosis frequently is the main cause of cardiovascular illnesses, such as myocardial infarction, atrial fibrillation, and associated mortality (McEwen, 2015). Despite advances in the identification and treatment of cardiovascular diseases, approximately half of all deaths are due to cardiovascular diseases (Wong et al., 2019). Many cardiovascular disorders are characterised by increased clotting activity; therefore, treatments include antithrombotic and anticoagulant drugs to prevent blood clotting (Lowe & Rumley, 2014). These drugs include antithrombotic agents, anticoagulants that stop the coagulation system and prevent clot expansion, antiplatelet agents that reduce platelet aggregation and prevent thrombus forma-

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tion, and fibrinolytic enzymes that directly dissolve thrombus. Anticoagulant drugs can have side effects, such as bleeding and thrombocytopenia (Harter, Levine, & Henderson, 2015). Given these potential side effects, studies investigating the use of herbal medicines against cardiovascular diseases are coming to the fore. Patients with cardiovascular diseases such as atherosclerosis and systolic hypertension tend to use herbal medicines (McEwen, 2015).

Zingiber officinale (Z. officinale, Ginger) is an herb that has been used as a spice and herbal medicine since ancient times. It contains a wide variety of active ingredients. The main types of gingerols are shogaol, paradol, quercetin, zingerone, and zingerone (Mao et al., 2019). There have been studies that demonstrate it possesses anti-oxidant (Abolaji et al., 2017), anti inflammatory (Teschke & Xuan, 2018), anti-microbial (Nassan & Mohamed, 2014), anti-cancer (El-Ashmawy, Khedr, El-Bahrawy, & Abo Mansour, 2018), and antiemetic (Bossi et al., 2017) properties. In addition to these functions, *in vitro* and *in vivo* studies have shown that *Z. officinale* may have an anticoagulant effect (Flynn, Rafferty, & Boctor, 1986; Koo, Ammit, Tran, Duke, & Roufogalis, 2001; Liao, Leu, Chan, Kuo, & Wu, 2012; Shih et al., 2014). Although there are studies on the anticoagulant effect, the results are inconclusive. This study aimed to determine the anticoagulant effect of *Z. officinale* in human blood. The effects of extracting *Z. officinale* in different solvents, such as methanol, ethanol, and water, on PT, aPTT, and INR in blood samples from healthy volunteers were investigated.

MATERIALS AND METHODS

Participants

The University of Health Sciences Turkey Hamidiye Scientific Research Ethics Committee (registration number: 21/563) approved the study. Fifty healthy volunteers from the Haydarpasa Numune Hospital were included in the study. Written consent to participate in the study was obtained from the participants. The study size was determined to be at least 30 people for the study group, with a G-power analysis of the effect size=80% at the α=0.05 significance level. Our study excluded individuals with diabetes, kidney dysfunction, liver dysfunction, hypertension, cardiovascular disorders, acute or chronic diseases, coagulation problems, or those taking medication.

Z. officinale extract preparation

Z. officinale samples were obtained from a grocery store (Istanbul, Türkiye). They were extracted using solvents. The inner and shell parts of 10 g of fresh *Z. officinale* were grinded and treated with 100 mL of 80% ethanol (Sigma, Darmstadt, Germany), 80% methanol (Isolab, Eschau, Germany), and 100% distilled water solutions, respectively, and mixed at room temperature for 24 h. Then, it was filtered using Whatman filter paper (0.45 µm). Solvents and water in the extract were removed using an evaporator and lyophilizer (Buchi Rotavapor R100, New Castle, USA). The extracts were stored in a refrigerator at -80°C until the study.

Blood Collection

Human blood was collected from fifty healthy volunteers. Approximately 5 mL of blood was taken from each volunteer in a sodium citrate tube (0.109 M Na_3Citrate).

Coagulation Analysis

Plasma coagulation analysis was performed using the STA Compact Max® automatic coagulation analyser (Asnières-sur-Seine, France). The aPTT and PT were measured in adherence to the protocols outlined by the manufacturer. The anticoagulant activity was quantified as the clotting time in seconds, with heparin serving as the reference.

Anticoagulant Activity

After blood was collected from each volunteer in a sodium citrate tube, the tube was turned upside down. Then, 0.1 mg/mL inner and shell parts of *Z. officinale* extract were added to whole blood samples in 3 different volumes as 100 µL, 150 µL, and 200 µL. After 15 min of incubation at room temperature, blood samples were collected separately and centrifuged at 3000 rpm for 10 min in a NUVE, NF 1200R centrifuge machine (Ankara, Türkiye), and plasmas were separated. Measurements were performed twice, before and after the *Z. officinale* treatment. PT and aPTT measurements were performed using the STA Compact Max® automatic coagulation analyser (Asnières-sur-Seine, France). International Normalised Ratio (INR) values were calculated using the formula INR = Patient PT/Control PT (Shikdar, Vashisht, & Bhattacharya, 2022).

Statistical Analysis

The IBM SPSS 25.0 software was used for statistical analyses. The data distribution was determined using the Kolmogorov-Smirnov test. Mean ± standard deviation values were used to express continuous variables. One-way analysis of variance (ANOVA) was used to determine differences between the means of two or more independent groups. Two-way ANOVA was used to examine differences in coagulation parameters before and after the *Z. officinale* extraction treatment. The 95% confidence intervals are used to show differences between groups. Statistical significance was defined as $p<0.05$.

RESULTS

Z. officinale extraction was performed on whole blood samples of the participants. Table 1-2 and Figure 1 compare the

PT, aPTT, and INR before and after the *Z. officinale* treatment. A statistically significant increase in PT values was observed after adding 100 µL IG (Inner of ginger)-Ethanol (3.0%), IG-Methanol (3.78%), IG-Water (2.3%), SG (Shell of ginger)-Ethanol (5.48%), SG-Methanol (2.07%), and SG-Water (0.74%) to the samples (p<0.001). As more *Z. officinale* extract was administered to the blood, increase in parameters was seen (p<0.001, For 200 µL extract: IG-Ethanol: 8.67%, IG-Methanol: 8.44%, IG-Water: 6.9%, SG-Ethanol: 10%, SG-Methanol: 5.9%, SG-Water: 2.37%). The greatest increase was observed after adding 200 µL of SG-ethanol (Pre-treatment: 13.50±1.02 second, post-treatment 14.90±1.02 second) (Figure 1.).

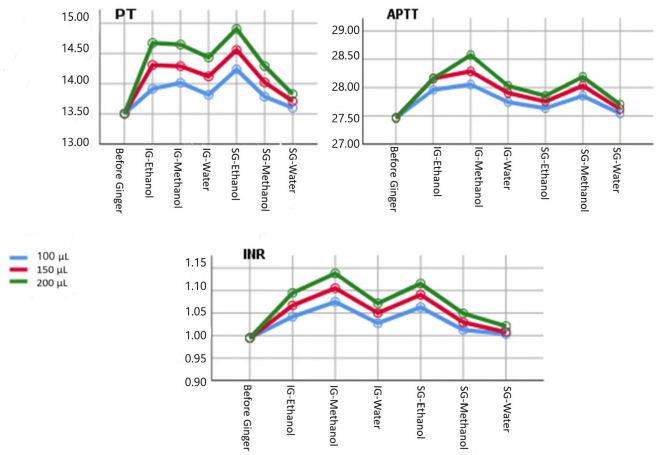

Figure 1. *Coagulation parameters before and after ginger supplementation PT: Prothrombin time, aPTT: Activated partial thromboplastin time, INR: International Normalised Ratio, IG: Inner of ginger, SG: Shell of Ginger*

Considering the difference in aPTT, a statistically significant increase was observed in all *Z. officinale* forms, except IG-Ethanol, compared with the pre-treatment (p<0.001). As the amount of *Z. officinale* extract added to the blood increased, aPTT values increased significantly (p<0.001). The most significant increase was observed after adding 200 µL of IG-Methanol (Pre-treatment: 27.46±3.28 second, post-treatment 28.57±3.36 second) (Figure 1.)

Similar to the other parameters, a statistically significant increase in INR was observed in all *Z. officinale* forms compared with the pre-treatment (Table 2, Figure 1) (p<0.001). As more *Z. officinale* extract was infused into the blood, INR levels increased significantly (p<0.001). Adding 200 µL of IG-methanol, similar to aPTT, resulted in the most significant rise (Pre-treatment: 0.99±1.06 second, post-treatment 1.13±0.09 second) (Figure 1.).

DISCUSSION

Coagulation, which is a part of the body's haemostasis mechanism, becomes a potentially fatal occurrence in pathological situations. Anticoagulant medications are commonly used to reduce blood clotting in various conditions, such as cardiovascular disease. Because anticoagulant medications may have adverse effects, researchers are looking for novel anticoagulants with fewer negative side effects (Ayodele, Onajobi, & Osoniyi, 2019). *Z. officinale* has been used in traditional medicine for centuries, and its potential medical properties are still being studied (Chrubasik, Pittler, & Roufogalis, 2005). This study found that *Z. officinale* extracts prepared in different solvents had anticoagulant effects by prolonging the PT, aPTT, and INR values in whole blood samples. The parameters were prolonged as the concentration of the *Z. officinale* extract increased. To the author's knowledge, this is the first study in the literature to show the effect of *Z. officinale's* inner and shell parts prepared in different solvents (water, ethanol, and methanol) and in different volumes (100, 150, 200 µL) on the coagulation parameters.

PT and aPTT are coagulation parameters used to determine the coagulation mechanism. The extrinsic coagulation cascade was assessed using PT, whereas intrinsic and common pathways were assessed using aPTT. In clinical evaluation, prolonged aPTT and/or PT indicates coagulation impairment. Prolonged PT and aPTT suggests that common pathway factors may be inhibited (V, X, and prothrombin) (Yang & Moosavi, 2022). In our study, *Z. officinale* showed anticoagulant effects by prolonging PT, aPTT, and INR. This effect may be caused by common pathway factors V, X, and prothrombin. More research is needed to understand the mechanism by which this mechanism is affected.

There have been studies showing that *Z. officinale* has an anticoagulant effect *in vitro*. In a study similar to our findings, researchers explored the impact of an aqueous extract from *Z. official* roots on blood PT *in vitro* and found a dose-dependent prolongation of PT (Eldin, Elmutalib, & Hamedelniel, 2016). In a study assessing the *in vitro* anticoagulant effect of ethanol extracts from *Z. officinale* roots, researchers observed prolonged PT, with no significant difference noted in aPTT (Ahmad, Mohammed, Mohamed Eltayeb, Mohammed Elmosaad, & Waggiallah, 2022). In an *in vitro* investigation, rat basophilic leukaemia 2H3 cells, which accurately reflect arachidonic acid metabolism, were used to demonstrate the antithrombotic efficacy of synthetically produced Gingerols. The effects of gingerols on arachidonic acid-induced platelet serotonin release and platelet aggregation were compared with aspirin, which has potent antiplatelet activity. According to these findings, gingerols can decrease the release and aggregation of platelets produced by arachidonic acid in human platelet-rich plasma. These changes were 2–4 times less effective than aspirin. Furthermore, in the same study, Prostaglandin D2 (PGD2), a result of arachi-

Table 1. Differences between before and after ginger treatmentfor PT and aPTT

Variable			Mean±SD	F	*p**	Difference
PT (sec)	Baseline		13.50±1.02	1496.633	**0.001**	4>3>2>1
	IG-Ethanol	100 µL	13.91±1.02			
		150 µL	14.30±1.02			
		200 µL	14.67±1.03			
	Baseline		13.50±1.02	591.528	**0.001**	4>3>2>1
	IG-Methanol	100 µL	14.01±1.01			
		150 µL	14.28±1.03			
		200 µL	14.64±1.04			
	Baseline		13.50±1.02	1056.928	**0.001**	4>3>2>1
	IG-Water	100 µL	13.81±1.02			
		150 µL	14.12±1.01			
		200 µL	14.43±1.00			
	Baseline		13.50±1.02	1084.970	**0.001**	4>3>2>1
	SG-Ethanol	100 µL	14.23±1.02			
		150 µL	14.55±1.03			
		200 µL	14.90±1.02			
	Baseline		13.50±1.02	787.703	**0.001**	4>3>2>1
	SG-Methanol	100 µL	13.78±1.02			
		150 µL	14.02±0.99			
		200 µL	14.29±0.99			
	Baseline		13.50±1.02	193.679	**0.001**	4>3>2>1
	SG-Water	100 µL	13.60±1.01			
		150 µL	13.71±1.01			
		200 µL	13.82±1.01			
aPTT (sec)	Baseline		27.46±3.29	10.187	0.338	3>4>2>1
	IG-Ethanol	100 µL	27.96±3.34			
		150 µL	28.16±3.34			
		200 µL	28.15±3.26			
	Baseline		27.46±3.29	397.998	**0.001**	4>3>2>1
	IG-Methanol	100 µL	28.05±3.38			
		150 µL	28.28±3.39			
		200 µL	28.57±3.36			
	Baseline		27.46±3.29	435.233	**0.001**	4>3>2>1
	IG-Water	100 µL	27.74±3.32			
		150 µL	27.90±3.34			
		200 µL	28.03±3.32			
	Baseline		27.46±3.29	236.510	**0.001**	4>3>2>1
	SG-Ethanol	100 µL	27.63±3.30			
		150 µL	27.75±3.29			
		200 µL	27.85±3.28			
	Baseline		27.46±3.29	532.898	**0.001**	4>3>2>1
	SG-Methanol	100 µL	27.85±3.34			
		150 µL	28.03±3.32			
		200 µL	28.18±3.32			
	Baseline		27.46±3.29	70.582	**0.001**	4>3>2>1
	SG-Water	100 µL	27.54±3.30			
		150 µL	27.62±3.27			
		200 µL	27.70±3.28			

1=Pre-treatment; 2=100 µL; 3=150 µL; 4=200 µL PT: Prothrombin time, aPTT: Activated partial thromboplastin time, INR: International Normalised Ratio, IG: Inner of ginger, SG: Shell of Ginger, *: Two-way analysis of variance (two-way ANOVA).

Table 2. Differences between before and after ginger treatmenton INR

Variable			Mean±SD	F	*p**	Difference
INR	Baseline		0.99±0.10			
	IG-Ethanol	100 µL	1.04±0.10	373.943	**0.001**	4>3>2>1
		150 µL	1.06±0.10			
		200 µL	1.09±0.10			
	Baseline		0.99±0.10			
	IG-Methanol	100 µL	1.07±0.10	711.178	**0.001**	4>3>2>1
		150 µL	1.10±0.10			
		200 µL	1,13±0,09			
	Baseline		0.99±0.10			
	IG-Water	100 µL	1.02±0.10	423.455	**0.001**	4>3>2>1
		150 µL	1.05±0.10			
		200 µL	1.07±0.10			
	Baseline		0.99±0.10			
	SG-Ethanol	100 µL	1.06±0.10	606.383	**0.001**	4>3>2>1
		150 µL	1.09±0.10			
		200 µL	1.11±0.10			
	Baseline		0.99±0.10			
	SG-Methanol	100 µL	1.01±0.10	252.143	**0.001**	4>3>2>1
		150 µL	1.02±0.10			
		200 µL	1.04±0.10			
	Baseline		0.99±0.10			
	SG-Water	100 µL	1.00±0.10	74.761	**0.001**	4>3>2>1
		150 µL	1.00±0.10			
		200 µL	1.02±0.10			

1=Pre-treatment; 2=100 µL; 3=150 µL; 4=200 µL

INR: International Normalised Ratio, IG: Inner of ginger, SG: Shell of Ginger, *: Two-way analysis of variance (two-way ANOVA).

donic acid metabolism, was measured in RBL-2H3 cells, and gingerols were found to suppress COX (cyclooxygenase) activity (Koo et al., 2001). The COX enzyme converts arachidonic acid, an omega-6 fatty acid, into prostaglandins (PGD2, PGE2, PGF2, PGI2) and thromboxanes (TXA2, TXB2). These lipid mediators, synthesised and released from endothelial cells and platelets, are associated with platelet aggregation and inflammation (Wang et al., 2021). In another study, gingerdione, a component of ginger, was found to suppress the synthesis of 5-hydroxyeicosatetraenoic acid (5-HETE) and PGE2 from arachidonic acid in human neutrophil cells. Furthermore, shogaol in ginger inhibits 5-HETE and gingerol and dehydroparadol inhibit COX (Flynn et al., 1986; Thomson et al., 2002). *In vitro* inhibition of arachidonic acid-induced platelet activation in whole human blood examined by Effie et al. using 20 active components of *Z. officinale*. It has been observed that the components of *Z. officinale* have much greater antiplatelet activity than do aspirin. Additionally, [8]-Paradol was shown to be the substance that inhibits COX most (Nurtjahja-Tjendraputra, Ammit, Roufogalis, Tran, & Duke, 2003).

Z. officinale antithrombotic properties have been studied in animals. Thomson et al. administered 500 mg/kg aqueous extract of *Z. officinale* to rats for 4 weeks in an *ex vivo* study. A 50% reduction in TXB2 levels was observed (Thomson et al., 2002). In rabbits, [6]-Paradol prevented arachidonic acid-induced platelet aggregation (Shih et al., 2014). Similarly, Liao et al. (2012) reported that [6]-Gingerol and [6]-Shogaol exhibit antiplatelet activity in rabbits (Liao et al., 2012). A reduction in platelet adenosine deaminase activity and an increase in adenosine levels were observed in a hypertensive rat study following the administration of *Z. officinale* root according to these find-

ings, *Z. officinale* may help reduce hypertension-related problems induced by platelet activity (Akinyemi et al., 2016).

While *in vitro* research on *Z. officinale*'s anti-coagulant activity is promising, human studies are conflicting. In a placebo-controlled study, 30 patients with myocardial infarction were administered *Z. officinale* capsules for 3 months, and fibrinogen, fibrinolytic activity, and platelet aggregation were measured. Significant reductions in adenosine diphosphate (ADP) and adrenaline-induced platelet aggregation were observed 4 h after the administration of 4 g of *Z. officinale* (Bordia, Verma, & Srivastava, 1997). ADP, adrenaline, and TXA2 stimulate platelet activation (McEwen, 2015). Contrary to these findings, Janssen et al. administered raw and cooked *Z. officinale* to healthy volunteers for 2 weeks in a placebo-controlled study. TXA2 levels were measured on days 12 and 14, and there was no significant reduction compared with placebo (Janssen, Meyboom, van Staveren, de Vegt, & Katan, 1996).

Studies conducted both *in vivo* and *in vitro* revealed that reduced TXA2 or PG endoperoxide synthesis causes antiplatelet activity. It has been reported that this may be caused by the inhibition of platelet COX enzymes and/or the antioxidants found in *Z. officinale* (Bordia et al., 1997). It is thought that the carbonyl functional group in 3.C of the paradol and diarylheptanoid series in the content of *Z. officinale* may exert antithrombotic effects by inhibiting COX-1 (Nurtjahja-Tjendraputra et al., 2003). In the structure-activity relationship (SAR) analysis, it was revealed that the phenolic compounds in *Z. officinale* have an inhibitory effect on the COX-2 enzyme due to the lipophilic feature of the alkyl side chain, the position of the hydroxy and carbonic groups in the side chain, and the hydroxy and methoxy groups in the aromatic side chain (Tjendraputra, Tran, Liu-Brennan, Roufogalis, & Duke, 2001).

As a result, in our study, extracts of fresh *Z. officinale* obtained with water, ethanol, and methanol solvents prolonged the coagulation parameters in human whole blood samples. PT, aPTT, and INR continued to be prolonged as the concentration of *Z. officinale* extracts added to the samples increased. Based on these findings, *Z. officinale* appears to influence coagulation. Its potential therapeutic and pharmacological effects, along with the underlying mechanisms, require further investigation through various study designs.

Study Limitations: *Z. officinale* employed in this investigation lacks comprehensive characterisation. Consequently, due to the absence of standardised characteristics, it is not suitable to assess its effects using only this study. Furthermore, the active constituents of the used plant have not been isolated or subjected to analysis. Identification of the specific molecule within its composition that is responsible for the primary effect remains uncertain based on the present study. Hence, we propose that future studies should aim to determine the active ingredients of *Z. officinale* and evaluate its anticoagulant activity.

Ethics Committee Approval: The University of Health Sciences Hamidiye Scientific Research Ethics Committee (registration number: 21/563) approved the study.

Informed Consent: Written consent to participate in the study was obtained from the participants.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study: E.M.G., M.M.Y., M.S.K., E.Ş.Ö., S.K.; Data Acquisition: E.Ş.Ö., S.K., D.Ö., F.G.; Data Analysis/Interpretation: S.A., E.P.H., E.M.G.., S.K; Drafting Manuscript: E.Ş.Ö., S.K., D.Ö., E.M.G.; Critical Revision of Manuscript: E.M.G., S.A., M.M.Y., E.P.H., M.S.K., F.G.; Final Approval and Accountability: E.M.G., S.A., M.M.Y., E.P.H., M.S.K., F.G., E.Ş.Ö., S.K., D.Ö.

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Original Article

First screening of volatile constituents and antibacterial, antibiofilm, and anti-quorum sensing activities of *Cinclidotus* species

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ABSTRACT

Background and Aims: Bryophytes are the center of interest for natural sources due to their medicinal and traditional usage in various ailments with their interesting phytochemicals. However, there are very few studies on this subject. In the present study, we aimed to investigate mosses belonging to the genus *Cinclidotus* (Cinclidotaceae), such as *C. pachyloma, C. bistratosus, C. riparius, C. pachylomoides, C. fontinaloides* and *C. aquaticus* regarding their volatile components and biological activities.

Methods: The mosses were collected from various locations in Türkiye and then extracted with ether. The volatile components were identified and semi-quantified using GC-TQMS (Gas Chromatography and a Triple Quadrupole Mass Spectrometer). The MICs were evaluated using the broth microdilution method. A microplate-based biofilm model was used against *P. aeruginosa* PAO1 using the crystal violet assay to determine the antibiofilm activity. The anti-quorum sensing activity was carried out using the disc diffusion method.

Results: The initial screening of the selected mosses to confirm the significant potential of their volatile phytochemicals was investigated for the first time in this study. The main components were determined as linoelaidic acid, glycerol 2-hexadecanoate, and campesterol in diverse types of *Cinclidotus* species by GC/TQMS. In addition, the potency of the antibacterial, antibiofilm, and anti-quorum sensing activities of these species exhibited moderate to highly effective results.

Conclusion: All results showed that mosses are rich sources of natural compounds and good samples for biological activities. Mosses are promising candidates that could be useful in preventing or treating various pathological conditions. However, further *in vitro* and *in vivo* studies should focus on a single component or the mechanisms.

Keywords: Bryophyte, Metabolites, Moss, Phytochemicals, Activity, Volatile

INTRODUCTION

New drug molecules are discovered based on natural products (Dushenkov & Raskin, 2008). Investigations into medicinal plants have steadily increased, providing reasonable and essential information on plants and candidate molecules in drug development (Cragg & Newman, 2013). Records of Bryophytes date back to ancient times, with the first being in the first century; more Bryophyte taxa are considered medicinal plants (Drobnik & Stebel, 2014; 2015; 2018). Bryophytes are the second largest group behind only the flowering plants that grow almost all over the world and stand out with their extraordinary chemical properties and biological activities (Sabovljević, Bijelović, & Grubišić, 2001; Klavina et al., 2015; Aruna & Krishnappa, 2018), used as a remedy for many ailments in many forms in traditional medicine in China, Europe, North America, and India (Saxena & Harinder, 2004; Glime, 2007; Karim, Suleiman, Rahmat, & Abu-Bakar, 2014; Klavina et al., 2015; Nilsu et al., 2018; Ludwiczuk & Asakawa, 2019). However, the phytochemistry of Bryophytes has been neglected for a long time due to their small size and difficulties in the identification and collection of the sample in pure forms (Saxena & Harinder, 2004; Adebiyi, Oyedeji, Chikwendu, & Fatoke, 2012), but in recent years, interest in bryophytes has been increasing due to

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their rich source of novel active compounds (Asakawa, 2007; Xie & Lou, 2009; Asakawa, Ludwiczuk, & Nagashima, 2013a; 2013b). All these features of bryophytes mentioned here indicate that they are potentially important biomaterials (Klavina et al., 2015).

The term bryophyte is a common name used to express liverworts (Marchantiophyta), hornworts (Anthocerotophyta), and mosses (Bryophyta), which are taxonomically closely related groups. Today, they represent about 20.000–25.000 taxa globally and show an almost global distribution (Crum, 2001; Patiño & Vanderpoorten, 2018). According to a study by Kürschner and Erdağ in 2023, the bryophytes of Turkey are represented by 1244 taxa (1025 mosses, 215 liverworts, and 4 hornworts), of which 1% are endemic. This number tends to increase with intensive floristic studies conducted by Turkish scientists (Erdağ & Kürschner, 2017; Erata, Batan, Alataş, & Özen, 2020; Ursavaş, Keçeli, Uyar, & Ören, 2020; Kırmacı, Özenoğlu, Armağan, Aslan, & Çatak, 2022; Özenoğlu & Kırmacı, 2022). Mosses, the second largest group of the plant kingdom (more than 14.000 taxa), are not woody and are very simple land plants with typically small sizes (maximum of 60 cm) (Asakawa et al., 2013b). They contain various phytochemicals, besides important medicinal uses in different fields (Saxena & Harinder, 2004; Klavina et al., 2015). The North American tribes used mosses as medicinal plants to treat wounds, burns, tuberculosis, pneumonia, neurasthenia, and other diseases. In China, several mosses are used medicinally for burns, bruises, external injuries, snake bites, pulmonary tuberculosis, neurasthenia, fractures, convulsions, scalds, uropathy, and pneumonia. (Saxena & Harinder, 2004; Klavina et al., 2015). The mosses have also been used for medicinal purposes in Malaysia; in Egypt, some are used to prepare medicinal tea primarily for treating colds (Glime, 2017). In Germany, *Ceratodon purpureus* (Hedw.) Brid. and *Bryum argenteum* Hedw. are the remedies for fungal infections of horses besides anti-leukemic and anticancer effects (Glime, 2013; Aslanbaba, Yilmaz, Yayıntaş, Özyurt, & Öztürk, 2017). *Polytrichum commune* Hedw., a traditional Chinese moss, has been used especially for lymphocytic leukemia besides high fever, injuries related to pneumonia, and prolapsed uterine (Cheng et al., 2013).

Antibiotic resistance is a major global health concern as new strains of resistant bacteria continue to emerge, causing significant morbidity and mortality. New compounds are urgently needed to combat bacterial infections caused by resistant bacteria. The inability to discover new groups of antibiotics and the inevitable emergence of resistance to existing antibiotics has prompted scientists to explore alternative treatment options (Islam et al., 2017; WHO, 2017). Researchers have been searching for new molecules that target different mechanisms of pathogenicity because antibiotics are ineffective against resistant bacteria. Biofilm formation, one of these mechanisms, can increase the pathogenicity of the bacteria and protect them from external factors. The other mechanism is quorum sensing (QS). QS plays a role in synthesizing virulence factors, which contribute to the formation of biofilms and pathogenicity. Recently, scientists have shifted their focus towards researching antibiofilm and anti-quorum sensing molecules as alternatives to antibacterials for treating bacterial infectious diseases. Based on current research, anti-quorum sensing, and antibiofilm compounds, especially those found in natural resources, would be effective in controlling the resistance problem (Uroz, Dessaux, & Oger, 2009; Nithya, Begum, & Pandian, 2010).

The genus *Cinclidotus* from mosses, which constitutes the main material of this study, is represented by 9 taxa, 3 of which are endemic to our country (Erdağ & Kürschner, 2011; Ursavaş & Çetin, 2014). According to Erdağ and Kürschner (2011), the southern part of Türkiye is an excellent site condition for a main speciation center for this hygrophytic complex. Recently, there have been limited studies on the biologically active chemical components of Bryophytes. According to the literature review, there was no detailed study on *Cinclidotus* (Cinclidotaceae) species from Bryophytes. The only research on the genus investigating the allelopathic effects of *C. pachylomoides* Bizot on pepper and corn plants (Turkyılmaz Unal, İşlek, Ezer, & Düzelten, 2017). Therefore, the study aimed to analyze the volatile components using GC/TQMS and to evaluate the antibacterial, antibiofilm, and anti-quorum sensing activities of *Cinclidotus* species.

MATERIALS AND METHODS

Plant materials

The species were collected from different localities in Türkiye. The authentic samples were deposited in the Herbarium Aydın Adnan Menderes University (AYDN). The collection sites and herbarium number of the species are displayed in Table 1.

Extraction of the samples

The mosses (*Cinclidotus* spp.) were extracted with ether (500 mL) via maceration in a 2 L Erlenmeyer and shaken randomly in a cool and dark place for approximately two months. Then, the extracts were filtered through filter paper and in a small column with a small amount of Celite. The samples were weighed in a precision balance (Table 2).

Volatile compounds

The volatile profile was determined according to Issa-Issa, Hernández, Lipan, López-Lluch, and Carbonell-Barrachineta, (2021) with slight modifications. The identification and semi-quantification of the volatile compounds were carried out using a Shimadzu GC2030 gas chromatograph and a TQ8040 NX triple quadrupole mass spectrometer as the detector, GC-MS (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA)

Table 1. The list of *Cinclidotus* species and their localities

No	Species name	Collector no: (M. Kırmacı)	Herbarium no:	Locality*
C1	*C. pachyloma* E.S. Salmon	MKIR 8579	AYDN 4009	1*
C2	*C. bistratosus* Kurschner & Lübenau-Nestle	MKIR 8578	AYDN 4008	2*
C3	*C. riparius* (Host ex Brid.) Arn.	MKIR 8573	AYDN 4003	1*
C4	*C. fontinaloides* (Hedw.) P. Beauv.	MKIR 8575	AYDN 4005	1*
C5	*C. aquaticus* (Hedw.) Bruch and Schimp.	MKIR 8574	AYDN 4004	1*
C6	*C. pachylomoides* Bizot	MKIR 8572	AYDN 4002	1*

(*) Locality 1: Antalya/Manavgat/ Köprülü Canyon, Köprü River, on Limestone Rock, 200 m, 18/06/2019 N 37° 11' 13,75" E 31° 10' 50,95" (*) Locality 2: Isparta/Sütcüler/ Kesme, Between Kesme-Sütçüler, in the stream, on Limestone Rock, 960 m, 19/06/2019 N 37° 27' 50,73" E 31° 14' 56,39"

Table 2. The extracted number of *Cinclidotus* species

No	Species name	The Plant List*	Plant amount g	Ether extract mg	Yield %
C1	*C. pachyloma* E.S. Salmon	Accepted	130.8	832.5	0.64
C2	*C. bistratosus* Kurschner & Lübenau-Nestle	Accepted	141.97	1292.9	0.91
C3	*C. riparius* (Host ex Brid.) Arn.	Accepted	57.61	227.5	0.39
C4	*C. fontinaloides* (Hedw.) P. Beauv.	Accepted	69.07	490	0.71
C5	*C. aquaticus* (Hedw.) Bruch and Schimp.	Accepted	145.64	4260.4	2.92
C6	*C. pachylomoides* Bizot	Accepted	74.22	667.6	0.90

(*) http://www.theplantlist.org/

with an AOC- 6000Plus. A SAPIENS X5MS column, 30.0 m length × 0.25 mm inner diameter × 0.25 µm film thickness (Teknokroma, Barcelona, Spain) was used. For the analysis of the samples, approximately 1.0-2.5 mg of essential oil was dissolved in 1 mL of hexane, and 1 µL was injected. Helium was used as the carrier gas at a pressure of 53.5 kPa, a total flow of 12.0 mL/min, and the flow control mode was adjusted at a linear velocity of 36.3 cm/s, and a split ratio of 1:5 was used. The temperature was 50°C, then 5°C/min ramp to 300°C. The injection temperature was 280°C, the ion source temperature was 200 °C, and the interface temperature was 250°C. Mass spectra were obtained from electron ionization (EI) at 70 eV, with an even time of 0.2 s and a spectral range of 45–400 m/z. Most volatiles were identified according to the retention indices calculated using the C7 to C16 n-alkane mixture (Sigma-Aldrich, Steinheim, Germany), the retention indices of the standards, and comparing the mass spectra obtained with those of the standards from the NIST 14 and Wiley 229 spectrum libraries. All analyses were performed in triplicate, and the results were expressed as a percentage of the relative area (Table 3).

Antibacterial activity

In the antibacterial activity tests, *Staphylococcus aureus* ATCC 25923, methicillin-resistant *S. aureus* ATCC 43300 (MRSA), *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 13883 were used as test bacteria. The extracts were dissolved in dimethyl sulfoxide (10% DMSO). The minimum inhibitory concentration (MIC) values were determined using the broth microdilution method (CLSI, 2018). Dilutions ranging from 10 mg/mL to 0.078 mg/mL were prepared in Mueller Hinton Broth (MHB) (Difco, Detroit, MI, USA). The final test concentration of the bacteria was adjusted to 5×10^5 CFU/mL with the overnight subcultures. The microplates were incubated at 35°C for 18-24 hours. After the incubation period, the MIC values (mg/mL) were noted as the last well that completely inhibited the visual bacterial growth. MHB and 10% DMSO were used as the negative controls. Ciprofloxacin (Sigma, USA), ampicillin (Sigma, USA), and ofloxacin (Sigma, USA) were used as reference drugs.

Antibiofilm activity

Before performing the antibiofilm activity test, the MIC values of the extracts against *Pseudomonas aeruginosa* PAO1 were detected. No antibacterial activity was observed. The antibiofilm activity was determined by an *in vitro* microplate-based biofilm model against *P. aeruginosa* PAO1 using the crystal violet assay (Bali, Türkmen, Erdönmez, Sağlam, 2019; Eryılmaz, Kart, Gürpınar, 2019; Jardak, Mnif, Ayed, Rezgui, Aifa, 2021).

Table 3. Concentrations (%) of volatile compounds in the *Cinclidotus* species

Code	Compounds	RT	*C. riparius*	*C. fontinaloides*	*C. aquaticus*	*C. pachylomoides*	*C. pachyloma*	*C. bistratosus*
1	Heptanal	5.883	0.01	-	-	-	-	0.02
2	4-Methyl-3-Hexanol	5.96	0.01	-	-	-	0.17	-
3	3-Hexanol	6.873	0.20	-	0.21	0.20	0.15	0.33
4	4-Methyl-2-Pentanol	7.112	0.21	-	-	0.22	0.02	0.37
5	2-Heptenal	7.257	0.01	-	-	-	-	-
6	6-Undecanone	7.379	-	-	-	-	-	-
7	Benzaldehyde	7.424	-	-	-	-	-	-
8	2,4-Dimethyl-2-pentene	7.492	-	-	-	-	-	0.05
9	Hexanoic acid	7.561	-	-	-	-	-	-
10	4-Ethylcyclohexene	8.325	0.02	-	-	-	-	-
11	Octanal	8.496	-	-	-	-	-	-
12	2-Ethoxy-butane	8.967	-	-	-	-	-	-
13	*o*-Cymene	9.15	0.02	0.05	0.02	0.12	-	-
14	Limonene	9.297	0.10	0.28	0.09	0.70	0.09	0.03
15	Eucalyptol	9.381	0.01	0.01	-	0.06	-	-
16	γ-Terpinene	10.118	0.01	0.02	-	0.04	-	-
17	Heptanoic acid	10.299	-	-	-	-	-	-
18	Linalool	11.267	0.08	0.07	-	-	0.02	-
19	Nonanal	11.403	0.02	-	-	0.02	0.01	0.04
20	Camphor	11.507	0.02	-	-	-	-	0.03
21	2,4-Dimethyl-3-pentanol	12.107	-	-	-	-	-	-
22	3-Methyl-3-pentanol	12.255	-	-	-	-	-	-
23	2-Hydroxy-2-methyl-butyric acid	12.637	-	-	-	-	-	-
24	2-Bornanone	12.722	0.42	0.76	-	2.45	0.15	-
25	Octanoic acid	13.134	0.01	-	-	-	-	-
26	3-Methyl-3-heptanol	13.176	-	-	-	-	-	-
27	Borneol	13.442	0.07	0.03	-	-	-	-
28	Levomenthol	13585	0.01	-	-	-	-	-
29	Terpinen-4-ol	13688	0.02	0.01	-	-	-	-
30	1-(2-Methylphenyl)-ethanone	13.797	-	-	-	-	-	-

Table 3. Continued

31	*α*-Terpineol	14091	0.01	-	-	-	-	-
32	Estragole	14.149	0.05	0.02	-	-	-	-
33	Decanal	14.36	-	-	-	-	-	-
34	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	15.009	-	-	-	-	-	-
35	Linalyl acetate	15.593	0.33	0.09	-	-	0.03	-
36	Nonanoic acid	15.938	0.06	-	0.04	0.06	0.03	0.04
37	Anethole	16.654	0.11	0.02	-	-	-	-
38	2,4-Decadienal	16.866	0.03	0.03	0.40	0.15	0.05	-
39	Thymol	16.948	0.06	-	-	-	-	-
40	Tridecan-1-ol	17.309	-	-	0.02	-	-	-
41	2-Ethyl-hexanoic acid	17.427	-	-	-	-	-	-
42	Fenchol	17.519	0.04	-	-	-	-	-
43	3,5-Dimethyl-3-hexanol	17.653	-	-	-	-	-	-
44	*α*-Terpinyl acetate	18324	0.09	-	-	-	-	-
45	Eugenol	18.454	0.02	-	0.02	-	-	-
46	Hexyl nonanoate	18.622	-	-	-	-	-	-
47	2-Ethyl-3-hydroxyhexyl 2-methylpropanoate	18.988	0.07	-	-	0.06	-	-
48	Tetradecane	19.763	0.01	-	-	-	-	-
49	Caryophyllene	20.389	0.08	-	-	-	-	-
50	*o*-Benzoquinone	21.244	-	0.03	-	-	-	-
51	*β*-Ionone	21.792	-	-	0.03	-	-	-
52	Dihydroactinidiolide	23.041	0.04	0.04	0.00	0.00	0.06	0.03
53	Dodecanoic acid	23.665	0.27	0.05	0.04	0.41	0.81	0.09
54	2,2,4-Trimethyl-1,3-pentanediol di-isobutyrate	24.445	0.06	-	-	-	-	-
55	Tetradecanal	25.025	-	0.01	-	-	-	-
56	Methyl didecanoate	25.263	0.11	0.12	0.14	-	0.12	-
57	1-Tetradecanol	26.502	-	0.02	-	-	-	-
58	1-Nonadecene	26.9	0.23	0.12	-	6.12	-	-
59	Hexadecanal	27.368	-	-	-	-	-	-
60	1-Heptacosanol	28.112	-	-	-	-	-	-
61	Nonacosane	28.186	-	-	-	-	-	-
62	Pentadecanoic acid	28.276	0.37	0.35	0.17	-	-	-

Table 3. Continued

63	2-Butyl-2-octenal	28.477	-	0.32	0.25	-	-	-
64	1-Hexadecanol	28.761	-	-	0.22	-	-	0.13
65	Ethyl eicosanoate	29.054	-	-	-	-	-	-
66	1-Octadecene	29.125	-	-	-	-	-	-
67	Isopropyl myristate	29.728	-	-	0.03	-	-	-
68	Tetradecanoic acid	29.809	-	-	-	0.58	0.98	-
69	3,7,11-Trimethyl-1-dodecanol	29.897	-	-	-	-	-	-
70	9-Eicosen-1-ol	30.026	-	1.47	1.24	1.38	0.33	-
71	6,10,14-Trimethyl-2-pentadecanone	30.106	3.44	-	0.14	1.19	-	-
72	3,7,11,15-Tetramethyl-1-Hexadecanol	30.162	1.51	0.28	0.49	0.74	-	-
73	3-Methyl-heptadecane	30.599	-	-	-	-	-	-
74	Octadecanal	30.917	0.25	-	-	-	-	-
75	9-Tricosene	31.106	-	-	-	-	-	-
76	*β*-Farnesene	31.579	1.18	1.12	0.10	0.49	-	0.04
77	9,12,15-Octadecatrienoic acid	31.973	-	-	0.17	-	-	-
78	Palmitoleic acid	32.075	-	1.74	0.55	-	5.86	-
79	Hexadecanoic acid	32.571	4.03	10.22	12.34	4.29	9.83	7.18
80	Ethyl hexadecanoate	33.171	0.30	0.46	5.50	-	0.29	0.62
81	Arachidonic acid	34.217	5.70	3.77	2.06	3.05	2.02	2.39
82	Tetracosanoic acid	34.441	-	-	-	-	-	-
83	Octacosanol	34.929	-	-	-	-	-	-
84	Isopropyl palmitate	35.022	-	-	-	-	-	-
85	Phytol	35.349	1.81	4.63	1.59	3.54	2.96	2.52
86	Linoelaidic acid	35.857	2.86	12.89	19.27	4.31	12.96	7.84
87	9,12-Tetradecanyl acetate	35.966	3.64	11.50	15.66	1.31	10.24	12.31
88	9-Octadecenoic acid	36.037	3.42	4.11	-	1.63	2.94	3.51
89	Octadecanoic acid	36.345	0.92	1.17	-	1.07	-	0.94
90	Ethyl 9,12,15-octadecatrienoate	36.421	0.30	0.68	-	-	-	0.70
91	Ethyl Oleate	36.552	0.24	0.46	0.48	-	-	-
92	17-methyl-Octadecanoate	36.924	-	-	-	-	-	-
93	Methyl arachidonate	38.835	-	-	-	-	0.88	-

Table 3. Continued

94	8,11,14-Eicosatrienoic acid	39.005	-	-	-	-	-	-
95	*β*-Carotene	39.837	-	0.14	-	-	-	-
96	Glycerol 2-hexadecanoate	42.23	8.45	6.27	10.36	12.82	15.71	19.08
97	Isopropyl linoleate	45.032	-	1.32	-	-	-	-
98	1-Glyceryl stearate	45.391	4.79	1.74	-	6.86	4.02	10.32
99	Squalene	46.806	0.42	0.30	0.37	-	0.63	0.69
100	2-Hexyl-1-decanol	48.023	0.19	-	0.23	0.32	-	0.79
101	Batilol	48.439	-	-	-	-	-	-
102	*α*-Tocopherol	49.985	5.64	3.63	3.08	7.68	2.59	3.02
103	Cholesterol	50.980	0.68	0.70	-	-	1.03	-
104	16-Dehydropregnenolone	52.533	2.69	-	0.53	1.44	1.39	0.53
105	Campesterol	52.68	26.79	16.26	11.19	19.22	11.96	12.45
106	Stigmasterol	53.121	-	2.90	1.83	3.69	0.87	3.49
107	*β*-Sitosterol	54.257	17.41	9.78	11.14	13.79	10.79	10.44
	Total		**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

[†]n.d: not detected

Biofilm formation

Pseudomonas aeruginosa PAO1 was incubated in Brain Heart Infusion (BHI) Broth for 24 h at 37°C. A final inoculum was prepared in BHI Broth with 2% sucrose, containing approximately 106 CFU/mL of P. aeruginosa.100 µL of the bacterial suspensions were added to 96-well microtiter plates for each test condition. The plates were incubated at 37°C for 72 h to form mature biofilms.

Treating the biofilm cells with the extracts

After the mature biofilms formed, the wells were washed with sterile phosphate-buffered saline (PBS, pH 7.2) to remove non-adhered cells. Then, the extracts (10 mg/mL) were transferred into the mature biofilm wells. The plates were incubated at 37°C for 24 h. After the incubation, the contents of the wells were aspirated and washed with PBS. The plates were dried at room temperature for 1 h. Then, 100 µL of 0.5% crystal violet solution was added to each well to stain the biofilm cells. After 30 min, the wells were washed with PBS, followed by adding a solution of acetone-alcohol (30:70 v/v) into the wells to dissolve the dye bound within the biofilm matrix. BHI Broth with 2% sucrose and 10% DMSO were used as negative controls. The optical density of the dissolved crystal violet dye was measured using a microplate reader (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Vantaa, Finland) at 620 nm (OD 620 nm). The percentage biofilm inhibition values were calculated according to the following formula:

% Biofilm inhibition = [(OD (growth control)/ OD (sample)) /OD (growth control)] x 100

Anti-quorum sensing activity

The anti-quorum sensing activity was determined using the disc diffusion method with *Chromobacterium violaceum* ATCC 12472 as the reporter bacteria (Gajdács & Spengler, 2020; Batohi et al. 2021). The bacterial suspension was adjusted to $1.5x10^8$ CFU/mL with the overnight culture and inoculated on Luria Bertani Agar. Then, sterile blank discs (6 mm diameter; Bioanalyse®, Ankara, Türkiye) impregnated with 20 L twenty microliters of the extracts (10 mg/mL) were placed on the medium. After incubation at 30°C for 24 h, the plates were observed for a zone of violacein inhibition. The formation of an inhibition zone around the disc was noted as the potential anti-quorum sensing activity.

RESULTS AND DISCUSSION

Bryophytes are used in traditional medicine for many health disorders (Singh, Singh, Nath, Sahu, & Singh Rawat, 2011). the main purpose of this study was to determine the volatile components using GC/MS (Table 3) and analyze the antibacterial,

antibiofilm, and anti-quorum sensing activities of *Cinclidotus* species.

In previous studies, several p-terphenyl derivatives from the ethyl acetate extract of *Homalia trichomanoides* (Hedw.) Brid. displayed antifungal activity (MIC: 2.0, 2.0, and 0.6 µg/mL) against *Candida albicans* (Wang, Yu, & Lou, 2005). In another study, the methanol extracts of the mosses exhibited moderate antimicrobial activity against Gram-negative and Gram-positive bacteria (Dulger, Yayıntaş, & Gonuz, 2005). The ethanol extract of *Bryum argenteum* against all bacteria and fungi exhibited an antibacterial effect by the microdilution method (Sabovljevic, Sokovic, Sabovljevic, & Grubisic, 2006). The mosses were highly active against Gram-positive and Gram-negative bacterial and fungal strains (Singh, Rawat, & Govindarajan, 2007). The methanol extracts (80%) of some mosses also showed antibacterial activity, especially *Hylocomium splendens* (Hedw.) Schimp. extract was the strongest (Kang, Kim, Liu, Jovel, Towers, 2007). However, some are used as a remedy for infections and skin diseases in humans with marked antimicrobial effects (Singh et al., 2007). The antioxidant activity of ethanol extracts from *Atrichum undulatum* (Hedw.) P. Beauv. and *Polytrichum formosum* Hedw. was stronger than that of *Pleurozium schreberi* (Willd. ex Brid.) Mitt. and *Thuidium tamariscinum* (Hedw.) Schimp. (Chobot, Kubicová, Nabbout, Jahodář, Hadacek, 2008). The water extract of *Ptychostomum moravicum* (Podp.) Ros&Mazimpaka were found to have moderate antioxidant activity (Pejin, Bogdanovic-Pristov, Pejin, & Sabovljevic, 2013). The water extracts of the Peat moss called *Sphagnum* sp. showed anti-inflammatory and antioxidant effects, suggesting that they can suppress inflammation and prevent oxidative stress and cellular damage (Choi et al., 2014). The ethanolic extract from the Chilean native

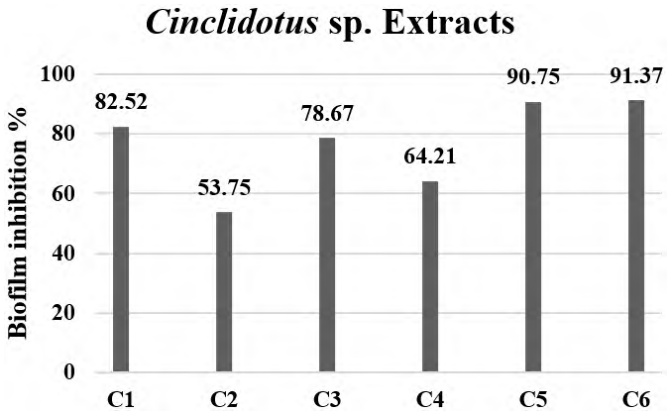

Figure 1. Antibiofilm activity of *Cinclidotus* spp. Extracts C1: *C. pachloma*, C2: *C. bistratosus*, C3: *C. riparius*, C4: *C. fontinaloides*, C5 C5: *C. aquaticus*, C6: *C. pachylomoides*

moss *Sphagnum magellanicum* Brid. created inhibitory effects against Gram-negative and Gram-positive bacteria. In addition, the presence of vanillic, chlorogenic, syringe, caffeic, gallic,

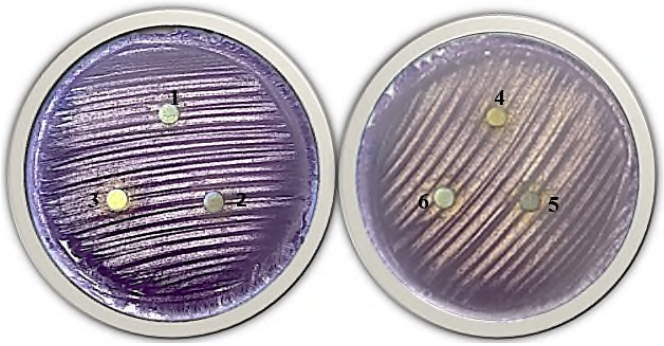

Figure 2. QS inhibitory activity of the *Cinclidotus* spp. Extracts C1: *C. pachloma*, C2: *C. bistratosus*, C3: *C. riparius*, C4: *C. fontinaloides*, C5: *C. aquaticus*, C6: *C. pachylomoides*

3-4 hydroxybenzoic, p-coumaric, and salicylic acids was determined by RP-HPLC (Montenegro, Portaluppi, Salas, Diaz, 2009). The butanol fractions of some mosses have significant effects against Gram-positive bacteria, especially against *Staphylococcus aureus* (Singh et al., 2011). The ethanol and chloroform extracts of the common mosses from Northern Europe exhibited antimicrobial activity against several bacterial strains, but the most potent one was *Polytrichum commune* Hedw. (Klavina et al., 2015). Ethanol and water extracts of *Cinclidotus fontinaloides* and *Palustriella commutata* (Hedw.) Ochyra exhibited a significant antibacterial effect, especially in the *C. fontinaloides* and *P. commutata* ethanol extracts. In addition, the potent antioxidant effects of these mosses were also observed (Yayıntaş, Alpaslan, Karagul, Yilmaz, & Sahiner, 2017). The extract from *Ptychostomum capillare* (Hedw.) Holyoak & N. Pedersen showed a 3-5% biofilm inhibition against *S. epidermidis*; and reduced the effect of H2O2 (Onbasli & Yuvali, 2021). Mosses from the Anatolian flora were also evaluated for their potential antimicrobial, antioxidant, anthocyanin, and allelopathic effects (Yayıntaş et al., 2017; Turkyılmaz Ünal et al., 2017). All results showed that mosses are rich sources of natural compounds and good samples for biological activities. Therefore, this preliminary information shows that it is noteworthy to investigate the *Cinclidotus* species. Consequently, among the tested extracts, C1 and C2 exhibited an antibacterial effect against S. aureus ATCC 25923 and S. aureus ATCC 43300 (MRSA) with a MIC value of 10 mg/mL (Table 4). Nevertheless, none of the extracts showed antibacterial activity against the test bacteria except for these. The percentage biofilm inhibition values of C1, C2, C3, C4, C5, and C6 were determined as 82.52%-53.75%-78.67%-64.21%-90.75% and 91.37%, respectively (Figure 1). The appearance of a transparent inhibition zone around the disc indicated the potential occurrence of QS inhibitory activity (Figure 2). However, the other tested Cinclidotus sp. extracts exhibited anti-quorum sensing activity.

Table 4. Minimum inhibitory concentration (MIC) values (mg/mL) of *Cinclidotus* species extracts against the tested bacteria

Cinclidotus sp. Extracts	Gram-positive Bacteria			Gram-negative Bacteria		
	***Staphylococcus aureus* ATCC 25923**	***Staphylococcus aureus* ATCC 43300(MRSA)**	***Enterococcus faecalis* ATCC 29212**	***Escherichia coli* ATCC 25922**	***Klebsiella pneumoniae* ATCC 13883**	***Pseudomonas aeruginosa* ATCC 27853**
C1	10	10	-	-	-	-
C2	10	10	-	-	-	-
C3	-	-	-	-	-	-
C4	-	-	-	-	-	-
C5	-	-	-	-	-	-
C6	-	-	-	-	-	-
Ampicillin	0.0016	0.05	NT	NT	NT	NT
Ciprofloxacin	NT	NT	0.0625	NT	0.0625	NT
Ofloxacin	NT	NT	NT	0.001	NT	0.008
DMSO (10%)	-	-	-	-	-	-

"-": represents no activity. C1: *Cinclidotus pachloma,* C2: *C. bistratosus,* C3: *C. riparius,* C4: *C. fontinaloides, C5* C5: *C. aquaticus,* C6: *C. pachylomoides* NT: not tested

CONCLUSION

In this study, the volatile compounds of the tested *Cinclidotus* species collected from different localities of Türkiye were analyzed by GC/MS and examined in terms of their biological activity. The result of the phytochemical analysis in this study is that there are bioactive components in ether extracts. The investigated extracts showed reasonable activity at the concentrations evaluated. Based on the data presented, it was concluded that mosses are promising candidates that could be useful in the prevention or treatment of various pathological conditions. However, further *in vitro* and *in vivo* studies should focus on a single component or the mechanisms.

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Original Article

Differences in genotoxicity and cytotoxicity potentials of green and chemically synthesized silver nanoparticles

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ABSTRACT

Background and Aims: In recent years, metal nanoparticles have been extensively synthesized for a variety of applications and have been used in large-scale research in various fields, such as chemistry, physics, life science, material science, medical science, and engineering, depending on their size and shape adjustment properties. In this study, we aimed to compare the effects of silver nanoparticles synthesized using two different methods on DNA damage and cell viability in human lymphocyte cultures.

Methods: We introduced a green and simple method for the synthesis of AgNPs using endemic *Onosma papillosa* Riedl leaf extract as a reducing agent for the first time. Blood samples were collected in heparinized tubes from four healthy males, non-smokers, and healthy male. In this study, we used comet assay [Genetic Damage Index (GDI) and Damaged Cell Percentage (DCP)] and flow cytometry methods for genotoxicity and cytotoxicity.
For comparison, commercially obtained AgNPs synthesized by chemical methods were used, with consideration given to the size of AgNPs synthesized via the green method.

Results: Based on the results, it was determined that DNA damage caused by AgNPs synthesized through the green method in human lymphocyte cultures was not statistically significant compared with the negative control. AgNPs obtained by chemical synthesis caused, however, a statistically significant increase in the frequency of DNA damage compared with the negative control ($p<0.001$). The percentage of necrotic cells was 13.55±3.37 and 25.37±14.53 in cultures obtained by green and chemically synthesized AgNPs, respectively.

Conclusion: Essentially, green synthesis can be recommended for use because of its lower toxicity compared with chemical synthesis.

Keywords: *Onosma papillosa*, Green synthesis, Chemical synthesis, Comet assay, Flow cytometry, Genotoxicity, Cytotoxicity

INTRODUCTION

Nanotechnology has received great attention and significant progress in many fields of science such as medicine and in the production of commonly used materials (Nikalje, 2015). This innovative technology has a significant impact on society, influencing numerous sectors such as communication, textiles, medicine, engineering, agricultural products, and food technology (Francisco and Garcia-Estepa, 2018). Among the myriad of products that incorporate nanomaterials, silver nanoparticles (AgNPs) are among the most widely utilized. Because of their unique antibacterial properties, AgNPs can be found in medical bandages, surgical devices, and medical masks to minimise microbial functions (Das et al., 2020; Gkika, Vordos, Magafas, L., Mitropoulos, and Kyzas, 2021; Buzea, Pacheco, and Robbie, et al., 2007).

In recent years, metal nanoparticles have been extensively synthesized for a variety of applications and have been used in large-scale research in various fields, such as chemistry, physics, life science, material science, medical science, and engineering, depending on their size and shape adjustment properties. Silver is one of the most mercantile nanomaterials, with a half million tonnes of silver nanoparticle output per year (Larue et al., 2014). In addition, metal nanoparticles play a profound role in the fields of high-precision biomolecular identification, catalysis, biosensors, and medicine. They have been recognized for their robust inhibitory and bactericidal effects as

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well as their antifungal, anti-inflammatory, and antiangiogenesis activities (Palencia, Berrio, and Palencia, 2017; Veerasamy et al. 2011). Veerasamy et al., (2011) demonstrated that the use of a natural, low-cost biological reducing agent, *Garcinia mangostana* L. leaf extracts (aqueous), can produce metal nanostructures through efficient green nanochemistry methodology, avoiding the presence of hazardous and toxic solvents and waste. Biosynthesized silver nanoparticles using mangosteen leaf extract showed excellent antimicrobial activity. The AgNPs used in the study performed by Veerasamy (2011) et al. have a size of approximately 30 nm.

The effects of AgNPs on viability have been reported in many *in vitro* and *in vivo* studies. Moreover, the toxicity of AgNPs is critically determined by NP size. The AgNPs used in this study have a size of approximately 30 nm. As reported by Avalos et al., (2014), AgNPs with a smaller size exhibit a higher toxicity than larger ones, as they can more easily enter cells and promote reactive oxygen species (ROS) generation. Phagocytosis of AgNPs by macrophage cells results in increased ROS. Therefore, it generates an inflammatory signal and subsequently activates macrophage cells, which induce the secretion of TNF-α. Increment of TNF-α levels cause cell membrane damage and apoptosis (Mikhailova, 2020). It has been reported that silver nanoparticles did not cause any change in biochemical parameters in rats administered a dose of 5 mg/kg. However, significant increases were observed in biochemical parameters on the 29^{th} day in rats administered a dose of 10 mg/kg for 28 days (Nakkala, Mata, Sadras, 2017).

The chemical synthesis of metal nanoparticles requires the use of hazardous chemicals such as sodium borohydride (Alexandridis, 2011) and hydrazine (Iravani, Korbekandi, Mirmohammadi, and Zolfaghari, 2014) as reducing agents to convert metal ions into metal nanoparticles. Green synthesis, unlike chemical synthesis, is an environmentally friendly method in which environmentally friendly compounds are used as reducing agents instead of hazardous chemicals. In green synthesis, bacteria, fungi, plants, algae, and other microorganisms that are part of the biological system are used as reducing agents (Kahraman, Binzet, Turunc, Dogen, and Arslan, 2018). This new approach has emerged as non-toxic, eco-friendly, clean, and cost-effective. It also offers the possibility to be performed under mild conditions (Bharathi, Vasantharaj, Bhuvaneshwari, 2018; Tharani, Bharathi, Ranjithkumar, 2020; Nandana, Christeena, Bharathi, 2022; Hawar et al., 2022; Khane et al., 2022). Green synthesis can indeed be regarded as an alternative method to produce biocompatible nanomaterials, representing an optimal convergence of materials science and biotechnology (Rashidipour and Heydari, 2015; Heydari and Rashidipour, 2015; Heydari, 2017; Heydari, Koudehi, Pourmortazavi, 2019; Arya, Mishra, and Chundawat, 2019).

In this study, we aimed to compare the effects of AgNPs synthesised by chemical and green methods using the endemic *O. papillosa* Riedl plant on oxidative DNA damage using single-cell gel electrophoresis (comet) and cell viability using flow cytometry in human lymphocyte cells. The purpose of using the *O. papillosa* plant in this study is that it is an endemic species in Türkiye, and to the best of our knowledge, it has not yet been used in nanoparticle synthesis.

MATERIALS AND METHODS

Chemicals

Chemically synthesized AgNPs was purchased from SkySpring Nanomaterials, Inc 2935 Westhallow Dr., Houston, TX 77082 (15 nm, purity of Ag: 99.99. Product#0127SH; Lot#0127-031314). Roswell Park Memorial Institute (RPMI) medium, phosphate buffer solution (PBS), normal melting agarose (NMA), Trisma base, Triton X-100, ethylene diamine tetra acetic acid (EDTA), and ethidium bromide (EtBr) were purchased from Sigma. Sodium chloride (NaCl), sodium hydroxide (NaOH), and hydrogen peroxide (H_2O_2) were purchased from Merck. Low melting agarose (LMA), phytohemagglutinin (PHA), penicillin-streptomycin, Fetal Calf Serum were purchased from Bioshop and Biochrom AG, respectively.

Plant material

The *O. papillosa* samples were collected from Niğde province (Locality: Niğde, Ulukışla-Aksaray 8 km (37º 34' N 34º 25' E), slopes, open field and roadside, 1380 m. in June 2017 (Figure 1). The voucher specimen was identified by Dr. Rıza Binzet (2018) and has been deposited in the herbarium of Mersin University (MERA), Mersin Province.

Figure 1. ***Onosma papillosa***

Experimental design I: chemico-biotechnical analysis

Preparation of the leaf extract from O. papillosa

O. papillosa leaves were washed 2-3 times with water and left to dry in the shade at ambient temperature. The dried leaves were ground in a mill to obtain fine powder (Waring 8011, USA) for extraction. The fine powder (5 g) was placed into 250 mL round bottom flask and boiled under stirring for 30 min. in 150 mL of ultrapure water. At the end of the specified period, the mixture was cooled to room temperature, filtered through Whatman No. 1, and the filtrate was stored at 4 °C before use.

Green synthesis of silver nanoparticles

The green synthesis of AgNPs was performed by placing into flask 100 mL of 1.0 mM $AgNO_3$ and 100 mL of *O. papillosa* leaf extract. The reaction mixture was stirred vigorously at 60 ºC. The colour change from yellowish brown to dark brown ratifies the formation of AgNPs. The mixture was allowed to cool to room temperature. For characterization of AgNPs, the reaction mixture was centrifuged at 14000 rpm for 15 min. The supernatant was removed, and the resultant pellet was washed three times, centrifuged, dried, and stored for further characterization.

Structural characterization of silver nanoparticles

The formation of AgNPs was determined by monitoring the color change using a UV-Vis spectrometer (Shimadzu, Japan). The crystallinity nature of the nanoparticles was identified using an X-ray powder diffraction pattern (XRD) (Rikagu, Japan). FT-IR spectroscopy (PerkinElmer, USA), Scanning electron microscopy (SEM) (Zeiss, Germany), Energy-dispersive X-ray spectroscopy (EDX) (Zeiss, Germany), and dynamic light scattering (DLS) (Malvern, England) were applied to determine the morphology, composition, size, and stability of the AgNPs, respectively.

FT-IR analysis

It is stated that plant extracts play a dual role in nanoparticle synthesis. FT-IR analyses were conducted to determine the functional groups of phytochemicals that are thought to be involved in the synthesis of nanoparticles in the *O. papillosa* extract and in providing stable structures of the synthesized nanoparticles.

SEM/EDX analysis

SEM and EDX analyses were performed to determine the surface morphology and chemical composition of AgNPs synthesized using *O. papillosa* extract. For comparison, SEM analyses of chemically supplied AgNPs were also performed.

Preparation of the AgNP solutions

Five mg of both the AgNPs obtained via green synthesis and the chemically synthesized were weighed and dissolved in 10 mL sterile distilled water. The final concentrations were adjusted to 150 µg/mL (Battal et al., 2015).

Experimental design II: Toxicity and genotoxicity evaluation

Collection of blood samples

The blood samples (3 mL) used in this study were taken from four healthy male non-smoking donors (mean age, 24.75 ± 0.82 years). During blood collection, heparinized tubes were used. *The Mersin University Ethical Committee approved the experiments described in this study (06.04.2022-2022/241).*

Lymphocyte isolation, cell growth, and exposure to AgNPs in culture media

Human peripheral lymphocytes (HPL) are noncycling primary cells (G0 cells). They are easily collectable by venipuncture. In the presence of suitable culture media and stimulants *in vitro*, HPL enters the cell cycle and undergoes mitotic division (Johannes and Obe, 2019). Blood samples were collected from heparinized tubes from four healthy male non-smokers. Lymphocyte cultures were prepared according to previous studies (Moorhead, Nowell, Mellman, Battips, and Hungerford, 1960; Sınacı, Çelik, Yetkin, Çevik, S., and Güler, 2023). 5 mL of whole blood was added to 5 mL of Histopak-1077 and centrifuged for 30 min at 2000 rpm. Lymphocytes were separated from the medium using a micropipette. A mixture of 500 µL PBS and 500 µL lymphocytes was prepared. Subsequently, 300 µL of this mixture was transferred into labelled tubes containing 5 mL of RPMI 1640 medium. PHA (0.2 mL) was added to the lymphocyte culture. Subsequently, solutions of 150 µg/mL of each AgNP obtained from chemical and green synthesis were added. In the positive control group, 10 mM H2O2 was used. No chemicals were used in the negative control groups. The tubes were closed and incubated at 37 °C at an angle of 45° for 72 h. Subsequently, single-cell gel electrophoresis and flow cytometry were performed.

Application of the single cell gel electrophoresis (comet) method and slide scoring

Single-cell gel electrophoresis (comet) was performed with lymphocytes from four donors following the protocol outlined by Singh et al. (1988). Briefly, 100 µL of lymphocyte cell suspension and 100 µL of 2% low-melting-point agarose were mixed at 37 °C and then placed on a slide recoated with a thin layer of 0.5% normal-melting-point agarose. The cell suspension was immediately covered with a cover glass, and the slides

were held for 5 min at 4 °C to solidify the agarose. After removing the cover glass, the cells were placed in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4 ºC. After washing in distilled water, the slides were placed in a horizontal gel electrophoresis chamber filled with a cold electrophoretic buffer (1 mM EDTA, 300 mM NaOH, pH = 13), and the slides were kept at 4 ºC for 20 min to allow the DNA to unwind. Then, electrophoresis was conducted at 20 °C using 25 V and 300 mA for 20 min. After electrophoresis, the slides were treated three times with a neutralisation buffer (0.4 M Tris, pH = 7.5). All preparative steps were conducted in the dark to prevent undesired DNA damage. Finally, the slides were stained with ethidium bromide (0.1 mg/mL, 1:4) and analysed using a fluorescence microscope (Olympus BX 51) equipped with a CCD-4230 video camera.

To determine the DNA damage in each experimental sample, two slides and 50 cells per slide were evaluated. A researcher (G.G) blindly scored slides to prevent bias. Damages in cells were classified by eye into five categories on the basis of the extent of DNA migration as undamaged (*Type0*), very little damage (*TypeI*), moderate damage (*TypeII*), high damage (*TypeIII*), and ultra-high damage (*TypeIV*). In the comet assay, two different parameters were evaluated. i. Genetic damage index ii. Damaged cell percentage, in formula below;

Genetic damage index (GDI)=0xType0+1xTypeI+2xTypeII+ 3xTypeIII+4xTypeIV
Dameged cell percentage (DCP)=TypeII+TypeIII+TypeIV

Flow cytometry method/apoptosis assay

The Annexin V-FITC apoptosis detection kit with PI was utilized to specify cellular apoptosis or necrosis. This kit is based on the observation of phosphatidylserine translocation from the inner to outer surface of the plasma membrane, which is easily detected by staining with the fluorescent dye Annexin V. Annexin V, which has a high affinity for phosphatidylserine, was conjugated to FITC for visualization. In flow cytometry analyses, late and early apoptotic cells, live cells, and necrotic cells were counted. Early apoptotic cells are not stained with PI but with annexin V. Late apoptotic cells stained with Annexin V and PI. While necrotic cells are stained with PI, they are not stained with Annexin V. PI and Annexin V dyes do not penetrate living cells (Sliwinska et al., 2015). Approximately $4x10^5$ cells were analysed via flow cytometry to determine the apoptotic response of blood lymphocytes exposed to two different types of AgNPs.

Statistical analysis

Normality control for all parameters was performed using the Shapiro–Wilk test. The STATISTICA 13.0 analysis programme was used for statistical evaluation of the data obtained because of the experiment protocols. To examine if there was a statistically significant difference between the results, the concentrations applied were compared both among themselves and between the positive control values and the negative control values. The averages of the data obtained from the experimental protocols were used for all analyses. One-way ANOVA was used because the data showed normal distribution according to the Shapiro-Wilk test result. The One-Way ANOVA test statistic was employed to compare the averages of more than two independent groups. In case of a significant difference detected with ANOVA, Tukey's test was used as a Post Hoc test. The p value (confidence interval) was set at 0.05 for evaluation. When examining the relationships between Continuous Measurement parameters, the "Pearson Correlation" coefficient was used. "Spearman Correlation" coefficient was used for analyzing the relationships between continuous ordinary measurement parameters.

RESULTS

Structural characterisation of nanoparticles

UV-Vis spectroscopy

The formation of AgNPs synthesized by the green method was determined using UV-Vis spectroscopy. Figure 2a shows the UV-Vis spectrum of the precursor salt $AgNO_3$ and AgNPs. When examining the spectrum, no absorption peak of the precursor salt was observed in the wavelength range of 300–800 nm. It was determined that the absorption peak observed at 430 nm in the same wavelength range is related to the formation of AgNPs. In metal nanoparticles, the conduction and valance bands are so close that electrons move freely. These free electrons of AgNPs, in resonance with light, promote the colour of the AgNPs appearing dark brown. It is known that alkaloids, terpenoids, and polyphenolic compounds in the plant content play a role in the formation of nanoparticles. Flavonoids are polyphenolic compounds containing various groups that are involved in the formation of nanoparticles.

X-ray diffraction method (XRD)

The crystal structure of AgNPs synthesized using *O. papillosa* plant extract was determined by XRD analysis and is shown in Figure 2b. When examining Figure 2b, four characteristic 2θ values between 0° and 80° were determined. According to the results obtained from the XRD spectrum of AgNPs, diffraction peaks corresponding to (111), (200), (220), and (311) crystal structures were observed at 38.09°, 44.26°, 64.53°, and 77.35° angles. It has been determined that the results obtained are compatible with the card number 01-071-9155 obtained from library scanning.

Dynamic light scattering analysis

The particle size of AgNPs synthesised using *O. papillosa* plant extract was measured using DLS. The average particle size and zeta potential of the synthesised AgNPs were determined to be 14 nm and -20.5 mV, respectively (Fig. 2c and 2d). The negative values of the zeta potential indicate that the synthesised AgNPs have high stability. In addition, negatively charged groups on the surface of nanoparticles are believed to prevent the aggregation of nanoparticles.

FT-IR analysis

As depicted in Figure 2e, the band observed at 3272 cm^{-1} in *O. papillosa* extract corresponds to -OH stretching vibrations. In the synthesized AgNPs, a stretching vibration band was observed at 3230 cm^{-1} (Fig. 2e). The bands observed at 2919 and 2849 cm^{-1} in plant extract belong to aliphatic C-H stretches, and these bands shifted to 2916 and 2853 cm^{-1} values in AgNPs (Fig. 2f). The C=O stretching vibration band observed at 1734 cm^{-1} in plant extract was not observed in the spectrum of AgNPs. The bands observed at 1603, 1423 and 1012 cm^{-1} in the plant extract belong to aromatic C=C stretching vibration, asymmetrical C-H deformation and C-O-C bending, respectively. These values are 1600, 1414 and 978 cm^{-1} in AgNPs.

SEM/EDX analysis

As depicted in Figure 3a, AgNPs synthesised with *O. papillosa* extract were spherical and homogeneously dispersed. In addition, upon examination of the SEM images of commercially available AgNPs (Figure 3b), it is evident that they possess a spherical structure similar to the nanoparticles synthesized with plant extract. To determine the composition of the biosynthesize nanomaterial, EDX analysis was performed. As illustrated in Figure 3c, the synthesized nanomaterial consists of Ag, C, N, and O, indicating the natural product. The presence of Pt is attributed to the sample plating.

Evaluation of the genotoxicity and cytotoxicity of nanoparticles

In this study, the effects of AgNPs obtained using *O. papillosa* and chemically obtained AgNPs on oxidative DNA damage and cell viability in human lymphocyte cells were assessed. The study aimed to compare the effects of AgNPs obtained through two different methods. Oxidative damage to DNA was evaluated using single-cell gel electrophoresis and cell viability was assessed using flow cytometry.

Results obtained by single-cell gel electrophoresis

A total of 100 cells were counted from samples of each dose, and the counted cells were classified according to their tail length as *Type 0,Type I,Type II,Type III,Type IV*. Data obtained from single-cell gel electrophoresis are shown in Table 1.

According to data from single-cell gel electrophoresis studies, the genetic damage index and the percentage of damaged cells caused by AgNPs obtained by green synthesis in human lymphocyte cultures were not statistically significant compared with the negative control ($p = 0.124$ and $p = 0.613$). The genetic damage index and the percentage of damaged cells caused by the AgNPs obtained through chemical synthesis reached 165.25 ±27.72 and 50.50 ±12.66, respectively, and for the positive control, these values reached 322.50 ±16.36 and 90.75 ±3.94, respectively. The genetic damage index and the percentage of damaged cells caused by AgNPs obtained through chemical synthesis and by the positive control were statistically significant when compared with the negative control ($p < 0.01$). When comparing green synthesis and chemical synthesis, the genetic damage index and the percentage of damaged cells caused by the nanoparticles obtained by chemical synthesis reached 165.25 ±27.72 and 50.50 ±12.66, on the other side, the genetic damage index and the percentage of damaged cells caused by the nanoparticles obtained by green synthesis reached 80.50 ±9.46 and 17.50 ±5.80, respectively. The increase in the genetic damage index value in cultures exposed to AgNPs obtained by chemical synthesis increased 2-fold compared with that in cultures exposed to AgNPs obtained by green synthesis. The percentage of damaged cells in cultures exposed to AgNPs obtained by chemical synthesis increased 3-3.5-fold compared to this percentage in cultures exposed to AgNPs obtained by green synthesis. This increase is statistically significant ($p < 0.001$). Comet formation of lymphocyte cells observed under fluorescence images is shown in Figure 4 a-e.

Results of the flow cytometry analysis

Table 2 presents data related to flow cytometry for the four donors. According to the data from flow cytometry studies, the number of early apoptotic cells increased in human lymphocyte cultures treated with silver nanoparticles obtained through green and chemical synthesis compared with the negative control. It is found that this increase is not statistically significant. The number of late apoptotic cells in cultures treated with silver nanoparticles obtained through green and chemical synthesis reached to 13.57±4.78 and 17.95±6.85, respectively, whereas it reached to 0.025±0.05 for negative control. There was a significant difference between negative control cultures and cultures treated with silver nanoparticles obtained through green ($p < 0.01$) and chemical ($p = 0.01$) synthesis for the number of late apoptotic cells. When comparing the green synthesis and chemical synthesis groups, the number of late apoptotic cells in lymphocyte cultures treated with AgNPs obtained through chemical synthesis was higher (Figure S1). While number of surviving cells in negative control cultures is 99.8±0.08, this value decreased to 59.82±9.27 and 44.57±11.86, green synthesis and chemical synthesis cultures, respectively. A 1.67-fold decrease in the number of cells in cultures exposed to AgNPs obtained from green synthesis was observed compared with

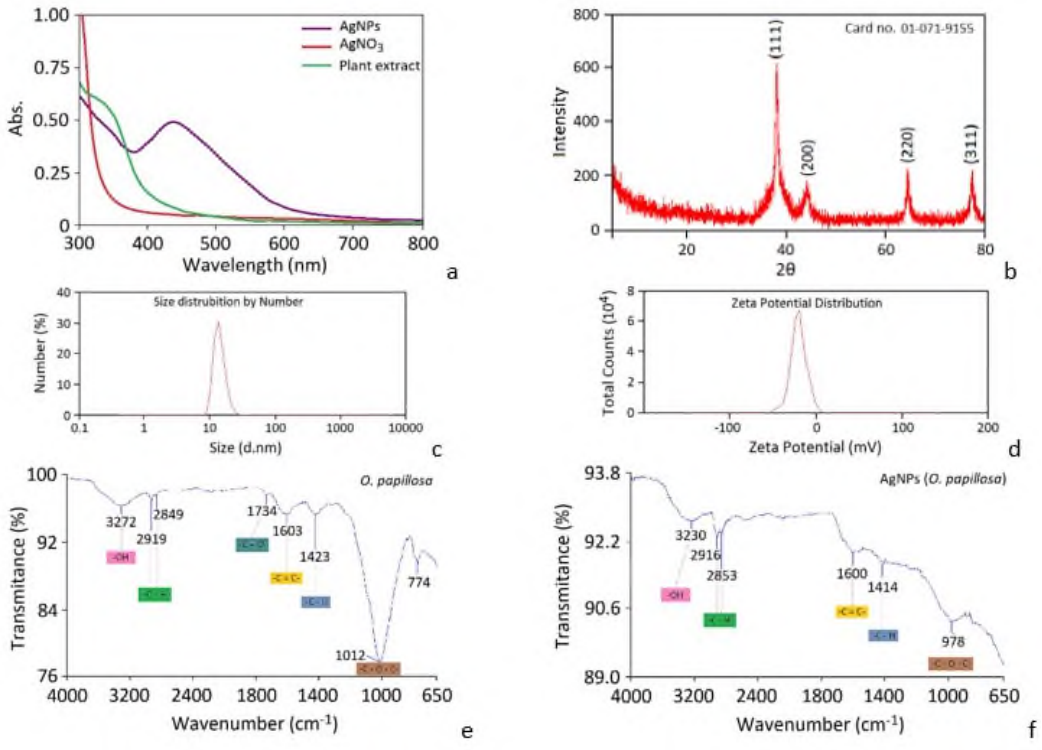

Figure 2. (a) UV-Vis spectrum of plant extract, precursor $AgNO_3$ salt and AgNPs, (b) X-ray diffraction pattern, (c) The particle size, (d) the zeta potential of green synthesized AgNPs, (e) FT-IR analysis of *O. papillosa* extract and (f) FT-IR analysis of green synthesized AgNPs.

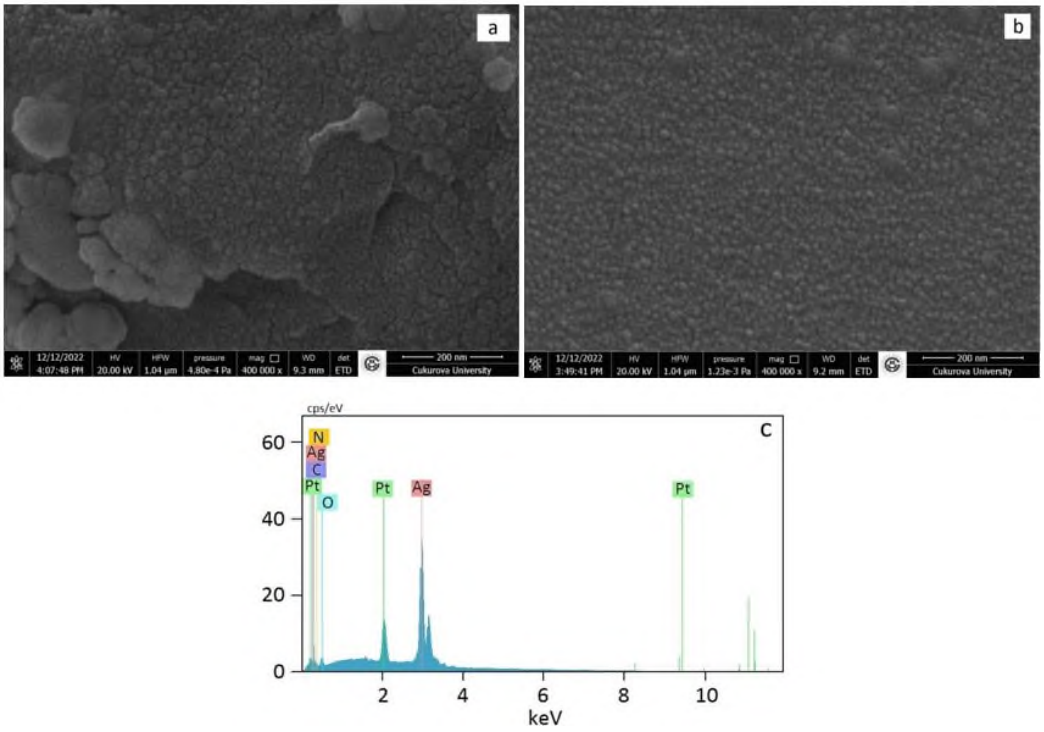

Figure 3. SEM image of AgNPs synthesized with *O papillosa* extract (a), Commercially available AgNPs (b), EDX profile of AgNPs synthesized with *O papillosa* extract.

Table 1. Data of comet assay analysis and statistical results for Genetic Damage Index and Damaged Cell Percentage in human lymphocytes cell treated with AgNPs.

	Donors	Type 0	Type I	Type II	Type III	Type IV	GDI	DCP
Negative Control	**Donor 1**	62	22	13	3	0	57	16
	Donor 2	61	25	10	4	0	57	14
	Donor 3	62	30	6	2	0	48	8
	Donor 4	64	31	4	1	0	42	5
Mean ±S.E.							**51.00 ±7.34**	**10.75 ±5.12**
Green synthesized AgNPs (150 µg/mL)	**Donor 1**	50	26	13	8	3	88	24
	Donor 2	44	39	7	7	3	86	17
	Donor 3	49	41	6	2	2	67	10
	Donor 4	48	33	12	4	3	81	19
Mean ±S.E.							**80.50 ±9.46**	**17.50 ±5.80**
Chemical synthesized AgNPs (150 µg/mL)	**Donor 1**	8	39	29	17	7	176	53
	Donor 2	0	39	40	15	5	184	60
	Donor 3	26	42	19	8	5	124	32
	Donor 4	4	39	35	20	2	177	57
Mean ±S.E.							**165.25 ±27.72*** **	**50.50 ±12.66*** **
Positive control (10mM) (H_2O_2)	**Donor 1**	4	11	13	25	47	300	85
	Donor 2	2	4	8	26	60	338	94
	Donor 3	2	6	8	28	56	330	92
	Donor 4	1	7	14	25	53	322	92
Mean ±S.E.							**322.50 ±16.36*** **	**90.75 ±3.94*** **

***p < 0.001 compared with negative control, GDI: Genetic damage index, DCP: Damaged cell percentage

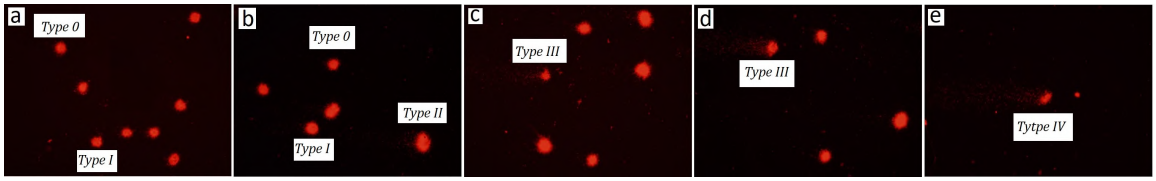

Figure 4. Comet formation of lymphocyte cells observed under fluorescent microscopy. (a:Negative control, b:Green synthesis, c:chemical synthesis, d-e:Positive control)

the negative control. In chemical synthesis, this decrease was observed to be 2.25 times. It was found that there was a significant difference between negative control cultures and cultures treated with AgNPs obtained by green and chemical synthesis compared with the negative control for the number of live cells ($p < 0.001$). The AgNPs obtained by chemical synthesis decreased the number of living cells compared with those obtained from green synthesis. While the number of necrotic cells in negative control cultures is 0.12±0.05, this value increased to 13.55±3.37 and 25.37±14.53, green synthesis and chemical synthesis cultures, respectively. The increase in necrotic cells in cultures exposed to silver nanoparticles obtained by chemical synthesis is 1.92 times higher than that in cultures exposed to silver nanoparticles obtained by chemical synthesis. The AgNPs obtained from both chemical and green synthesis increased the number of necrotic cells. This increase is not statistically significant for the AgNPs obtained by green synthesis, but it is statistically significant for AgNPs obtained by chemical synthesis compared with the negative control ($p < 0.01$) (Table 2).

Table 2. Statistical results for flow cytometry analysis in human lymphocyte treated with AgNPs.

		Examined cells	Early Apoptotic cell (%)	Late Apoptotic cell (%)	Live cell (%)	Necrotic cell (%)
Negative Control	**Donor 1**	2959	0.1	0.1	99.7	0.1
	Donor 2	2905	0	0	99.9	0.1
	Donor 3	3343	0	0	99.8	0.2
	Donor 4	3110	0.1	0	99.8	0.1
Mean ±S.E.		**3079.25±196.07**	**0.05±0.05**	**0.025±0.05**	**99.8±0.08**	**0.12±0.05**
Green synthesized AgNPs	**Donor 1**	3421	22.0	18.7	49.9	9.4
	Donor 2	3396	17.2	16.6	54	12.2
	Donor 3	3638	7.3	9.8	66.8	16.2
	Donor 4	3425	5.9	9.2	68.6	16.4
Mean ±S.E.		**3470±112.73**	**13.1±7.77**	**13.57±4.78****	**59.82±9.27*****	**13.55±3.37**
Chemical synthesized AgNPs	**Donor 1**	3654	32.6	28.2	31.3	7.9
	Donor 2	3562	0.9	15	41.4	42.7
	Donor 3	3732	4.7	13.7	59.9	21.7
	Donor 4	3261	10.2	14.9	45.7	29.2
Mean ±S.E.		**3552.25±206.22**	**12.1±14.18**	**17.95±6.85****	**44.57±11.86*****	**25.37±14.53****
Positive Control (10 mM) H_2O_2	**Donor 1**	2889	4.5	3.4	76.3	15.8
	Donor 2	3697	12	12.4	62.1	13.6
	Donor 3	3453	7.5	11.4	60.2	20.9
	Donor 4	2776	1.8	3.2	72.8	22.1
Mean ±S.E.		**3203.75±442.51**	**6.45±4.37**	**7.6±4.98**	**67.85±7.90*****	**18.1±4.05***

*p < 0.05, **p < 0.01, ***p < 0.001 compared with negative control.

Correlation results between flow cytometry and comet analysis data

Because of the correlation between flow cytometry data, a very high negative correlation was found between live and late apoptotic cell data (r = -0.943). In addition, a high positive correlation was found between early and late apoptotic cell numbers (r = 0.869). There was a moderate negative correlation between live and necrotic cell numbers (0.664). There was a medium-level correlation between live cell and early apoptotic cell numbers (r = -0.720). In addition, a very high positive correlation was found between the genetic damage index and the percentage of damaged cells (0.992).

DISCUSSION

In this study, the genotoxic and apoptotic effects of AgNPs (~ 14-15 nm) obtained using *O. papillosa* and by chemical methods were investigated in human lymphocyte cell cultures. In Comet analysis, an increase in GDI and DCP values was observed in human lymphocyte cell cultures treated with AgNPs obtained from the chemical method. This increase was found to be statistically significant ($p < 0.001$). However, it was determined that the increase in GDI and DCP values of AgNPs obtained from green synthesis in human lymphocyte cell cultures was not statistically significant.

In flow cytometry analysis, it was determined that AgNPs obtained by both methods increased the number of early, late apoptotic, and necrotic cells. The increase in the number of late apoptotic cells was found to be statistically significant ($p < 0.01$). While the increase in the number of necrotic cells was significant ($p < 0.01$) for chemical AgNPs, it was not significant for AgNPs synthesized by the green method. In addition, it was determined that AgNPs caused a decrease in the number of viable cells, and this decrease reached a statistically significant level ($p < 0.001$).

This study is the first to compare the genotoxic and apoptotic effects of AgNPs synthesised by different methods (green and chemical synthesis). Ghosh et al. (2012) investigated the *in vitro* and *in vivo* genotoxic and cytotoxic effects of AgNPs in the 90-180 nm size range using chromosome aberration, comet, and flow cytometry tests. According to the data obtained, it was concluded that AgNPs caused a statistically significant increase in chromosome aberration and comet parameters in mouse bone marrow cells and induced oxidative damage. On the basis of the data obtained from comet test studies in lymphocyte cultures, it was reported that the increase in tail length and reactive oxygen species was dose dependent. Based on the data obtained from comet test studies in plants, it was determined that there was an increase in tail length depending on the dose. The results obtained in our study are consistent with those of Ghosh et al. (2012). Josie et al. (2016) investigated the effects on chromosome aberration, micronucleus induction, repair of double chain fractures, cell renewal potential, and lipid peroxidation caused by different concentrations of AgNPs with sizes varying between 2, 3, 4, 6, and 7 nm in human lymphocytes. As a result of the studies conducted, it was reported that nanoparticles with sizes of 3, 4, 6, and 7 nm cause a clastogenic effect, and nanoparticles with a size of 2 nm cause cell proliferation by increasing insulin-like growth factor concentration. Although the sizes of AgNPs differ between the two studies, they support each other in terms of DNA damage. Jiravova et al. (2016) investigated the genotoxic and cytotoxic effects of AgNPs with a size size of 27 nm in two different mammalian cell cultures (NIH3T3, SVK14). AgNPs were determined to have effects in both cell cultures. However, because NIH3T3 cells are more sensitive to AgNPs, they cause more damage to DNA. They also reported that AgNPs increased apoptotic and necrotic cell numbers. In our study, it is understood from the comet and flow cytometry data that human lymphocytes are sensitive to AgNPs obtained from chemical synthesis. Both studies support each other because they both observe increases in apoptotic and necrotic cell numbers and DNA damage. Dobrzyńska et al. (2014) investigated the genotoxic effects of AgNPs of 20 nm (at doses of 5 mg/kg and 10 mg/kg) and 200 nm (at a dose of 5 mg/kg) sizes in rat bone marrow cells using the comet and micronucleus test methods. The data obtained from the comet test emphasised that AgNPs increased the comet tail length, but this increase was not statistically significant. They reported that the data obtained from the micronucleus test showed genotoxic damage. In our study, AgNPs caused genetic damage in the comet assay. However, we concluded that the increase in this statistically significant value may have resulted from the different cell types and study types between the two studies. Li *et al*. (2012) tested the genotoxic effect of 5-nm AgNPs using Ames (0.15 - 76.8 µg/plate) and TK6 cell cultures (10-30 µg/mL). In the Ames test, they determined that AgNPs did not exhibit a mutagenic effect at doses ranging from 2.4 to 38.4 µg/plate, but they did show an effect at higher doses. In TK6 cell cultures, they reported that AgNPs increased micronucleus frequency at concentrations of 5, 10, 15, 20 µg/mL, with the increase at concentrations of 25 and 30 µg/mL reaching statistical significance compared with the negative control. In our study, demonstrating the genotoxic damage of AgNPs obtained by the chemical method in human lymphocyte cultures is valuable in terms of supporting the data of the two studies. However, the lack of a significant effect on human lymphocytes by AgNPs obtained through green synthesis makes the study unique in terms of showing the advantages of green synthesis. Nakkala et al. (2017) evaluated the serum levels of ALT, AST, LDH, IL-6 and TNF-α to assess the effects of toxicity in an *in vivo* (rat) study of approximately 21-nm AgNPs obtained by green synthesis using *Ficus religiosa* plant for 28 days. On the 29th day following the monitored application, they determined significant increases in these values. They reported that these values completely recovered on the 89th day and determined that AgNPs accumulated in the liver, brain, and lung, respectively, using the ICP-OES technique. In their *in vitro* experimental studies using A549, HeLa, Hep2, COLO 205, and SH-SY5Y cell lines, the authors evaluated the effects of AgNPs on oxidative stress parameters, apoptotic staining techniques, apoptotic cell death, and apoptotic gene expression (caspase 8, 3, 9). It causes apoptotic changes in A549 and Hep2 cells through both extrinsic and intrinsic apoptotic pathways. No changes in biochemical parameters were reported at low concentrations in rats. Considering these data, our study supports the demonstration that AgNPs synthesised by "green synthesis" are suitable for use in the nanomedicine field and can be evaluated as an environmentally friendly material. Rajanahalli et al. (2015) attempted to determine whether two differently coated AgNPs and uncoated AgNPs cause delays in the cell cycle and their effects on the formation of ROS in mouse embryonic stem cells. In our study, AgNPs obtained via chemical synthesis led to genotoxicity of DNA according to comet assay data. In the data from the study by Rajanahalli et al, the result that AgNPs increase ROS in the cell overlaps with the comet data characterising the damage in DNA we obtained in our study. Okafor et al. (2013) tested the cytotoxicity of AgNPs produced using extracts of *Aloe, Magnolia, and Eucalyptus* leaves at concentrations of 2, 4, and 15 ppm on Human Embryonic Kidney 293 cells-HEK293 using automated InQ Plus equipment. They reported that AgNPs with concentrations of 2 and 4 ppm were not toxic to human healthy cells but inhibited bacterial growth.

CONCLUSION

In conclusion, the current study established an easy, economical, and inexpensive eco-friendly protocol for the synthesis of AgNPs using *O. papillosa* leaf extract. UV-Vis spectroscopy, DLS, SEM, and XRD measurements confirmed the formation of green synthesised silver nanoparticles. It was observed that green silver nanoparticles prepared at certain concentrations prevented DNA damage. The results obtained in this work con-

tribute to the increase in knowledge of the effects of AgNPs on mammalian cell systems. The fact that nanoparticles obtained by the green method do not show genotoxic effects in mammalian cell cultures seems to reduce concerns about their use in many industrial and medical areas. The information presented in this study will shed light on nanogenotoxicology, showing that the genotoxic and apoptotic effects of AgNPs are not only related to the size of the nanoparticle but also to the method of obtaining it. The average particle size of the synthesised nanoparticles was measured to be 14 nm with a zeta potential of -20.5 mV. However, further detailed studies are needed to develop biological applications of biosynthesize AgNPs. This study will form the basis for detailed in vivo studies and future projects.

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Original Article

Allomaltol derivatives as Antimycobacterial agents: *In vitro* and *in silico* evaluations with potential protein targets

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ABSTRACT

Background and Aims: Mycobacterium species cause life-threatening pulmonary and extrapulmonary diseases in humans. This study aimed to evaluate the potential antimycobacterial activity of allomaltol derivatives in the Mannich base structure *in vitro* and *in silico.*

Methods: The antimycobacterial activity of each compound against *Mycobacterium tuberculosis* and *Mycobacterium avium* was tested using a resazurin microplate assay, and cytotoxicity was assessed using human MRC-5 and He-La cells. Using the SwissTarget tool, Rip1 protease, the metallo-beta-lactamase (MBL) superfamily protein, the serine protease Rv3671c, and zinc metalloprotease 1 (ZMP1) were identified as potential targets. Blind docking was performed for compound 14 using CB-Dock to identify and assess the most probable binding sites on the target proteins. Defined docking was performed with Flare to determine the best binding pose at the predicted binding pocket. The druglikeness of hit compounds, including the partition coefficient, number of hydrogen bond donors/acceptors, molecular refractivity, topological polar surface area (PSA), and gastrointestinal and blood-brain barrier absorption, were evaluated using the SwissADME tool.

Results: Compounds with methyl-substituted piperidine groups were found to have antimycobacterial activity (MICs: 2 µg/mL) against M. avium, which was as potent as the clinically used drugs ethambutol and streptomycin. The predicted physicochemical properties of the four hit compounds were satisfactory. According to the docking results, the binding energies of compound 14, which showed the best overall antimycobacterial activity, ranged from -8.14 to -5.97 kcal/mol, with ZMP1 showing the lowest binding energy.

Conclusion: The results of this study provide evidence that allomaltol derivatives are promising antimycobacterial agents with satisfactory drug profiles.

Keywords: Allomaltol, Tuberculosis, Molecular Docking, Druglikeness

INTRODUCTION

Tuberculosis (TB), a life-threatening disease caused by *Mycobacterium tuberculosis*, is one of the most deadly respiratory bacterial diseases and the second leading cause of death from infectious agents, following COVID-19. War, immigration, social status, poverty, gender, HIV infection, and homelessness are significant risk factors for tuberculosis, as well as other public health problems (Amiri, Siami, & Khaledi, 2018). Moreover, the development of drug resistance and the presence of comorbidities, such as acquired immunodeficiency syndrome (AIDS) and diabetes mellitus, increase the morbidity and mortality of the disease (Venugopala et al., 2021). Non-tuberculous mycobacteria (NTM) are opportunistic pathogens that can be found in natural water and soil, posing a serious threat to human health, particularly for patients who are immunocompromised or with pre-existing lung diseases. Among these, *Mycobacterium avium* is the most clinically significant pathogen. The substantial levels of inherent drug resistance in NTM contribute to unsatisfactory treatment outcomes, necessitating the development of novel drugs and therapeutic regimens (Portell-Buj et al., 2019; O. Falkinham, 2018). Therefore, *Mycobacterium* spp. have attracted the attention of medicinal chemists, encouraging them to search for novel compounds with improved bioactivities.

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Kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one), is a natural secondary metabolite produced by different types of fungi during aerobic fermentation. Owing to the unique structure of its scaffold, many synthetic derivatives have been prepared and investigated for different biological activities (Brtko, 2022; Zilles et al., 2022; Saeedi, Eslamifar, & Khezri , 2019). Since 3-Hydroxy-4(1*H*)-pyrones and their analogues are extensively utilised as foundational components for biologically active substances, allomaltol (5-hydroxy-2-methyl-4H-pyran-4-one) has drawn the attention of researchers (Milyutin et al., 2023; Ercan et al., 2020; Kandioller et al., 2011).

Nitrogen-containing heterocyclic compounds such as piperazine and piperidine rings have been widely used for the development of many drugs and other bioactive substances, including several of the currently used antitubercular drugs (Girase et al., 2021). Mannich reactions, also known as aminomethylation reactions, facilitate the synthesis of chemical entities with basic nitrogen atoms, making them valuable tools for drug discovery (Roman, 2022). Our research group has previously reported the anticonvulsant effects of several Mannich bases of allomaltol with piperazine moieties (Aytemir, Çaliş, & Özalp 2004; M. Aytemir & Çalış, 2006) (Figure1). In another study, Mannich bases of kojic acid and allomaltol were used to synthesize piperazine and piperidine analogues. These derivatives were examined for their effects on seizures induced by scMet and MES (maximal electroshock) (Aytemir & Çalış, 2010). The results show that piperidine-containing allomaltol derivatives have enhanced activity compared with piperazine-containing derivatives.

The primary objective of drug discovery is to develop bioactive compounds that are effective, selective, and have low toxicity. Most of the compounds bearing 3-Hydroxy-4(1*H*)-pyrone-containing Mannich bases designed and synthesized by our research group exhibited important bioactivities (Karakaya et al., 2019; Karakaya et al., 2019; Oncul et al., 2019). Because nitrogen-containing heterocyclic derivatives of allomaltol exhibit a wide spectrum of biological activities, our current goal is to use these derivatives to further develop bioactive compounds, particularly those with anticancer and antimicrobial properties, that do not harm healthy cells. These compounds have shown promising and uniform results and can be used as leads for drug development in the future.

Therefore, in the present study, we aimed to investigate the activities of 19 allomaltol derivatives against *M. tuberculosis* and *M. avium* and their cytotoxic effects on healthy (MRC-5) and carcinogenic (He-La) cell lines. Molecular docking studies were conducted with these compounds against several potential antimycobacterial drug targets. In addition, the drug-likeness properties of the most promising compounds were predicted in silico.

MATERIALS AND METHODS

Antimicrobial agents

All the compounds were dissolved in dimethylsulphoxide (3%) or d-H_2O at a final concentration of 512 µg /ml, sterilised by filtration using 0.22 µm Millipore (MA 01730, USA), and used as stock solutions. The standard antimycobacterial agents were isoniazid, ethambutol, and streptomycin. All compounds were purchased from Sigma Chemical Co. The stock solutions of the agents were prepared in accordance with the guidelines for the preparation of solutions outlined by the CLSI (Clinical and Laboratory Standards Institute) (Ozcelik et al. 2013; Aytemir & Özçelik, 2011; CLSI 2006).

Antimycobacterial Activity

The antimycobacterial activities of the compounds against M. tuberculosis and M. avium were determined using the Resazurin Microtiter Assay (REMA). M. tuberculosis H37Rv ATCC 27294 reference strain and M. avium ATCC 15769 strains were maintained on Lowenstein–Jensen medium, subcultured on Middlebrook 7H11 agar (Becton Dickinson), and resuspended in 7H9-S broth medium supplemented with 10% OADC (0.1% casitone, 0.5% glycerol, supplemented oleic acid, albumin, dextrose, and catalase), 0.2% glycerol, and 0.1% bactocasitone (Difco). Suspensions were prepared in 0.04% (v/v) Tween 80–0.2%+bovine serum albumin and adjusted to McFarland tube number 1. This was diluted to 1:20, and a 100-L aliquot was used as the inoculum. 100 µL of Middlebrook 7H9 broth (10% OADC, 0.5% glycerol, 0.1% casitone, Becton-Dickinson) was dispensed in each well of a sterile flat-bottom 96-well plate, and serial two-fold dilutions (256-0.06 µg /mL) of each compound were prepared. 100 µL of inoculum was added to each well. Growth and sterile control were also included for each isolate. Sterile water was added to prevent evaporation. The plate was then covered and incubated at 37 °C under a normal atmosphere. After 7 days of incubation, 10 µg /mL of resazurin solution was added, and the plate was reincubate overnight. Any change in colour from blue to pink indicated the growth of bacteria, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration of the drug that prevented change in colour (Ozcelik et al. 2013; Aytemir & Özçelik, 2011).

Cytotoxicity assay

MRC-5 and He-La cell cultures were grown in Eagle's Minimal Essential Medium (Seromed; Biochrom; Berlin; Germany) enriched with 10% foetal calf serum (Biochrom, Germany). Streptomycin at a concentration of 100 mg/ml and penicillin at a concentration of 100 IU/ml in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were harvested using trypsin solution (BibcoLife Technologies, UK). The Maximum non-toxic

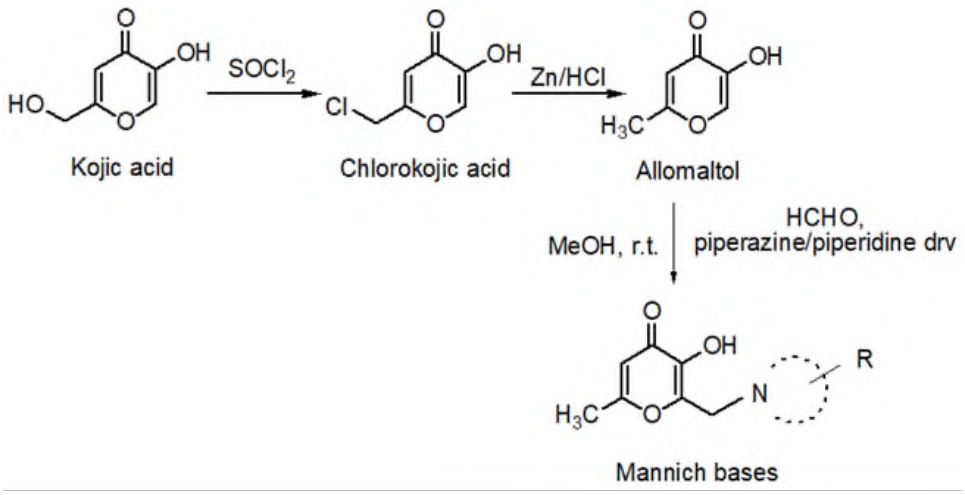


Figure 1. Synthesis and chemical structure of allomaltol derivatives.

concentrations (MNTCs) were determined on the basis of cellular morphologic alteration (Aytemir, Özçelik, & Karakaya, 2013; Karakaya et al., 2013). Several concentrations of each sample were placed in contact with confluent cell monolayers and incubated in 5% CO2, at 37 °C for 48 h. After incubation, non-toxic drug concentrations were evaluated and compared with those of untreated cells. The rows causing cell damage were evaluated as toxicity. In addition, maximum drug concentrations that did not affect the cells were evaluated as nontoxic concentrations. The MNTCs of the compounds were determined by comparing treated and untreated cultures.

In silico Studies

The structures were downloaded from the protein data bank (https://www.rcsb.org) and processed with UCSF Chimaera software. The structures of the compounds were drawn using ChemSketch. First, blind docking was performed using the online tool CB-Dock, which was developed using AutoDock Vina. This approach was used to identify and assess the most probable binding sites on target proteins via cavity detection (CurPocket) (Liu et al., 2020). Next, we performed defined docking using Flare version 6.0 software (Cresset, UK) to determine the best binding pose at the predicted binding pocket. Hydrogen atoms were included, and the optimal ionisation states were assigned to each residue. The chemical structure of compound 14 was uploaded in SDF format and processed using default settings. The grid was selected to include the binding site, which was determined using blind docking. The binding poses of each protein with the best scores and analysed for its interaction with compound 14.

RESULTS AND DISCUSSION

Chemistry

All of the compounds that were evaluated for their antimycobacterial activity in this study have been synthesised and characterised in our previous studies (Aytemir & Çalış, 2010; Aytemir, 2007; Aytemir & Çalış, 2006; Aytemir et al., 2004). Kojic acid was used as the starting compound in a two-step reaction including chlorination of kojic acid and subsequently reduction with zinc dust in conc. HCl, to gain allomaltol.

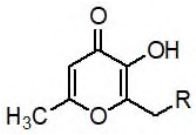
In vitro antimycobacterial effects of the compounds

Infectious diseases, particularly tuberculosis, pose a serious public health threat, and the emergence of antimicrobial resistance has limited clinical treatment options (Lv et al. 2024). Therefore, the discovery of new anti-TB agents is required.

Herein, REMA was performed to determine the antimycobacterial activity of all compounds against *M. tuberculosis* H37Rv and *M. avium* ATCC 15769)using isoniazid and ethambutol as control agents. The mechanism of action of ethambutol is not fully understood, whereas isoniazid inhibits the formation of the mycobacterial cell wall. A bacterial enzyme called "KatG" activates the drug, and the complex product inhibits the synthesis of mycolic acid, which is an essential cell wall component (Suarez et al., 2009). MICs (Table 1) of studied compounds against *M. tuberculosis* and *M. avium* were in the range of 2–128 µg/ml.

All 21 compounds exhibited antimycobacterial activity, including the synthesis starting materials. Among these compounds, 3, 10, 11 and 19 were found to be equally effective against both species, whereas the other compounds were more effective against *M. avium*.

Table 1. Anti-Mycobacterium activities coupled with the cytotoxicity of the compounds as MICs and MNTCs (µg/mL).



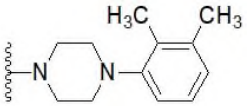
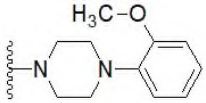
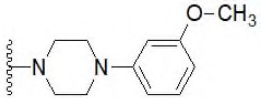
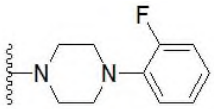
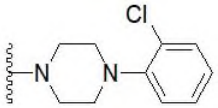
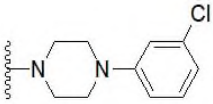
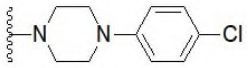
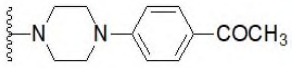
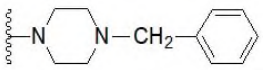
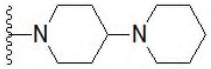
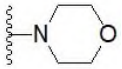
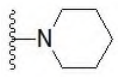
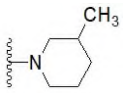
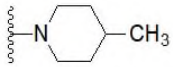
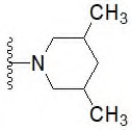
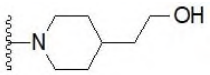
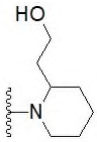
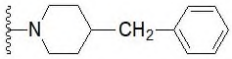
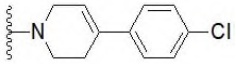
Comp. No.	R	MICs		Cytotoxicity (MNTCs)	
		M. tuberculosis ATCC 27294	*M.avium* ATCC15769	MRC-5	He-La
1[a]		64	16	≥512	≥512
2[b]		64	16	≥512	≥512
3[b]		16	16	≥512	≥512
4[b]		64	16	≥512	≥512
5[b]		64	16	≥512	≥256
6[a]		64	16	≥512	≥512
7[b]		64	16	≥512	≥256
8[b]		64	16	≥256	≥256
9[b]		64	16	≥256	≥256
10[a]		16	16	≥256	≥256
11[a]		16	16	≥256	≥256
12[c]		32	16	≥256	≥256

Table 1. continued

13[c]		**16**	**2**	≥256	≥256
14[c]		**16**	**2**	≥256	≥256
15[c]		**32**	**2**	≥256	≥256
16[c]		**8**	**4**	≥256	≥256
17[c]		64	128	≥256	≥256
18[c]		64	16	≥256	≥256
19[d]		16	16	≥256	≥256
Kojic acid		32	16	≥256	≥256
Allomaltol		32	16	≥256	≥256
Isoniazide		0.125	0.125		
Ethambutol		2	2		
Streptomycin		1	2		

a: (M. Aytemir & Çalış, 2006), b:(M. D. Aytemir et al., 2004), c:(Aytemir, M. D., Çalış, Ü., 2007), d: (M. D. Aytemir & Çalış 2010); MNTCs: Maximum non-toxic concentrations

Compound 16, which includes a 4-(2-hydroxyethyl) piperidine-1-yl group, was the most effective compound against *M. tuberculosis* (MIC = 8 µg/ml) and was four times more active than kojic acid and allomaltol (MIC = 32 µg/ml). However, compounds 12 and 15 showed similar activities to those of kojic acid and allomaltol.

Since it is known that bioactivity is not the only parameter required for a good drug candidate, drug-like properties were improved by switching to Mannich bases, which will be discussed in detail in the next section.

All compounds except compound 17 were more active against *M. avium*. In particular, compounds 13, 14, and 15 were the best derivatives in the series (MIC = 2 µg/ml) and were eight times more active than kojic acid and allomaltol (MIC = 16 µg/ml). More importantly, these three compounds were found to have the same antimycobacterial activity as the reference drug ethambutol and streptomycin. Compound 16 also showed good activity against *M. avium* (MIC = 4 µg/ml).

Correlating the chemical structures of compounds with their bioactivities is an important area of medicinal chemistry. The chemical structures of the compounds tested in this study differ according to the type of amine groups added by the Mannich reaction. The molecular modification was performed by adding secondary amine groups. Hence, the synthesised derivatives can be categorised into those bearing substituted phenyl piperazine and piperidine groups.

According to the electronic characteristics of the substituents in the compounds bearing the phenylpiperazine group, there was no difference between the groups in terms of electron-donating or electron-withdrawing properties. In addition, the position of the substituent does not have any effect, which can be easily observed from the lack of difference in the activities of compounds 5, 6, and 7, which are positional isomers with respect to chlorine atoms. Furthermore, the introduction of a methylene bridge between the piperazine and phenyl groups, as in the structure of compound 9, did not alter the activity. The antimycobacterial activity of compound 18, an isomer with

a piperidine ring instead of a piperazine ring based on the structure of compound 9, remained the same.

By evaluating all activity results, we found that the most potent compounds in the series were piperidine derivatives bearing -CH3 at different structural positions. Although the MIC values of compounds 13, 14, and 15 bearing 3-CH_3, 4-CH_3, and 3,5-diCH_3, respectively, were 16 µg/ml, 16 µg/ml, and 32 µg/ml, respectively, against *M. tuberculosis*, their activity against *M. avium* was the same, with an MIC of 2 µg/ml, similar to the reference drugs ethambutol and streptomycin. By comparing the antimycobacterial activity of compound 12, a nonsubstituted piperidine derivative without a methyl group, it can be assumed that the methyl group, which provides electrons to the ring inductively, significantly contributes to the bioactivity.

In contrast to the piperazine derivatives, the positions of the hydroxyethyl groups in the piperidine derivatives significantly affected their bioactivity. In this case, the hydroxyethyl group at the 4-position resulted in the second most active compound in the series, whereas the hydroxyethyl group at the 2-position resulted in the weakest compound.

Cytotoxicity of the compounds

Allomaltol derivatives were evaluated for their cytotoxic effects against normal MRC-5 (human lung fibroblast) and cancer HeLa (Human cervix epithelial carcinoma) cells using a previously described method (M. D. Aytemir et al., 2013; Karakaya et al., 2013). The MNTCs were determined based on cellular morphologic alterations and were designated as either ≥128 or ≥512 µg/mL. According to the cytotoxicity results, we observed no selectivity between the two groups. All compounds were bioactive at non-toxic concentrations (≥256 µg/mL). Except for compounds 8 and 9, piperazine containing Mannich bases had higher MNTCs (512 µg/mL) than kojic acid, allomaltol, and piperidine analogues (MNTCs: ≥256 µg/mL).

In silico studies

Target Prediction Analysis Results

The protein groups with a high likelihood of binding, as determined by the SwissTarget prediction analysis, a web-based tool, are depicted in the graph below (Figure2) (Daina et al., 2019). This tool predicts the protein targets with which the investigated compound may interact. In this study, Rip1 protease, metallo-beta-lactamase (MBL) superfamily protein, serine protease Rv3671c, and zinc metalloprotease 1 (ZMP1) were predicted as potential targets of compound 14 and were selected for molecular docking. Rip1 is a metalloprotease and an important virulence factor in mycobacteria. It cleaves anti-sigma factors K, L, and M, which negatively regulate the corresponding sigma factors SigK, SigL, and SigM, respectively. These sigma factors are involved in activating the expression of several other virulence factors (Schneider, Sklar, & Glickman, 2014). The MBL superfamily includes a group of enzymes that inactivate a broad spectrum of -lactam antibiotics, except for monobactam. They are not affected by most beta-lactamase inhibitors, such as clavulanate, sulbactam, and tazobactam (Boyd et al. 2020). Rv3671c is a serine protease that enables mycobacteria to persist within phagolysosome (Biswas et al. 2010). Zinc metalloprotease-1 (Zmp1) is an important target and virulence factor in mycobacteria. It is a zinc-containing peptidase that interferes with phagosome maturation in macrophages, possibly by inhibiting the secretion of caspase-1/interleukin-1β and suppressing the formation of inflammasome and phagolysosomes (Ferraris et al. 2011). Cyclic di-GMP (c-di-GMP) phosphodiesterase (CDP) hydrolyses c-di-GMP to yield two GMP molecules. It regulates biofilm formation, cell motility, and virulence in mycobacteria (Hull et al. 2012). Therefore, these five enzymes represent attractive therapeutic targets for mycobacteria, and their inhibition may attenuate virulence.

Molecular Docking Results

According to the literature, only a few publications have reported the targets and mechanism of action of synthetic antimycobacterium compounds. The use of computational chemistry accelerates the discovery and design of new compounds with improved potency while also reducing the synthesis cost. Molecular docking simulation is a crucial tool for structure-based drug design (SBDD) because it can predict the binding affinity as well as the binding pose of the ligand with the active sites of a target (Abdullahi et al., 2020). It is an optimisation process in which the main goal is to find the most stable binding position of the ligand with the target molecule. The binding affinity of each possible orientation of the ligand within the active sites forming the complex was calculated by sampling the 3D coordinate space of the binding site on the target. Thus, the pose with the lowest binding energy is predicted as the most stable conformation of the protein-ligand complex.

In this study, we subjected compound 14 to two rounds of molecular docking. The first round was a blind docking approach used to identify and assess the most probable binding sites on the target proteins. The second round was a defined docking approach used to determine the best binding pose at the predicted binding pocket. The binding energies, shown in Table 2, ranged from -8.14 to -5.97, kcal/mol, with ZMP1 exhibiting the lowest binding energy. These results suggest that compound 14 binds with high affinity to each of the predicted targets. The predicted binding interactions showed that compound 14 formed one hydrogen bond with ALA 631 in ZMP1, two hydrogen bonds with SER 442 and GLN 445 in CDP, one hydrogen bond with SER 397 in the serine protease Rv3671c, one hydrogen bond with ASP 76 and one aromatic interaction with PHE 175 in the MBL superfamily protein, and two hy-

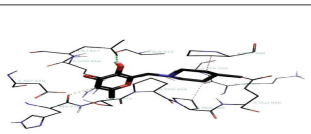
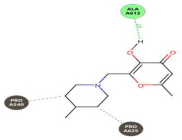
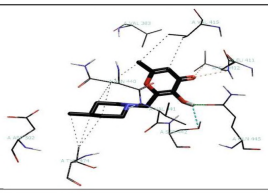
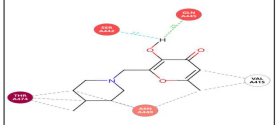
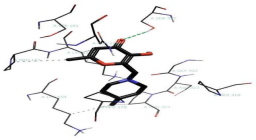
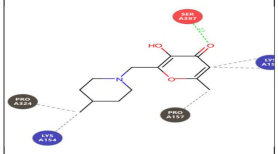
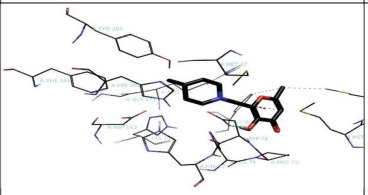
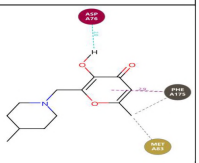
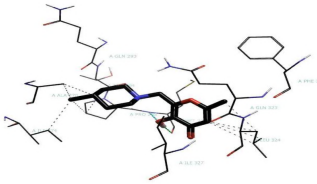
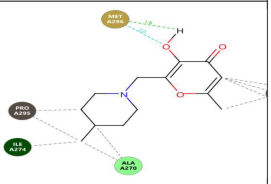
Target	Docked view of the target active site	2D Interactions
ZINC METALLOPROTEASE ZMP1 (PDB: 3ZUK)		
Cyclic diguanylate phosphodiesterase (EAL) domain protein (A0A0H2ZTL9)		
Serine protease Rv3671c **(P9WHR8)**		
Metallo-beta-lactamase superfamily protein (P96924)		
Zinc metalloprotease Rip1 (Q9CBU4)		

Figure 2. 2D and 3D binding poses of compound 14

Table 2. Docking scores for compound 14 for predicted targets

Protein	**UniProt Acc. (No.**	**Docking Score (kcal/mol)**
Zinc metalloprotease Rip1 (PDB:3ZUK)	Q9CBU4	-6.83
Metallo-beta-lactamase superfamily proteins	P96924	-5.97
Serine protease Rv3671c	P9WHR8	-6.77
Zinc metalloprotease ZMP1 (PDB:6XLY)	O53649	-8.14
c-di-GMP phosphodiesterase	A0H2ZTL9	-6.16

Table 3. Predicted ADME and physicochemical parameters of the compounds

Comp. no	MW (g/mol)	Consensus logP	HBD	HBA	MR	TPSA (Å2)	GI Absorption	BBB Permanent
13	237.29	1.68	1	4	70.47	53.68	High	Yes
14	237.29	1.67	1	4	70.47	53.68	High	Yes
15	251.32	1.91	1	4	75.28	53.68	High	Yes
16	267.32	1.19	2	5	76.44	73.91	High	No

Table 4. Drug-likeness filters of the compounds

Comp. no	Lipinski	Those	Veber	Egan	Muegge	Bioavailability score
	MW ≤ 500; Mean logP ≤ 4.15; HBA ≤ 10; HBD ≤ 5	60 ≤ MW ≤ 480; − 0.4 ≤ WlogP ≤ 5.6; 40 ≤ MR ≤ 130; 20 ≤ atoms ≤ 70	Rotatable Bonds ≤ 10; TPSA ≤ 140 Å2	WLOGP ≤ 5.88; TPSA ≤ 131.6	200 ≤ MW ≤ 600, − 2 ≤ XLOGP ≤ 5; TPSA ≤ 157; HBA ≤ 10; HBD ≤ 5; RB ≤ 15; Number of rings ≤ 7; Number of carbons > 4; Number of heteroatoms > 1	Probability of F > %10 in rats
13	Yes	Yes	Yes	Yes	Yes	0.55
14	Yes	Yes	Yes	Yes	Yes	0.55
15	Yes	Yes	Yes	Yes	Yes	0.55
16	Yes	Yes	Yes	Yes	Yes	0.55

drogen bonds with MET 296 in Rip1. Our molecular docking results provide further evidence that compound 14 might exert its anti-TB effect by inhibiting all five enzymes, particularly ZMP1. However, further in vitro studies are required to confirm this result.

Druglikeness of the compounds

In principle, the optimisation of lead compounds into drug candidates should address their potency and selectivity and improve their ADME profiles. Insufficient ADME and physicochemical properties led to the withdrawal of drug candidates from preclinical studies. Therefore, it is useful to evaluate the ADME properties of the compounds in the first step. Compounds 13, 14, 15, and 16 were selected to predict ADME properties because they exhibited the highest antimycobacterial activity.

The druglikeness of a molecule is determined by balancing hydrophobicity, electronic distribution, size, and flexibility. These properties influence the bioavailability, toxicity, metabolic stability, affinity to proteins, and transport properties of compounds in living organisms.

In a previous study, 1271 synthetic anti-tubercular compounds were evaluated based on their physicochemical properties (Motamen & Quinn, 2020). Calculated partition coefficient (clog P), molecular weight (MW), and polar surface area (PSA) as the three important properties arising from the analysis. In addition, a new TB space with more appropriate values of MW ≤ 500, -4 ≤ clog P ≤ 3, and 30 ≤ PSA ≤ 140 Å is proposed which may be a useful guide for designing new compounds against *Mycobacterium* species.

In this study, the drug-likeness properties of the selected derivatives were evaluated using the SwissADME web-based tool (Daina et al., 2017). In silico prediction of physicochemical and pharmacokinetic properties such as hydrogen bond donor/acceptor (HBD/HBA), molecular refractivity (MR), partition coefficient (logP), topological PSA (tPSA) val-

ues, blood–brain barrier (BBB) transport, and gastrointestinal (GI) absorption are presented in Table 3.

The logP value, a valuable indicator of drug permeability, was estimated using different models, i.e., iLOGP, XLOGP3, MLOGP, SILICOS-IT, WLOGP, and the arithmetic mean of the logP values. The MR and tPSA polarity parameters indicate the transport of compounds in the body. Drug-likeness filters are also presented with their limitations in Table 4. All selected Mannich bases obeyed all considered filters.

CONCLUSION

The allomaltol derivatives tested in this study were effective against M. tuberculosis and M. avium and relatively non-toxic against human cells. In silico studies predicted that these compounds exhibit excellent drug-likeness and satisfactory physicochemical properties. In particular, compounds bearing methyl-substituted piperidine groups were found to be as effective as the drugs ethambutol and streptomycin, which are clinically used in these treatments. Furthermore, Rip1, MBL superfamily proteins Rv3671c, and ZMP1 were predicted to be potential targets of these compounds, with high binding affinity. Therefore, allomaltol derivatives are promising antimycobacterial agents that can be used to develop novel therapeutic agents for the treatment of tuberculosis and other mycobacterial infections with high safety profiles.

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Original Article

Synthesis, characterization, and crystal structure of a novel spirocyclic 2-indolinone bearing a 5-(trifluoromethyl)benzothiazoline moiety

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ABSTRACT

Background and Aims: Spirocyclic 2-indolinones are important and promising compounds because of their various biological activities in drug development studies. The main aim of this study is to determine spirocyclic, molecular and stereoisomeric structure of the new 5'-chloro-1',7'-dimethyl-5-(trifluoromethyl)-3*H*-spiro[1,3-benzothiazole-2,3'-indole]-2'-one (**4**) and to examine the contribution of the trifluoromethyl group.

Methods: Compound **4** was synthesized from the reaction of 2-amino-4-(trifluoromethyl)benzenethiol with 5-chloro-1,7-dimethyl-1*H*-indole-2,3-dione in ethanol. The purity and structure determination of compound **4** was carried out by elemental and spectral analyzes. The crystal structure of compound **4** was characterized by X-ray single crystal diffraction analysis method (SC-XRD). Additionally, compliance with Lipinski's rule of 5 (RO5) and some pharmacokinetic parameters of compound **4** were evaluated using the Qikprop modüle (Schrödinger).

Results: The molecular structure of **4** was confirmed by elemental and spectral (IR, ^{1}H NMR, ^{13}C NMR-APT, HSQC-2D, HMBC-2D and LCMS-APCI) data. The crystal, spirocyclic and stereoisomeric structure of compound **4** was elucidated by SC-XRD, and it was observed that N-H···O hydrogen bonding interactions take place within the molecular layers aligned parallel to the (010) plane. As a drug candidate, compound **4** exhibited physicochemical parameters consistent with Lipinski's RO5.

Conclusion: In the crystal, both intra- and intermolecular hydrogen bonds are present. The molecular packing is stabilized by intermolecular N— H···O hydrogen bonds.

Keywords: 2-Indolinone, Benzothiazoline, Crystal structure, Structural characterization, Synthesis.

INTRODUCTION

For pharmaceutical chemists, knowing the 3D structures of synthesized molecules are critical for obtaining and developing effective and safe drugs in the drug discovery and design process. Due to the interesting structure of spiro compounds and their importance in biological activity, there are many studies in the literature for the discovery of their 3D structures (Ding, Meazza, Guo, Yang & Rios, 2018). Spirocyclic compounds were first recognised in organic chemistry in the late 1800s and early 1900s and represent polycyclic structures in which one or more carbon atoms are common members of two or more different rings (Baeyer, 1900; Bariwal, Voskressensky & Van der Eycken, 2018). It is thought that polycyclic rings may pose compatibility problems and the presence of quaternary and generally chiral spiro atoms pose difficulties in terms of synthesising these compounds and determining their 3D structures.

Spirocyclic 2-indolinones derived from the 1*H*-indole-2,3-dione form polycyclic structures with relatively few compatibility problems. In particular, the presence of different heterocyclic rings fused at the position 3- of the 2-indolinone ring in bio-promicing natural products (the compounds **1-4**; Figure 1) has made spirocyclic 2-indolinone promising for drug discovery. Synthetic analogues of spirocyclic 2-indolinones have a broad therapeutic potential as antioxidants (Ermut, Karalı, Özsoy & Can, 2014 ; Karalı, Güzel, Özsoy, Özbey & Salman, 2010), antiviral (Jiang et al., 2006), anticancer (Altowyan et al., 2022; Abdelmouna et al., 2023) antimalarial (Schwertz et al., 2018; Rottmann et al., 2010), antimicrobial (Akdemir & Ermut, 2013), antidiabetic (Murugan, Anbazhagan & Narayanan,

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2009) and potent nonpeptide inhibitors of p53-MDM2 interaction (Shangary et al., 2008; Zhao et al., 2013) (the compounds **5-7**; Figure 1).

Reactions of 1*H*-indole-2,3-diones with 1,2-disubstituted benzene derivatives have been attempted using different catalysts in different solvents to synthesize these important heterocyclic compounds (Dowlatabadi et al., 2011; Jain, Sharma & Kumar, 2012; Popp, 1969). Data in the literature have shown that different compounds are obtained from the reaction of 1*H*-indole-2,3-diones with 2-aminobenzenethiol, depending on the substitution at the position 1- of 1*H*-indole-2,3-dione. It has been noted that when at the position 1- of 1*H*-indole-2,3-dione is unsubstituted, spirobenzothiazine, indolobenzothiazine and benzothiazinone are obtained, while only the spirocyclic compound is synthesized from the reaction of *N*-methyl-1*H*-indole-2,3-dione (Dandia, Khanna & Joshi, 1990; Joshi, Dandia & Khanna, 1990). In later studies, a single spirobenzothiazole compound was sythesized by the reaction of 1*H*-indole-2,3-dione with 2-aminobenzenethiol in ethanol (Alam & Nawwar, 2002; Karalı et al., 2010; Ermut et al., 2014). In addition, these findings were confirmed by single-crystal X-ray diffraction analyzes of the spirocyclic compounds synthesized from 1*H*-indole-2,3-dione and *N*-methyl-1*H*-indole-2,3-dione derivatives (Figure 2) (Karalı et al., 2010; Akkurt, Karaca, Ermut, Karalı & Büyükgüngör, 2010).

Considering these findings, we synthesized a novel spirocyclic 5-chloro-1,7-dimethyl-2-indolinone bearing a 5-(trifluoromethyl)benzothiazoline moiety and, characterized the crystal and spirocyclic structure of the compound by spectral and single crystal X-ray diffraction analyzes. Moreover, compliance with Lipinski's rule of 5 (RO5) and the ADME criteria was evaluated *in silico* using the Qikprop module. Unlike planar aromatic compounds, non-planar and especially rigid spiro heterocyclic systems exhibit stronger affinity for the three-dimensional regions of proteins that function as biological targets. In our current study, the spirooxindole derivative compound we synthesized is ideal candidate for various biological activities, including antiviral, antioxidant, and anticancer effects. In this context, comparisons with previous studies and similar molecules will be made to demonstrate the significant contribution of this compound.

MATERIALS AND METHODS

Chemistry

2-Amino-4-(trifluoromethyl)benzenethiol and methyliodide are purchased Sigma-Aldrich Chemical Co. (St. Louis, MO). 5-Chloro-7-methyl-1*H*-indole-2,3-dione is purchased Abcr. Devices used in analysis; IR spectra were determined on KBr discs using a Shimadzu IR Affinity-1 FTIR spectrophotometer. Elemental analysis was conducted on a Thermo Finnigan Flash EA 1112 elemental analyzer. ^{1}H NMR, ^{13}C NMR-APT, HSQC-2D and HMBC-2D spectra were obtained on a Bruker Avance III HD (600 MHz). The mass spectrum was confirmed on Agilent Infinity 1260 II LC-MS. The melting points were measured on the Büchi Melting Point B-540 and Büchi Melting Point M-560 instruments.

Synthesis of 1,7-dimethyl-5-chloro-1*H*-indole-2,3-dione (2)

A mixture of 5-chloro-7-methyl-1*H*-indole-2,3-dione (1) (5 mmol) and 0.97 g anhydrous K_2CO_3 (7 mmol) in 10 mL DMF was stirred at room temperature for 1 h. Methyliodide (15 mmol) and 0.17 g KI (1 mmol) as catalyst were added to the mixture and heated at 50-60ºC with refluxing and stirring continuously until the reaction was complete. The mixture was first evaporated to dryness at reduced pressure to give the crude product, then the solid product obtained was washed with water to remove excess K_2CO_3 and KI and purified by crystallisation with ethanol.

Brown powder (yield 91%), M.p.: 171-173 °C. **IR** (KBr) ν max (cm^{-1}): 3064, 3043 (aromatic C-H), 2951, 2889 (aliphatic C-H), 1737, 1687 (C=O), 1616, 1489, 1473 (C=C). **^{1}H NMR** (DMSO-d_6, 600 MHz) δ (ppm): 2.53 (s, 3H, ind. C_7-CH_3), 3.39 (s, 3H, ind. N-CH_3), 7.42 (d, J = 2.3 Hz, 1H, ind. C_6-H), 7.55 (d, J = 2.5 Hz, 1H, ind. C_4-H); **^{13}C NMR-APT** (DMSO-d_6, 126 MHz) δ (ppm): 18.35 (ind. C_7-CH_3), 29.61 (ind. N-CH_3), 119.93 (ind. C_{3a}), 122.05 (ind. C_4), 125.00 (ind. C_7), 127.61 (ind. C_5), 140.50 (ind. C_6), 148.23 (ind. C_{7a}), 159.22 (ind. C_3), 183.20 (ind. C_2).

Synthesis of 5'-chloro-1',7'-dimethyl-5-(trifluoromethyl)-3*H*-spiro[1,3-benzothiazole-2,3'-indol]-2'-on (4)

To a solution of 5-chloro-7-methyl-1*H*-indole-2,3-dione (2) (2.5 mmol) in absolute ethanol (20 mL) was added 2-amino-4-(trifluoromethyl)benzenethiol (3) (2.5 mmol). The mixture was heated in a water bath under a reversing cooler until the reaction was terminated. The product formed was separated by filtration and purified by crystallization from an ethanol- water mixture (Karalı et al., 2010).

Yellow powder (yield 77%), M.p.: 236-238 °C; **IR** (KBr) ν max (cm^{-1}): 3337 (N-H), 3064, 3044 (aromatic C-H), 2936, 2870 (aliphatic C-H), 1705 (C=O) 1599, 1581, 1458 (C=C); **^{1}H NMR** (DMSO-d_6, 600 MHz) δ (ppm): 2.55 (s, 3H, ind. C_7-CH_3), 3.39 (s, 3H, ind. N-CH_3), 6.77 (d, J = 1.8 Hz, 1H, b.t. C_4-H), 6.98 (dd, J = 8.0, 1.8 Hz, 1H, b.t. C_6-H), 7.28 (d, J = 8 Hz, b.t. C_7-H), 7.29 (d, J = 2.2 Hz, 1H, ind. C_6-H), 7.50 (d, J = 2.2 Hz, 1H, ind. C_4-H), 7.69 (s, 1H, b.t. N-H); **^{13}C NMR-APT** (126 MHz, DMSO-d_6) δ (ppm): 18.44 (ind. C_7-CH_3), 29.81 (ind. N-CH_3), 74.48 (spiro C), 104.21 (q, J= 3.6 Hz, b.t. C_4), 116.01 (q, J= 4.1 Hz, b.t. C_6), 121.88 (b.t. C_7), 123.42 (ind. C_7), 123.58 (ind. C_4), 124.94 (q, J= 272.2 Hz, CF_3), 127.22 (q, J= 31.4 Hz, b.t. C_5), 127.33 (ind. C_5), 129.98 (b.t. C_{7a}), 131.90

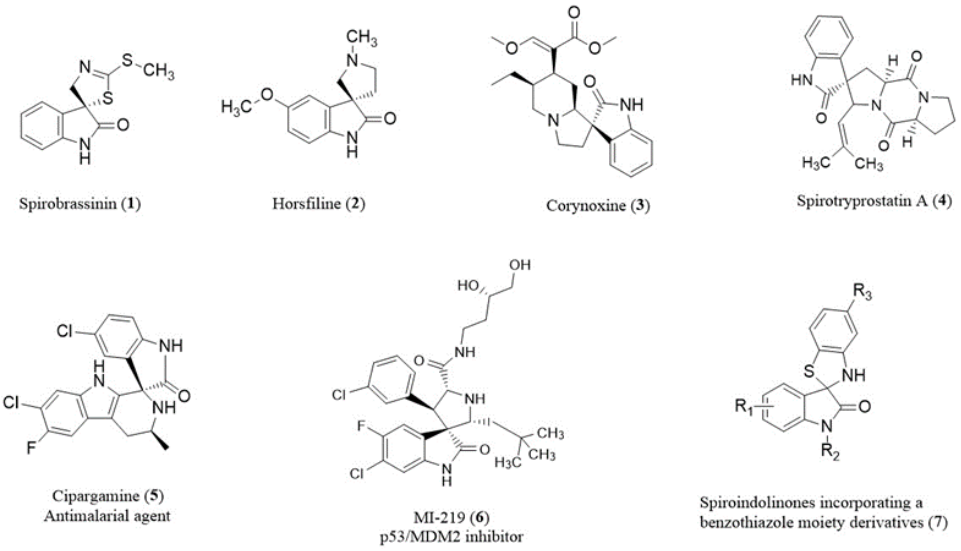

Figure 1. Chemical structures of spirocyclic 2-indolinone derivatives.

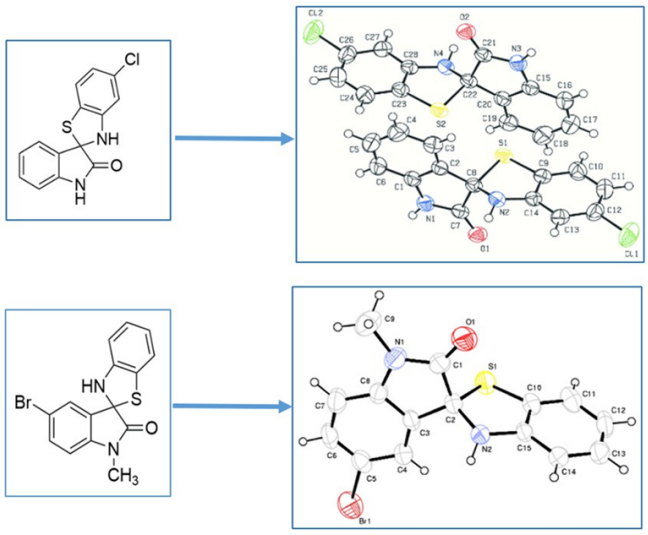

Figure 2. Spirocyclic 2-indolinone derivatives with confirmed crystal structures.

(ind. C_{3a}) , 134.00 (ind. C_6), 139.93 (ind. C_{7a}), 147.94 (b.t. C_{3a}), 175.02 (ind. C_2). **LC-MS (ESI)**: 407.0, 409.0 ([M+Na]$^+$; 100, 38), 385.0, 387.0 ([M+H]$^+$, 38, 13); **Analyses (%) cald for $C_{17}H_{12}ClF_3N_2OS$:** C, 53.06; H, 3.14; N, 7.28; S, 8.33. Found: C, 52.83; H, 3.11; N, 7.43; S, 8.27.

X-Ray Crystal Structure Determination and Refinement

The single-crystal X-ray data were collected on a STOE IPDS II image plate diffractometer at 293 K. Graphite-monochromated MoKα radiation (λ = 0.71073 Å) and the w-scan technique were used. Compound **4** was solved by direct methods using SHELXS-97 (Sheldrick, 1997) and refined through the full-matrix least-squares method using SHELXL-2014 (Sheldrick, 2015), implemented in the WinGX (Farrugia, 1999) programme suite. The non-hydrogen atoms were refined with the anisotropic displacement parameters.

Data collection and cell refinement were carried out using Stoe X-AREA (Stoe & Cie, 2002), while data reduction was conducted using Stoe X-RED (Stoe & Cie, 2002). The general-purpose crystallographic tool PLATON (Spek, 2009) was used for the structure analysis and presentation of the results. The dihedral angles were calculated using the PARST95 programme (Nardelli, 1995).

The hydrogen atom attached to the N2 atom was located using a difference Fourier map, and its coordinates and atomic displacement parameters were refined isotropically (N2-H2 = 0.81(2) Å). All remaining hydrogen atoms were placed geometrically, with C-H distances ranging from 0.93 to 0.97 Å, and Uiso(H) values set to 1.5 Ueq(C) for methyl hydrogen atoms and 1.2 Ueq(C) for other hydrogen atoms.

In silico analyses

Compliance with Lipinski's RO5 and some pharmacokinetic parameters of compound **4** were analysed *in silico* using the Qikprop module in Schrödinger (QikProp, Schrödinger, LLC, New York, NY 2018).

RESULTS AND DISCUSSION

Chemistry

5-Chloro-1,7-dimethyl-1*H*-indole-2,3-dione (**2**) was synthesized by methylation from the position 1- of indole ring with CH_3I of 5-chloro-7-methyl-1*H*-indole-2,3-

dione (**1**) using K_2CO_3 and KI as catalysts in DMF medium. New 5'-chloro-1',7'-dimethyl-5-(trifluoromethyl)-3*H*-spiro[1,3-benzothiazole-2,3'-indol]-2'-one (**4**) was obtained by the reaction of 5-chloro-1,7-dimethyl-1*H*-indole-2,3-dione (**2**) with 2-amino-4-(trifluoromethyl)benzenethiol (**3**) (Figure 3). The structure of the synthesized compound **4** was confirmed by spectral (IR, ^{1}H NMR, ^{13}C NMR-APT, HSQC-2D, HMBC-2D, and LC-MS) and analytical data.

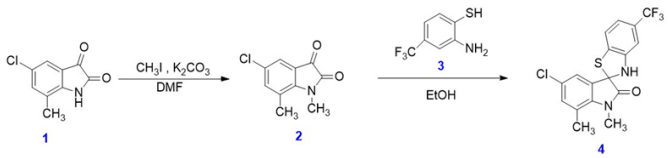

Figure 3. Synthesis of compound **4**.

Although the lactam group does not exhibit a strong nucleophilic character, it has the ability to undergo nucleophilic reactions in the enol form because of its weak acidic properties. K_2CO_3 used as a catalyst separates the proton of lactam and converts 5-chloro-7-methyl-1*H*-indole-2,3-dione (**1**) into the enol form. The anilide nitrogen of isatin, which is nucleophilic, attacks the carbon atom of iodomethane, forming an electrophilic centre. Because of this attack, a new bond is formed between the methyl carbon and the anilide nitrogen atoms and the iodide is separated to give 5-chlorine-1,7-dimethyl-1*H*-indole-2,3-dione (**2**). The condensation reaction between the amino group at the 2-amino-4-(trifluoromethyl)benzenethiol (**3**) and the ketone carbonyl of 5-chloro-1,7-dimethyl-1*H*-indole-2,3-dione (**2**) starts with the nucleophilic attack. In the intermediate product formed, (-) charged alkoxide group attacks the (+) charged amino group. This leads to the formation of the intermediate 3-hydroxy-3-anilino-5-chloro-1,7-dimethyl-1,3-dihydro-2*H*-indol-2-one, and by the elimination of a water molecule, 3-phenylimino-5-chloro-1,7-dimethyl-1,3-dihydro-2*H*-indol-2-one is formed. The 4-(trifluoromethyl)-benzenethiol sulfhydryl group attacks the imine carbon to form a new bond between the sulphur and carbon atoms. The (+) charged thioether group at the spirobenzothiazoline intermediate is neutralized by attracting electrons towards itself, while (-) charged secondary amine attacks the thioether proton. As a result of this reaction, compound **4** was obtained (Figure 4) (Dandia et al., 2006).

In the IR spectrum of compound **2**, ketone and lactam C=O stretching bands were observed at 1737 and 1687 cm^{-1}, respectively. The spectrum of compound **4** showed absorption bands at 3337 and 1705 cm^{-1} resulting from the benzothiazoline NH and lactam C=O functions, respectively, and the ketone C=O band was not observed (Allam & Nawwar, 2002; Castineiras, Gómez & Sevillano, 2000). The benzothiazoline NH proton signal was observed as a singlet at δ 7.69 ppm in the ^{1}H NMR spectrum of compound **4**. The benzothiazoline C_4 and C_6 proton signals due to the more shielding effect of the amine group on the protons at the positions *o*- and *p*- of the phenyl

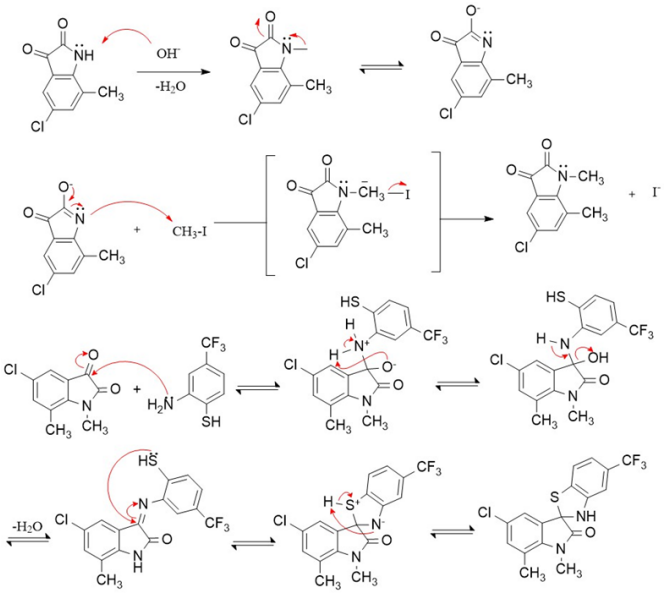

Figure 4. Possible synthesis mechanism of compound 4.

ring were determined at δ 6.77 (doublet, *m*-pairing) and 6.98 (doublet doublet, *o*- and *m*- pairings) ppm, respectively. The benzothiazoline C_7 proton resonated more downfield than the benzothiazoline C_4 and C_6 protons as a result of the deshielding effect of the thioether and trifluoromethyl groups and signaled as a doublet (*o*-pairing) at δ 7.28 ppm. The chemical shift values of the phenyl protons of 3-(trifluoromethyl)aniline (SDBS 3865) and the values calculated with the shift parameters of sulphur and amine groups confirmed the chemical shift values of the detected benzothiazoline ring protons and the substituent effects (Laatsch, Thomson & Cox, 1984; Santes, Rojas-Lima, Santillan & Farfàn, 1999; Ermut et al., 2014). In the spectrum of compound **2**, the indole C_4 and C_6 protons were observed as doublets (*m*- pairing) at δ 7.55 and 7.42 ppm, respectively. Whereas, in the spectra of compound **4**, indole C_4 and C_6 protons were shown as doublets (*m*- pairing) at δ 7.50 and 7.29 ppm, respectively. Methyl protons at positions 1- and 7- of the indole ring of compound 4 were observed as singlet at δ 3.39 and 2.55 ppm, respectively (Figure 5).

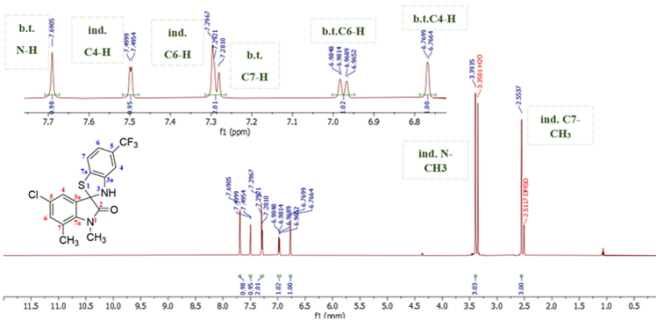

Figure 5. ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **4** (δ 0-12 ppm).

In the ^{13}C NMR-APT spectrum of compound **4**, signals (OCF_3, benzothiazoline C_5, C_6, and C_4) that showed the ^{13}C-^{19}F coupling were displayed as a quartet. These signals resonated at δ 124.94, 127.22, 116.01 and 104.21 ppm, respectively. The C_{3_a} signal, which resonates at the lowest field among the benzothiazoline carbons, was detected at δ 147.94 ppm, while the benzothiazoline C_{7_a} signal was seen at δ 129.98 ppm. The 2-indolinone C=O (δ 175.02 ppm), C_{7_a} (δ 139.93 ppm), C_6 (δ 134.00 ppm), C_{3_a} (δ 131.90 ppm), C_5 (δ 127.33 ppm), C_4 (δ 123.58 ppm), and C_7 (δ 123.42 ppm) signals were determined. Methyl protons at positions 1- and 7- of the indole ring of compound 4 showed at δ 29.81 and 18.44 ppm, respectively (Figure 6). The selected ^1H and ^{13}C NMR-APT chemical shift values (ppm) of compound **4** are shown in Figure 7.

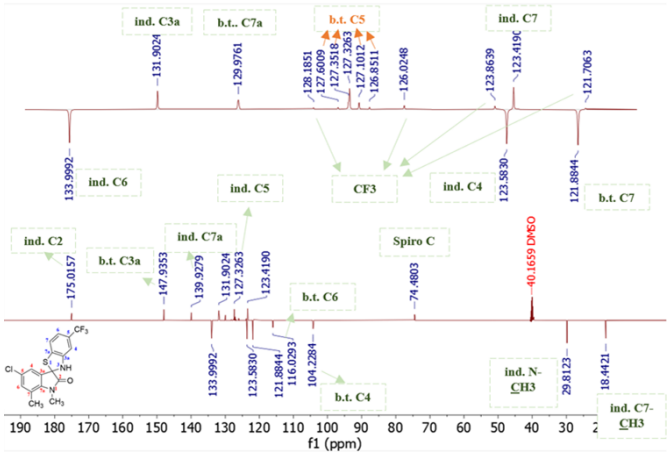

Figure 6. ^{13}C NMR-APT (126 MHz, DMSO-d_6) spectrum of compound **4** (δ 0-210 ppm).

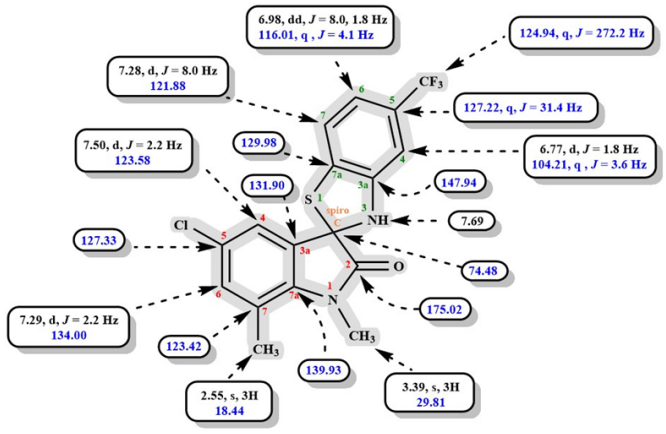

Figure 7. Selected ^1H and ^{13}C NMR-APT chemical shift values (ppm) of compound **4.**

Further verification was obtained from the HSQC-2D spectra of compound **4**, which clearly show the ^1H–^{13}C connections and allow definite assignment of the ^1H and ^{13}C resonances (Figure 8). The presence of spiro C (δ 74.48 ppm) in the ^{13}C NMR-APT and HMBC-2D spectra is evidence of a spirocyclic structure. These data are consistent with spiroindolinone studies in the literature (Figures 6 and 9) (Dandia et al., 1990; Dandia et al., 2004; Ermut et al., 2014; Karalı et al., 2010; Naumov & Anastasova, 2001).

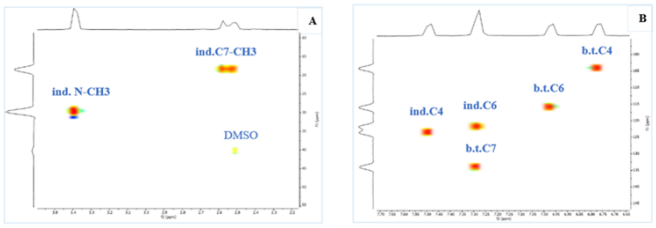

Figure 8. HSQC-2D (600 MHz, DMSO-d_6) spectra of compound **4** (**A**. δ 10-55 ppm; **B.** δ 90-150 ppm).

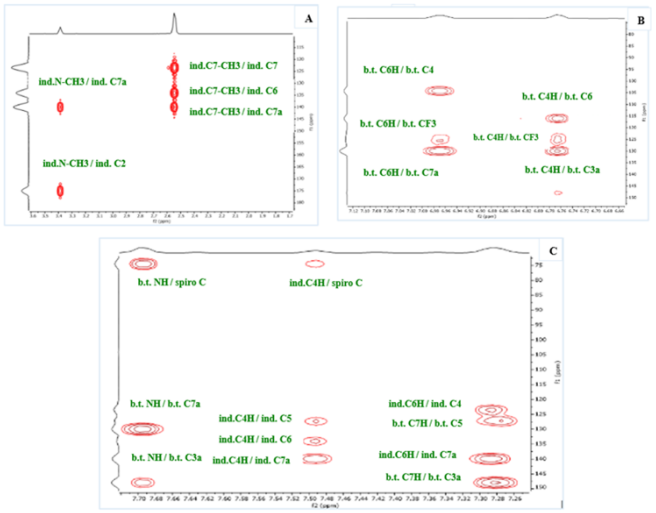

Figure 9. HMBC-2D (600 MHz, DMSO-d_6) spectra of compound **4** (**A**. δ 1.7-3.6 ppm; B. δ 6.65 – 7.12 ppm; C. δ 7.26-7.70 ppm).

When the HMBC-2D spectrum of compound **4** was examined, the interaction of the indole N-CH_3 protons with the indole C_{7_a} and C_2 carbons and the interaction of the indole C_7-CH_3 protons with the indole C_7, C_6 and C_{7_a} carbons were observed (Figure 9A). The benzothiazoline C_6 proton interacted with the benzothiazoline C_4, CF_3, and C_{7_a} carbons and the C_4 proton of benzothiazoline interacted with the benzothiazoline C_6, CF_3, and C_{3_a} carbons. (Figure 9B). The interaction of the spiro C signal with the benzothiazoline NH and indole C_4 proton signals supports the accuracy of the spiroindolinone structure. Furthermore, benzothiazoline NH interacted with benzothiazoline C_{7_a} and C_{3a} carbons, while the indole C_4 proton interacted with indole C_5, C_6, and C_{7_a} carbons. Additionally, it was determined that the indole C_6 proton interacted with the indole C_4 and C_{7_a} carbons, while the benzothiazoline C_7 proton interacted with the benzothiazoline C_5 and C_{3_a} carbons (Figure 9C).

In the LC-MS spectrum of compound **4** taken by positive ionization technique, the base peak was $[M+Na]^+$ (m/z 407.0) ion, and $[M+H]^+$ (m/z 385.0) peak was confirmed its molecular weight. Additionally, $[M+Na]^+$+2 and $[M+H]^+$+2 peaks resulting from ^{35}Cl and ^{37}Cl isotopes of the chlorine atom at the position 5- of the 2-indolinone ring were also observed in the spectrum at relative abundance ratios of 100:38 and 38:13, respectively.

X-ray single-crystal diffraction analysis

ORTEP 3 (Farrugia, 1997) drawing with atom numbering obtained by X-ray analysis of compound **4** confirms its molecular structure and atom connectivity, as depicted in Figure 10. Compound **4** consists of an indole ring [N1/C3-C10] and benzothiazole [S1/N2/C11-C16] ring connected to the trifluoromethyl group. The atoms O1 and C/1 are positioned at distances of -0.0683(16) and 0.0984(7) Å, respectively from the least square plane defined by all the atoms of the indole ring. In the molecule, the indole ring system is planar, with minimal deviations from the mean plane: -0.0269 Å for the C7 and 0.0259 Å for the C5 atoms, respectively. However, the indolinone group is not planar, and the O1 atom deviates from planarity by -0.068(2) Å. The benzothiazole ring exhibited non-planar characteristics, with the C10 atom deviating from the mean plane of S1/C12/C11/N2 by 0.518(2) Å. The indole ring system [N1/C3-C10] is at a dihedral angle of 89.48(5)° relative to the mean plane of the S1/N2/C11-C16 moiety, suggesting a near-perpendicular orientation between the indole ring [N1/C3-C10] and the benzothiazole ring system.

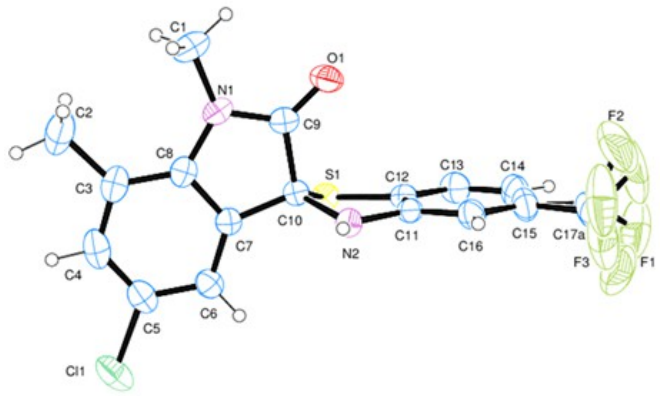

Figure 10. HMBC-2D (600 MHz, DMSO-d_6) spectra of compound **4** (View of compound **4** with the atom numbering scheme. Displacement ellipsoids for non-H atoms are drawn at the 30% probability level.

The structure exhibited disorder in the trifluoromethyl group connected to C17, with occupancy factors of 0.61(2) for component one and 0.39(2) for component a. The C17 and F atoms were found at two distinct positions, constrained by the SAME restriction. Despite this separation into two positions, the CF_3 group demonstrates continuous positional disorder, as evidenced by the significant thermal vibration parameters. The C17-F_3 bond lengths vary notably, ranging from 1.259 to 1.325 Å for one disordered component and from 1.260 to 1.315 Å for component a.

The crystal structure of compound **4** is stabilized by both intra- and intermolecular hydrogen bonds. The O1 atom within the indolinone moiety forms an intra-molecular hydrogen bond interaction [C1···O1 2.842(3) Å, C1-H1B 0.96(3) Å, and C1-H1B···O1 104.7(2)°]. The intermolecular hydrogen bond of N···O type between adjacent molecules is oriented along the c axis [N2···O1i 3.172(2) Å, N2-H2 0.81(2) Å, and N2-H2···O1i 150(2)° with symmetry code: (i) x, 1/2-y, 1/2+z]. Figures 11 and 12 show the intermolecular N-H···O hydrogen bonding interactions along the c axis, and Figure 12a shows the layers of molecules that are parallel to the (010) plane. A view of the crystal packing and hydrogen bonding of compound **4** along the a axis (a), b axis (b), and c axis (c) is provided in Figure 12a-c.

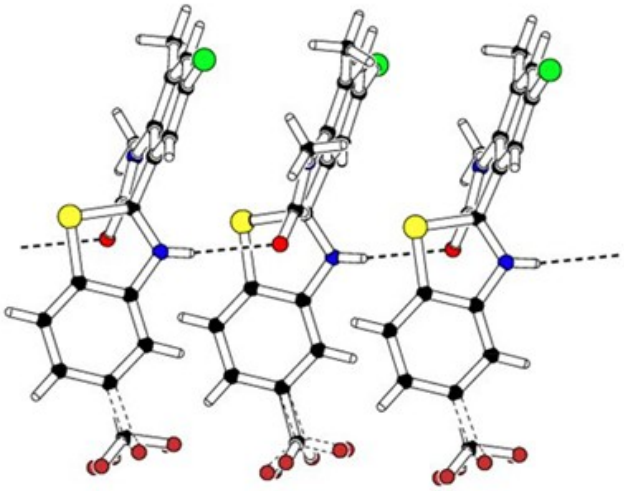

Figure 11. A view of N-H···O hydrogen bonding interactions of compound **4.**

All bond lengths and angles were within normal ranges and matched those documented in prior studies (Karalı et al., 2010; Akkurt et al. 2010). The torsion angle N1-C9-C10-N2 is measured at -132.3(2)°, indicating an -anti-clinal (-ac) conformation, while the C7–C10–N2–C11 torsion angle measures 156.9(2)°, indicating an antiperiplanar (ap) conformation. Details of the data collection conditions and the parameters of the refinement process are given in Table 1. The selected bond lengths and angles are presented in Table 2.

In silico analyzes

A factor considered for the synthesised compounds to be potential drug candidates is whether they have drug-like proper-

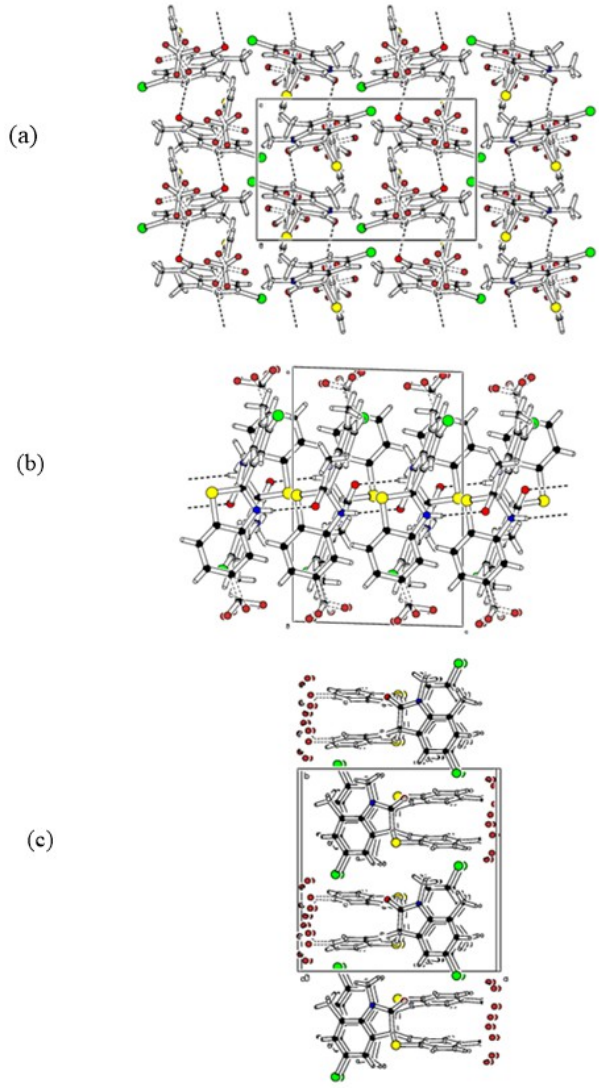

Figure 12. A view along the a axis (a), b axis (b), c axis (c) of the crystal packing and hydrogen bonding of compound **4.**

Table 1. Crystal data and structure refinement parameters for compound **4.**

Crystal data	
Chemical formula	$C_{17}H_{12}C/F_3N_2OS$
Formula weight	384.80
Crystal system, Space group	Monoclinic, $P2_1/c$
Temperature (K)	293(2)
***a*, *b*, *c* (Å)**	13.667(3), 14.063(2), 9.1013(19)
***β* (°)**	91.742 (17)
***V* (Å^3)**	1748.4 (6)
Z	4
Radiation type	Mo Kα
***μ* (mm^{-1})**	0.375
Crystal size (mm)	0.790 × 0.503 × 0.260
Data collection	
Diffractometer	STOE IPDS 2
Absorption correction	Integration
T_{min}, T_{max}	0.758, 0.906
No. of measured, independent and observed [I > 2σ(I)] reflections	13628, 3894, 2608
Rint	0.0731
(sin θ/λ)max (Å^{-1})	0.646
Refinement	
R[F^2 > 2σ(F^2)], wR(F^2), GOF	0.0427, 0.0930, 0.965
No. of reflections	3894
No. of parameters	269
No. of restraints	10
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.285, −0.324

Table 2. Selected bond lengths and angles of compound **4.**

Bond lengths (Å)			
S1–C10	1.8508(18)	C7–C8	1.395(3)
S1–C12	1.767(2)	N1–C8	1.423(3)
C11–C12	1.412(3)	N1–C9	1.361(3)
N2–C11	1.393(2)	N1–C1	1.457(3)
N2–C10	1.448(3)	O1–C9	1.221(2)
C10–C9	1.546(3)	C*l*1–C5	1.742(2)
C7–C10	1.507(3)	N2–H2	0.81(2)
Bond angles (°)			
C12–S1–C10	90.00(8)	O1–C9–N1	126.59(18)
N2–C10–S1	103.29(12)	O1–C9–C10	125.28(18)
C11–N2–C10	113.09(15)	C9–N1-C8	111.19(15)
O1–C9–C10	125.28(18)	C9–N1–C1	120.34(18)
N1–C9–C10	108.12(16)	C4–C5–C*l*1	119.03(18)
C4–C3–C2	119.2(2)	C6–C5–C*l*1	119.82(19)

ties. Lipinski's RO5 has traditionally been used for many years to evaluate the oral bioavailability and drug-like properties of compounds (Lipinski, Lombardo, Dominy & Feeney, 1997). According to Lipinski's RO5, a candidate molecule must fulfil these criteria in order to be orally usable and to have drug-like properties, and it is expected that at most one rule is not complied with. To evaluate the suitability of compound **4** to Lipinski's RO5, a comprehensive *in silico* study was performed using the Qikprop module (Schrödinger, LLC, New York, NY 2018), focusing on various physicochemical parameters.

To evaluate the suitability of compound **4** to Lipinski's RO5, a comprehensive *in silico* investigation was carried out using the Qikprop module (Schrödinger, LLC, New York, NY 2018), focusing on various physicochemical parameters and ADME criteria. The calculation of the molecular weight of compound **4** as 384.80 and logP value as 3.88 shows compliance with the criteria. In addition, it is predicted that compound **4** will have high biological activity with the determination of hydrogen bond donor and acceptor numbers as 1.0 and 4.5, respectively, which is a measure of its capacity to interact with target systems. With zero violations of the Lipinski's RO5, compound **4** demonstrated potential as a drug candidate (Table 3).

The water solubility of compounds is expressed by the "LogS" value and is extremely important for the oral absorption capacity of the drug (Di, Fish & Mano, 2012). When the logS value, which should be in the range of (-6.5)-0.5, was examined, this value was calculated as -4.902 for compound **4**. LogBB defines the blood-brain barrier partition coefficient and CNS defines the central nervous system activity. The CNS value should be in the range of (-2)-2 and LogBB value should be in the range of (-3)-1.2. The CNS activity of the compound whose LogBB value is close to negative values and CNS value

Table 3. Compatibility of compound **4** with Lipinski's RO5.

Molecular weight	QPlogP octanol/water	Number of hydrogen bond donors	Number of hydrogen bond acceptors	Number of RO5 not complied with
Ref. <500	**<5**	**<5**	**<10**	**≤1**
384.80	3.88	1	4.5	0

Table 4. Compatibility of compound **4** with some physicochemical parameters and ADME criteria.

QLogS [a]	QLogBB [b]	CNS [c]	QLogKhsa [d]	PMDCK [e]	Rot [f]	HOA(%) [g]
Ref. (-6.5)-0.5	**(-3)-1.2**	**(-2)-2**	**(-1.5)-1.5**	**<25 poor >500 very good**	**≤3**	**>80 high activity <25 weak activity**
-4.902	0.977	-2	0.509	6212.441	0	100

[a] Solubility in water
[b] Blood-brain barrier permeation capacity
[c] Central nervous system activity
[d] Binding rate to human serum albümin
[e] MDCK cell permeability
[f] Number of rotatable bonds
[g] Percentage of human oral absorption

is close to -2 decreases, thus it has difficulty in crossing the blood-brain barrier. The LogBB value of compound **4** was calculated as 0.977 and the CNS value as -2. This may help to reduce side effects and is important for the safety of the candidate compound. The logKhsa value, which describes the binding rate to human serum albumin, should be between (-1.5) and (1.5). This value was measured as 0.509 and fulfilled these criteria. Madin-Darby Canine Kidney (MDCK) cell permeability (PMDCK) helps assess the ability of a compound to pass through the cell membrane, aiding in determining the drug's bioavailability and efficacy. If this value is less than 25, it indicates poor cell permeability, while more than 500 signifies excellent cell permeability. For compound **4**, this value was calculated as 6212.441. The number of rotatable bonds (Rot) of compound **4** was determined to be zero. This indicates that the molecule is in a rigid structure. The percentage of oral absorption in men (HOA) was defined as > 80 for high oral activity and < 25 for poor oral activity. The calculation of this value as 100 for compound **4** indicates that the compound **4** has high oral absorption (Table 4).

CONCLUSION

The new 5'-chloro-1',7'-dimethyl-5-(trifluoromethyl)-3*H*-spiro[1,3-benzothiazole-2,3'-indol]-2'-on (**4**) was synthesized in good yield. The molecular, spirocyclic and stereoisomeric structure of the compound **4** was determined by spectral and X-ray single crystal diffraction analyses. In the crystal, both intra- and intermolecular hydrogen bonds stabilize the molecular packing. The intermolecular N-H···O hydrogen bonding interactions reveal layers of molecules that are parallel to the (010) plane. Additionally, in silico analyses showed that compound **4** has the appropriate biochemical properties for the desired pharmacokinetics and reduced toxicity in terms of drug-ability criteria.

Supplementary material

CCDC-2335656 contains the supplementary crystallographic data for the compound reported in this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/ retrieving.html [or from the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44(0)1223 336033; e-mail: deposit@ccdc.cam.ac.uk]. Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.saa.2013.08.054.

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Conflict of Interest: The authors have no conflict of interest to declare.

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Original Article

Optimization and validation of HPLC methods for *in vitro* and *ex vivo* analyses of bosentan monohydrate in FDA-recommended and biorelevant media

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ABSTRACT

Background and Aims: Bosentan (BOS) is an endothelin receptor antagonist indicated for the treatment of pulmonary arterial hypertension. It is a BCS Class II drug. This study aimed to apply and method validation the new HPLC methods for FDA-recommended and biorelevant media. These methods were used to assess the quantification of BOS in *in vitro* and *ex vivo* studies on lipid-based drug delivery systems (BOS-loaded self-nanoemulsifying drug delivery systems (SNEDDS), and solid self-nanoemulsifying drug delivery systems (S-SNEDDS)) compared to commercial products (Tracleer®). *In vitro* studies include assessments of content uniformity and dissolution in FDA-recommended and biorelevant media. The stability of S-SNEDDS tablets was evaluated in an FDA-recommended medium. The *ex vivo* study assessed the permeability of BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets in biorelevant media.

Methods: HPLC was operated using buffer solution: acetonitrile (45:55) with a flow rate of 1.5 mL/min at 220 nm. The injection volume was set at 100 µL. Separation was carried out using a Waters XSelect® HSS C18 column (250x4.6 mm, 5µm) at 25°C.

Results: HPLC methods were validated using ICH Q2(R2) and FDA guidelines. Retention times were found to be between 4.7 and 5.5 in different media. The validated methods were proved to be sensitive, simple, reproducible, rapid, and precise for determining BOS in pharmaceutical formulations and dosage forms.

Conclusion: These new HPLC methods were successfully applied and validated for FDA-recommended and biorelevant media *in in vitro, ex vivo*, and quality control tests of BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets.

Keywords: Bosentan monohydrate, HPLC, FDA-recommended and biorelevant media, Pharmaceutical dosage forms, Pharmaceutical formulations

INTRODUCTION

Bosentan monohydrate (BOS) is the first endothelin receptor antagonist to be approved for the treatment of pulmonary arterial hypertension (PAH) treatment (McLaughlin et al., 2005). Oral bosentan (Tracleer®) was approved for the treatment of pulmonary hypertension by the Food and Drug Administration (FDA) on November 20, 2001, and by the European Medicines Agency (EMA) on May 15, 2002. By blocking endothelin receptors, it acts as a vasodilator and neurohormonal blocker, improving left ventricular performance, reducing cardiac vascularization, and improving survival (Ioselevich, Nogid, & Rozenfeld, 2001).

BOS (Ro 47-0203), a substituted pyrimidine derivative without chiral centers, was developed by Hoffman-La-Roche (Basel, Switzerland) in 1994 (Ono & Matsumori, 2002; FDA, 2003). It is a solid, yellowish-white powder. It is very stable in the solid state, non-hygroscopic, does not show polymorphism, and is not affected by light. Slightly soluble in water (1.0 mg/100 mL). It has low solubility in low pH aqueous solutions (e.g., at pH 1.1 and 4.0: 0.1 mg/100 mL, at pH 5.0: 0.2 mg/100 mL) and at pH 7.5, its solubility is 43 mg/100 mL (FDA, 2003). BOS is slightly soluble in hexane, isopropanol, and methanol, soluble in ethyl acetate and ethanol, and freely soluble in dichloromethane and acetone (EMA, 2005). Other physicochemical properties are given in Table 1.

Ultraviolet (UV) spectrophotometric, high-performance thin-layer chromatography (HPTLC), and tandem mass spectrometry (LC-MS/MS) methods have been investigated in different studies for the analysis of BOS from bulk samples, tablet

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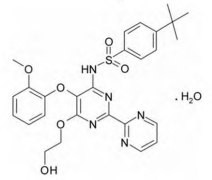
Table 1. Chemical structure and other physicochemical properties of BOS

Chemical structure and chemical name	Molecular formula	Molecular weight (g/mol)	Melting point	log P	pKa	log D
4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2 methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl]benzene-1-sulfonamide monohydrate* 	$C_{27}H_{29}N_5O_6S•H_2O$	569.64**	115°C***	3.1**** (pH 4)	5.5*****	1.3**** (pH 7.4)

* Kaur et al., 2013
** Cohen, Chahine, Hui & Mukherji, 2004
*** Jadhav & Pore, 2017
**** EMA, 2005
*****Roux, Breu, Ertel & Clozel, 1999

dosage forms, pharmaceutical formulations, and in vivo samples (Atila et al., 2014; Marolia, Shah, Bodiwala, Prajapati, & Jariwala, 2015). For BOS, UV spectrophotometry (Das, Narendra, Kumar, & Annapurna, 2010; Kumar, Kumar, & Sankar, 2011; Kumar, Sreenivas, Samal, Dey, & Priyanka, 2011; Narendra, Deepika, & Annapurna, 2012), HPLC (Jadhav et al., 2011; Lavudu, Rani, Chander, & Sekaran, 2013; Jatczak et al., 2016), and LC-MS/MS (Qiu, Zhao, Wang, Xu, & Xu, 2014) methods have been reported in the literature. However, most analytical studies use mass spectrometry and HPLC with UV detection as the methods used for the separation of BOS, its degradation products, and its metabolites in human plasma (Parekh, Shah, Sanyal, Yadav, & Shrivastav, 2012). The HPLC method was optimized using the USP Pending Monograph Version 1-Bosentan (USP, 2012). Since the desired results could not be obtained when using the monograph method, the method was adapted again by changing the column temperature (25°C) and mobile phase ratio (Acetonitrile: Buffer (55:45) in the current monograph method.

The *in vitro* media used in these studies vary, however there are not many studies on the formulation of BOS. The first source for dissolution studies is the FDA dissolution database. Dissolution study evaluations need to be conducted based on the information provided. However, there is no study in the literature on the specific quantitative analysis of BOS in biorelevant media, nor is there any application and method validation for this database. In our previous publications, the data regarding the application and method performed using HPLC were not discussed in detail. This publication is important in terms of providing guidance to people who want to perform their work in different media and drug content analyses, especially in formulation studies on BOS.

This study aimed to apply method validation specific, accurate, sensitive, and rapid HPLC method procedures for the determination of BOS based on the ICH Q2(R2) and FDA guidelines for the validation of analytical methods guidelines (ICH Q2(R2), 2023; FDA, 2001). FDA-recommended dissolution medium (1% SLS in distilled water) and biorelevant media (Fed State Simulating Intestinal Fluid (FeSSIF and its second version FeSSIF-V2) and Fasted State Simulating Intestinal Fluid (FaSSIF and its second version FaSSIF-V2)) were used as dissolution media for in vitro studies. In this respect, the present work is innovative because an HPLC method has not been developed for these media. These methods performed BOS analysis on pharmaceutical dosage form (SNEDDS, S-SNEDDS tablet formulations) samples (Table 2). All analyses were performed and compared with reference tablets (Yılmaz Usta, Timur, & Teksin, 2022; Yılmaz Usta, Olgac, Timur, & Teksin, 2023).

MATERIALS AND METHODS

Chemicals and reagents

Bosentan monohydrate was kindly supplied by Abdi İbrahim (Türkiye). Sodium lauryl sulfate, acetonitrile, and triethylamine were purchased from Merck (Germany). Phosphoric acid and methanol were obtained from Sigma-Aldrich (France). Biorelevant powder was purchased from Biorelevant.com (UK). All the reagents and chemicals were HPLC grade. The reference product is a Tracleer® 125 mg film-coated tablet (Johnson & Johnson, Switzerland) (Expiration date 10/20, Lot number IW067A0401).

HPLC method development and validation for BOS in in vitro and ex vivo samples

Instrumentation

The chromatographic system consisted of an Agilent Technologies Infinity Series 1220 LC (Germany) equipped with a UV diode array detector. XSelect® HSS C18, 5µm, 250 x 4.6 mm (Waters, Ireland) was used for chromatographic separation. The column was 25°C. Separation was carried out with the mo-

Table 2. HPLC methods

	HPLC				
	In vitro Dissolution studies		*Ex vivo* Permeability studies		Drug content analysis
	FDA-recommended medium	Biorelevant media	FDA-recommended medium	Biorelevant media	FDA-recommended medium
S	✓	✓	✓	✓	
S-ST	✓	✓	✓	✓	✓
RT	✓	✓	✓	✓	✓

S: SNEDDS, **S-ST:** S-SNEDDS tablet, **RT:** Reference tablet, **FDA-recommended medium:** 1% SLS in distilled water, **Biorelevant media:** FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2
SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

bile phase consisting of a buffer solution (1 mL of triethylamine added to 1 L of distilled water and the solution adjusted to a pH of 2.5 with phosphoric acid) and acetonitrile (45:55) at a flow rate of 1.5 mL min^{-1}. The Millipore 0.45 µm nylon filter was used for the filtration of the mobile phase and was degassed by sonication. The injection volume was set at 100 µL. The wavelength was adjusted to 220 nm. Chromatographic data were obtained from the peak area, which was automatically integrated using Agilent ChemStation software.

Preparation of stock solution

Stock solutions of BOS with concentrations of 40 µg/mL were prepared by dissolving 2 mg of BOS in 1% SLS and biorelevant media. Biorelevant media were prepared according to the manufacturer's instructions available on biorelevant.com (Biorelevant Media Prep Tool, 2022). These stock solutions were used to prepare working standard solutions with concentrations ranging from 0.0195–10 µg/mL for 1% SLS media, 0.5–20 µg/mL for all biorelevant media by appropriate dilutions. The Sartorius 0.45 µm nylon membrane filter was used for the filtration of all samples. Nylon filter membranes are composed of polyamide polymer filters with different pore sizes, and it is characterized by strong resistance to organic and alkali reagents, large specific surface area, and good permeability (Yue, Zhou, Peng & Zhao, 2022).

Preparation of the calibration curve

Calibration curves were generated using solutions of varying concentrations. The calibration curve was then plotted for 12 concentrations in the range of 0.0195–10 µg/mL for 1% SLS in distilled water, 0.5–20 µg/mL for all biorelevant media.

Method validation

Linearity: To evaluate the linearity parameter, three different stock solutions of BOS in 1% SLS and biorelevant media were prepared. Twelve different concentrations were prepared and injected into HPLC by making appropriate dilutions from these stock solutions. Three analyses were performed at each concentration. Peak areas corresponding to the concentrations were observed. The statistical parameters were calculated.

Precision: The interday precision (reproducibility) was determined on three different days at three different levels (0.625, 5, and 10 µg/mL for 1% SLS), (5, 10, and 16 µg/mL for FaSSIF and FaSSIF-V2), (6, 10, and 16 µg/mL for FeSSIF and FeSSIF-V2), and the intra-day precision (repeatability) study 10 different solutions of the same concentration (0.625 µg/mL for 1% SLS, 10 µg/mL for all biorelevant media) were prepared and analyzed three times in a day. Precision was evaluated using the mean, standard deviation, and relative standard deviation (RSD).

Accuracy (Recovery): Recovery studies were performed on different amounts (80%, 100%, 120%) of bulk BOS samples within the linearity range. The RSD% values were found to be less than 2%, indicating that the method is accurate.

Specificity: The specificity was checked to determine whether the excipients in the formulation showed absorbance at the same wavelength. For this purpose, the method specificity was evaluated by comparing the chromatograms of the BOS, media, and formulation components with those of the blank.

Limit of detection (LOD) and limit of quantification (LOQ): The LOD represents the lowest quantity level of an analyte in the sample. The LOQ represents the lowest quantity level reliably provided for a given signal-to-noise. The standard deviation of the intercepts and mean slope of the calibration curves of BOS were calculated for the LOD and LOQ of the developed method. The results demonstrate the sensitivity of the proposed method. The following equations were used to calculate LOD and LOQ:

$$\text{Detection limit} = 3.3\alpha/S \quad (1)$$

$$\text{Quantification limit} = 10\alpha/S \quad (2)$$

α and S are the response's standard deviation and the calibration curve's mean slope of the calibration curve, respectively.

Stability: The stability of the sample solutions for all media at 4°C, 25°C, and 37°C at 0 and 24 h was investigated. To evaluate the stability 10 µg/mL solutions of BOS were used in all media. Samples were analyzed by HPLC after 0 and 24 h.

Stability studies

The stability of S-SNEDDS tablets loaded with BOS was assessed under three conditions: 4°C, 25±2°C/60±5%, and 40±2°C/75±5%. The BOS quantity was assessed at 0, 1st, 3rd,

6th, and 12th months (Yılmaz Usta et al., 2023). The nine tablets selected at random were powdered, and they (equivalent to 30 mg of BOS) were accurately weighed. A 100 mL volume of 1% SLS in distilled water was added, and the mixture was mixed for 1 h in a magnetic stirrer. The samples were diluted to 20 µg/mL and filtered through a 0.45 µm nylon filter, and HPLC was used for analysis.

Dissolution studies

The *in vitro* dissolution studies were conducted using a USP apparatus II (Agilent 708-DS, USA) at 50 rpm at 37±0.5°C in 900 mL. The BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets were placed in 900 mL of biorelevant media and distilled water containing 1% SLS (Yılmaz Usta et al., 2022; Yılmaz Usta et al., 2023). The withdrawn samples of 5 mL were filtered using a 0.45 µm nylon filter, and samples were analyzed at 220 nm by HPLC. The percentages of cumulative amounts were evaluated.

Permeability studies

Franz diffusion cells were used in ex vivo permeability study. Biorelevant media were used as permeation media. The BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets were diluted to 1 mL using the appropriate media (1 mL of the samples equivalent to 7.5 mg BOS) (Yılmaz Usta et al., 2022; Yılmaz Usta et al., 2023). SNEDDS was used directly. Tests were carried out at 37°C for 24 h (60, 90, 120, 240, 360, 480, 600, and 1440 min). The samples of 2.5 mL were filtered using a 0.45 µm nylon filter, and samples were analyzed at 220 nm by HPLC. The flux (J) and permeability coefficient (P) values were calculated using Equations 3 and 4 as follows:

$$J = dQ/Adt(g/cm^2min) \quad (3)$$

Q, A, and t stand for substance crossing the goat intestine membrane, the contact area of the membrane, and the time of exposure, respectively.

$$P = J/C_o \text{ (cm/min)} \quad (4)$$

Where C_o is the initial drug concentration in the donor compartment and J is the flux value.

RESULTS AND DISCUSSION

HPLC method development and validation for BOS in *in vitro* and *ex vivo* samples

According to the ICH Q2(R2) guidelines, *in vitro* analysis methods should demonstrate that the relationship between concentration and peak area is linear. For accuracy, recovery, and precision, the RSD% should be below 2%, and it should be proven that it does not peak at the working wavelength for specificity and selectivity. The stability should be confirmed by stability studies at different temperatures (4°C, 25°C, and 37°C). The analysis methods developed to quantify BOS are described below.

Method validation

Linearity: The linearity of the method is the expression that the concentration of the active substance in the prepared sample is directly proportional to the concentration within a specific value range. At least five concentrations should be used to achieve linearity (ICH Q2(R2), 2023). Linearity was evaluated by plotting the concentration (x-axis) against the peak area (y-axis). The calibration curve showed higher regression coefficients (r^2>0.999) for all media. All calibration curves are shown in Figure 1. The other characteristic of the linearity results for BOS is given in Table 3.

Precision: Precision refers to the degree of closeness between successive measurements of a method. Intra-day (repeatability) and interday precision (reproducibility) studies were conducted to determine the precision of the analytical method. The RSD% values were found to be less than 2% (except for the four values in Table 5), which shows the developed method's precision. The results are summarized in Tables 4 and 5.

Accuracy (Recovery): The accuracy of an analysis method is the closeness of the value measured using that method to the known concentration value. The accuracy of the assay method depends on the percent recovery obtained by analyzing samples with known concentrations. The recovery of all media was within the acceptance criteria of 80.0–120.0%. The recoveries (1% SLS in distilled water: 97.7% to 101%, FaSSIF: 95.3% to 103%, FeSSIF: 97.0% to 103, FaSSIF-V2: 92.4% to 101%, FeSSIF-V2: 97.0% to 101%) of BOS were obtained at each concentration. The accuracy results are shown in Table 6.

Specificity: Specificity and selectivity refer to the ability to correctly distinguish the substances to be analyzed in the presence of other substances in the matrix, and the analysis method used should only detect the substance to be analyzed (Thompson, Ellison, & Wood, 2002). The method specificity was verified by comparing the chromatograms of the drug, media, and formulations (without active substance) with those obtained from the blank. There was no peak observed at the retention time of the active substance in the blank formulation samples or the media. Chromatograms are presented in Figures 2 and 3.

LOD and LOQ: The LOD and LOQ were evaluated based on α and S. The results were presented in microgram grades, indicating the method's sensitivity (Table 3).

Stability: The sample solutions of BOS for different media were stable at 4°C, 25°C, and 37°C for 24 h. The stability results are summarized in Table 7.

Stability studies

The HPLC method was used for the stability study (4°C, 25±2ºC/60±5% RH, and 40±2°C/75±5% RH) of BOS-loaded S-SNEDDS tablets. No excipient peaks were observed. The percentage of drug content was within the limits (85%-115%)

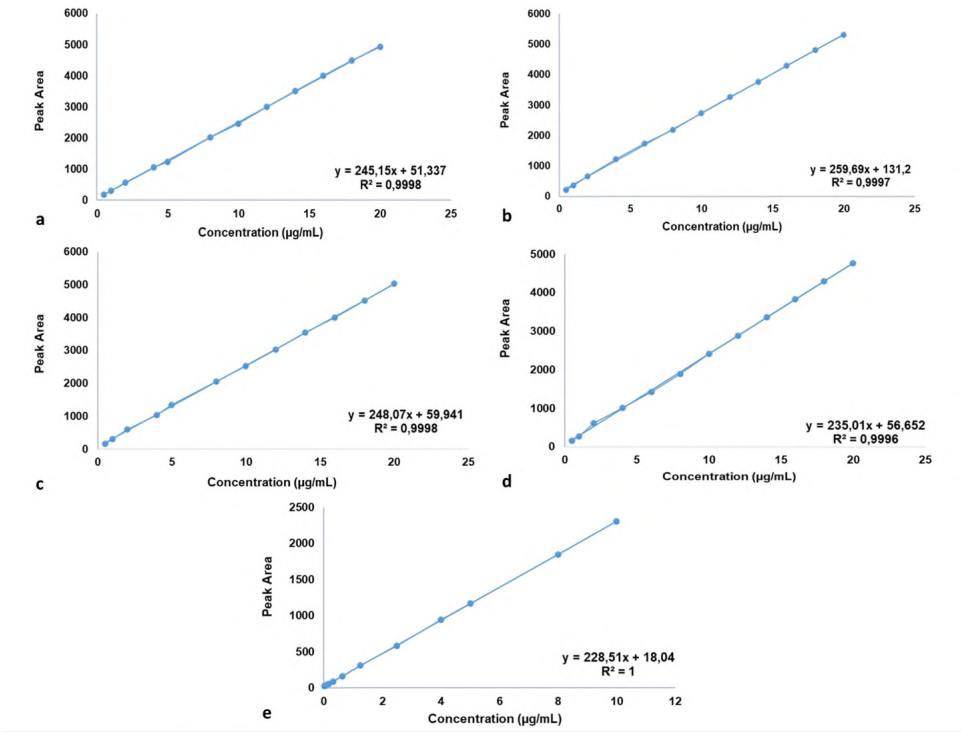


Figure 1. Calibration curves of a) FaSSIF, b) FeSSIF, c) FaSSIF-V2, d) FeSSIF-V2, and e) 1% SLS in distilled water

Table 3. Characteristic properties of the HPLC analysis methods of BOS

	1% SLS	FaSSIF	FeSSIF	FaSSIF-V2	FeSSIF-V2
Linearity range (µg/mL)	0.0195 - 10	5 - 20	5 - 20	5 - 20	5 - 20
Slope*	228.51	245.15	259.69	248.07	235.01
Intercept*	18.04	51.337	131.2	59.941	56.652
Correlation coefficient	1	0.9998	0.9997	0.9998	0.9996
Retention time (min)	5.5	5	4.7	5.1	4.9
LOD (µg/mL)	0.080	0.316	0.265	0.321	0.475
LOQ (µg/mL)	0.245	0.957	0.802	0.973	1.44
Peak height	406	580	627	434	483
Peak width	0.1389	0.1268	0.1238	0.1514	0.1671
Peak area	3652	4709	5173	4289	5258
Peak symmetry	0.971	0.917	0.912	0.846	0.911
Theoretical plate values	25087	24878	23061	18156	13758

*n=3, **SLS:** Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 4. Intra-day precision (repeatability) results of BOS

Injection time of the samples	1% SLS	FaSSIF	FeSSIF	FaSSIF-V2	FeSSIF-V2
1	0.615	10.6	9.71	9.29	11.1
2	0.599	10.7	9.62	9.84	11.0
3	0.617	10.7	9.63	9.43	11.0
4	0.607	10.6	9.67	9.78	11.0
5	0.622	10.7	9.64	9.73	11.1
6	0.621	10.7	9.68	9.97	11.1
7	0.594	10.7	9.67	9.50	11.0
8	0.606	10.8	9.70	9.57	11.0
9	0.591	10.6	9.74	10.1	11.0
10	0.603	10.6	9.67	9.95	11.1
Mean	0.608	10.7	9.67	9.71	11.0
SD	0.011	0.039	0.036	0.254	0.035
RSD%	1.80	0.368	0.375	2.62	0.316

SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 5. Interday precision (reproducibility) results of BOS

Amount added (µg/mL)	The calculated amount (µg/mL)			Mean	SD	RSD%
	1 day	2 day	3 day			
			1% SLS in distilled water			
0.625	0.639	0.669	0.648	0.655	0.012	1.85
5	5.02	5.13	5.05	5.07	0.059	1.16
10	10.1	9.82	9.83	9.92	0.167	1.68
			FaSSIF			
5	4.82	4.72	4.63	4.72	0.095	2.02
10	9.81	9.67	9.84	9.78	0.075	0.771
16	16.1	15.8	16.2	16.0	0.216	1.35
			FeSSIF			
6	5.91	5.98	5.80	5.90	0.092	1.55
10	9.73	9.87	9.87	9.82	0.080	0.816
16	15.7	15.7	15.9	15.8	0.131	0.830
			FaSSIF-V2			
5	5.15	5.13	4.67	4.98	0.269	5.40
10	10.1	9.99	9.72	9.93	0.196	1.97
16	15.3	15.5	14.0	14.9	0.818	5.47
			FeSSIF-V2			
6	5.96	6.14	5.51	5.87	0.325	5.54
10	10.5	10.5	9.34	10.1	0.686	6.77
16	16.0	15.9	16.1	16.0	0.108	0.674

SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 6. Accuracy and recovery results of BOS for all media

Level of recovery (%)	Amount added (µg/mL)	The calculated amount (Mean±SD) (µg/mL)	Recovery (%)	RSD (%)
		1% SLS in distilled water		
80	2	1.95 ± 0.04	97.7	1.79
100	2.5	2.52 ± 0.03	101	1.26
120	3	3.04 ± 0.03	101	1.02
		FaSSIF		
80	2	2.06 ± 0.01	103	0.525
100	2.5	2.42 ± 0.02	96.7	0.703
120	3	2.86 ± 0.07	95.3	2.46
		FeSSIF		
80	2	1.95 ± 0.01	97.6	0.496
100	2.5	2.58 ± 0.03	103	1.33
120	3	2.91 ± 0.03	97.0	0.851
		FaSSIF-V2		
80	2	2.01 ± 0.08	100.6	4.09
100	2.5	2.46 ± 0.10	98.2	4.09
120	3	2.77 ± 0.08	92.4	2.99
		FeSSIF-V2		
80	2	1.94 ± 0.03	97.0	1.46
100	2.5	2.45 ± 0.01	97.9	0.266
120	3	3.04 ± 0.21	101	0.214

SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

except for the 12th month at 40°C (Table 8). In the 12th month at 40°C, this out-of-limit result was related to the forced stability condition.

Dissolution studies

According to the FDA and EMA report, the solubility of BOS is a pH-dependent and poorly soluble drug. Hence, analysis and interpretation of the *in vitro* dissolution data is essential for predicting *in vivo* (FDA, 2003; EMA, 2005). The developed HPLC method can determine data with sufficient precision and accuracy. The percentages of cumulative BOS dissolution are presented in Table 9.

Permeability studies

The analysis method was found satisfactory. The flux and permeability coefficients were calculated and are presented in Table 10. The proposed method can be used to determine BOS.

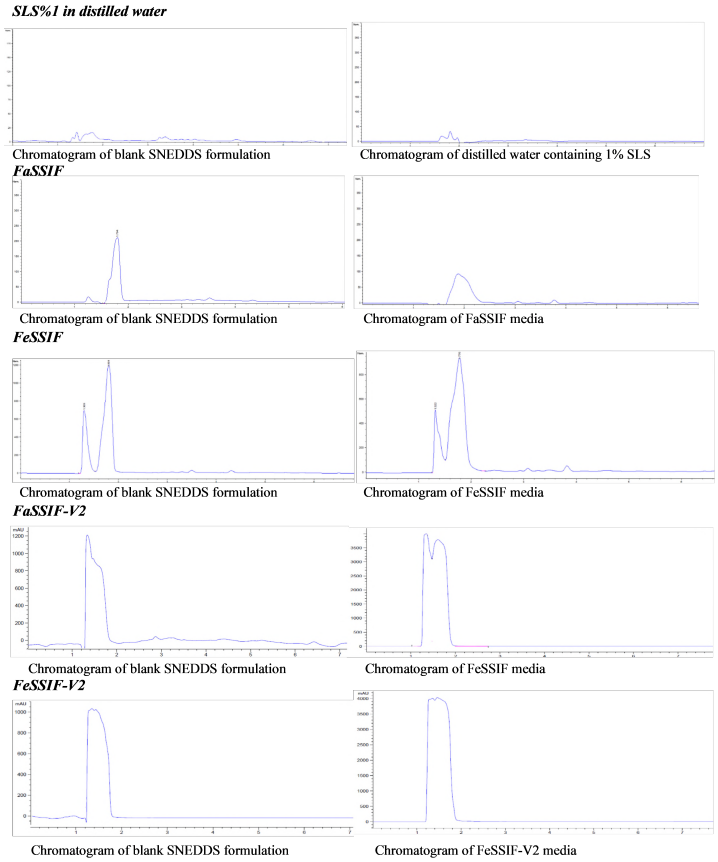


Figure 2. Chromatograms of FaSSIF, FeSSIF, FaSSIF-V2, FeSSIF-V2, and 1% SLS in distilled water and blank SNEDDS formulation in FaSSIF, FeSSIF, FaSSIF-V2, FeSSIF-V2, and 1% SLS in distilled water
SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 7. Stability results for BOS on all media

	Time (h)	Amount added (µg/mL)	The calculated amount (µg/mL)	Recovery (%)
		1% SLS in distilled water		
Initial		10	9.83	98.3
4ºC	24	10	9.66	96.6
25ºC	24	10	9.26	92.6
37ºC	24	10	9.73	97.3
		FaSSIF		
Initial		10	9.53	95.3
4ºC	24	10	9.28	92.8
25ºC	24	10	9.31	93.1
37ºC	24	10	9.38	93.8
		FeSSIF		
Initial		10	9.69	96.9
4ºC	24	10	9.65	96.5
25ºC	24	10	9.52	95.2
37ºC	24	10	9.59	95.9
		FaSSIF-V2		
Initial		10	10.1	101
4ºC	24	10	8.72	87.2
25ºC	24	10	8.91	89.1
37ºC	24	10	9.51	95.1
		FeSSIF-V2		
Initial		10	9.61	96.1
4ºC	24	10	10.3	103
25ºC	24	10	10.7	107
37ºC	24	10	10.5	105

SLS: Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

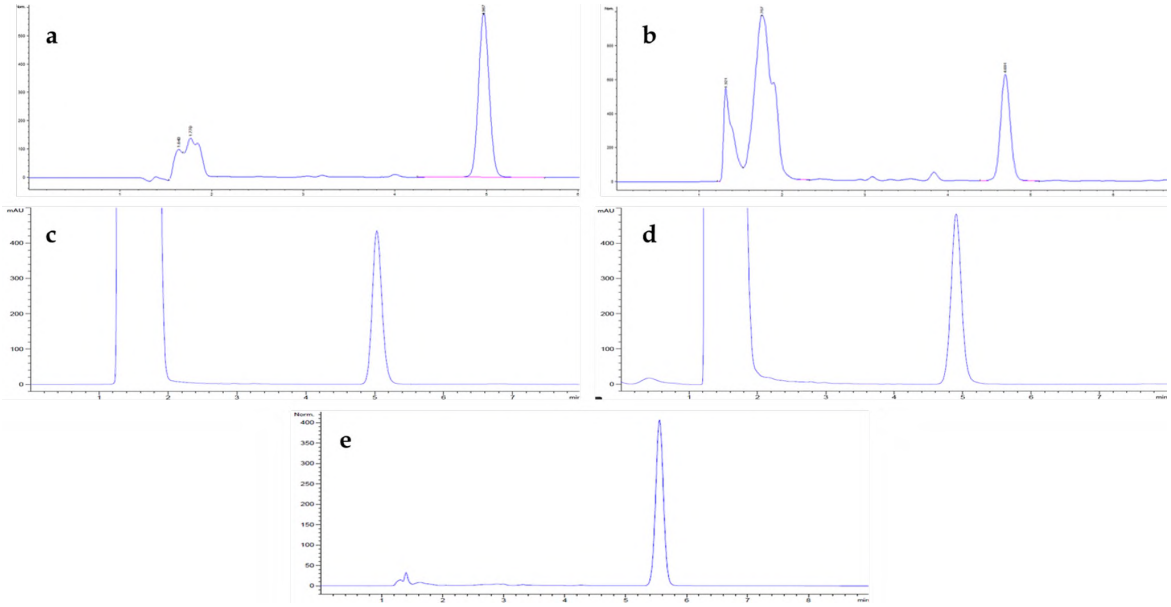


Figure 3. Chromatograms of BOS solutions in a) FaSSIF, b) FeSSIF, c) FaSSIF-V2, d) FeSSIF-V2, and e) 1% SLS in distilled water
FaSSIF: Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2,**FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2, **SLS:** Sodium Lauryl Sulfate

Table 8. Percentage of drug content of BOS for stability conditions

Time (month)	4°C	25±2°C/60±5% RH	40±2°C/75±5% RH
Initial		103%	
1	103%	99.7%	91.9%
3	102%	98.8%	90.9%
6	100%	97.1%	90.9%
12	102%	98.8%	81.4%

n=3

Table 9. Percentages of cumulative dissolution in 1% SLS in distilled water, FaSSIF, FeSSIF, FaSSIF-V2, and FeSSIF-V2 of the reference tablet, BOS-loaded SNEDDS, and BOS-loaded S-SNEDDS tablets

	15 min	30 min	45 min	60 min	90 min
			1% SLS in distilled water		
Reference tablet	90.2±2.6	96.9±5.4	100±2	101±2	102±1
BOS-loaded SNEDDS	80.2±19.9	103±0	104±0	104±1	104±1
BOS-loaded S-SNEDDS tablet	58.8±4.8	74.7±5.0	81.8±4.6	85.3±4.7	88.3±3.2
			FaSSIF		
Reference tablet	21.9±0.3	27.3±0.8	30.1±0.3	31.8±0.1	32.4±2.9
BOS-loaded SNEDDS	82.5±4.6	94.7±1.0	97.4±0.3	97.1±0.7	97.2±1.2
BOS-loaded S-SNEDDS tablet	44.9±0.9	59.8±0.5	67.1±4.1	72.4±3.1	80.0±4.1
			FeSSIF		
Reference tablet	8.72±0.9	10.1±0.1	10.7±0.9	10.7±0.5	11.0±0.1
BOS-loaded SNEDDS	70.8±6.1	84.6±1.3	86.7±1.4	87.3±2.1	87.7±1.0
BOS-loaded S-SNEDDS tablet	51.3±12.9	61.4±7.4	70.1±8.0	74.0±5.5	82.4±2.3
			FaSSIF-V2		
Reference tablet	14.9±0.4	17.9±0.5	19.7±0.8	20.5±0.2	22.3±0.8
BOS-loaded SNEDDS	81.5±11.3	89.0±2.4	93.1±0.5	94.1±0.4	94.1±0.8
BOS-loaded S-SNEDDS tablet	55.9±11.3	69.8±9.9	72.8±1.9	80.3±9.2	82.4±7.8
			FeSSIF-V2		
Reference tablet	17.0±0.2	18.5±0.5	19.3±1.7	19.3±0.2	20.1±2.6
BOS-loaded SNEDDS	91.5±10.2	93.6±3.3	98.7±2.9	99.5±1.9	99.1±1.9
BOS-loaded S-SNEDDS tablet	58.8±3.8	75.4±1.8	82.1±1.8	85.2±1.1	88.2±1.2

mean ± SD, n=3, **SLS:** Sodium Lauryl Sulfate, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

Table 10. Flux and permeability coefficient parameters of BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets in goat intestine membranes from different biorelevant media

		SNEDDS	S-SNEDDS tablet	Reference tablet
A	**Flux (μg cm^{-2} min^{-1})**	54.4±20.2	44.8±28.0	8.85±1.83
	Permeability coefficient (x 10^{-2} cm min^{-1})	0.181±0.067	0.597±0.374	0.118±0.024
E	**Flux (μg cm^{-2} min^{-1})**	208±26.6	3.2±2.89	2.69±0.317
	Permeability coefficient (x 10^{-2} cm min^{-1})	0.692±0.089	0.042±0.038	0.036±0.004
A-V2	**Flux (μg cm^{-2} min^{-1})**	417±262	24.2±19.2	6.36±0.816
	Permeability coefficient (x 10^{-2}cm min^{-1})	1.39±0.874	0.322±0.255	0.085±0.011
E-V2	**Flux (μg cm^{-2} min^{-1})**	229.±85.0	6.06±3.48	3.36±0.802
	Permeability coefficient (x 10^{-2} cm min^{-1})	0.763±0.283	0.081±0.046	0.045±0.011

mean±SD, n=3, **A:** FaSSIF, **E:** FeSSIF, **A-V2:** FaSSIF-V2, **E-V2:** FeSSIF-V2, **FaSSIF:** Fasted State Simulating Intestinal Fluid and its second version FaSSIF-V2, **FeSSIF:** Fed State Simulating Intestinal Fluid and its second version FeSSIF-V2

CONCLUSION

The validated HPLC methods for the quantitative determination of BOS were applied and method validation was successfully performed for pharmaceutical formulations and pharmaceutical dosage forms samples for FDA-recommended and biorelevant media in *in vitro* and *ex vivo* studies. The HPLC methods validation studies were conducted based on the ICH Q2(R2) guideline. The methods were applied and successfully validated for linearity, accuracy, precision, selectivity, specificity, LOD, LOQ, and repeatability for *in vitro* and *ex vivo* permeability studies. These methods can be used for *in vitro, ex vivo*, and quality control tests of BOS-loaded SNEDDS, S-SNEDDS tablets, and reference tablets.

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Original Article

Highly sensitive carboxyl group fluorimetric derivatization HPLC analysis for rosuvastatin content in tablets

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ABSTRACT

Background and Aims: The powerful antihyperlipidemic drug rosuvastatin blocks 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is essential for cholesterol formation. Statins are a more recent class of antihyperlipidemic medications. Accurate quantification methods are crucial because of low rosuvastatin levels in tablets. The following the International Conference on Harmonisation (ICH) guidelines, a sensitive and high-performance liquid chromatographic approach was established in this study for the accurate determination of rosuvastatin in tablet formulations using spectrofluorimetric detection.

Methods: The procedure requires one hour at room temperature and dark interaction between the acid group of rosuvastatin and the reagent 9-anthryldiazomethane. A C18 column (250 x 4.6 mm, 4 µm) was used for the gradient elution of an acetonitrile-water solution at a flow rate of 1.0 mL/min to achieve chromatographic separation. The internal reference was lovastatin. The excitation and emission wavelengths used for the detection were 366 and 410 nm, respectively.

Results: Calibration curves for standard solutions were established by plotting the ratio of concentration to peak area over the range 0.01–20.0 ng/mL. The limits of quantification (LOQ) and detection (LOD) were 0.0068 and 0.0023 ng/mL, respectively. The relative standard deviation values for interday and intraday measurements of the standard solutions ranged from 0.24% to 3.76%. The mean recoveries for 0.240. in the tablet formulation were calculated as 98.0-99.9%.

Conclusion: The developed method was used to determine the amount of rosuvastatin in tablets, and the results were compared with a 95% confidence level to those obtained using a literature method. The suggested approach works well for sensitive routine analysis and monitoring of drugs at low concentrations to investigate their bioavailability and bioequivalence.

Keywords: Derivatization, Fluorescence, HPLC, Rosuvastatin, Statin, Tablet

INTRODUCTION

Recently, humans have become more susceptible to hyperlipidaemia because of the rise in animal products and decline in physical activity caused by recent technological breakthroughs. Atherosclerosis and coronary heart disease (CHD) are linked to hyperlipidaemia. The goal of CHD treatment is to lower hyperlipidaemia. Because they block the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, statins are prescribed to people with high blood cholesterol who are at risk of cardiovascular disease. Since 1987, these medications have been the most successful means of treating hyperlipidaemia (Onat, Sansoy, Hergenç, Soydan, & Adalat, 2009). Early-stage CHD and high-risk patients without CHD respond well to statin treatment (Taylor et al., 2013).

The newest statin, rosuvastatin (ROS), decreases low-density lipoprotein cholesterol (LDL-C), total cholesterol, and triglycerides and raises high-density lipoprotein levels. It also has a stronger low-density lipoprotein (LDL)-lowering effect than other statins (Martin, Mitchell, & Schneck, 2002; Carswell , Plosker, & Jarvis, 2002). Figures 1 and 2, respectively, show the chemical structure, UV spectrum, and fourier transform infrared spectroscopy (FT-IR) spectrum of ROS in acetonitrile. ROS is soluble in acetonitrile, water, and methanol and has a pKa value of 3.8.

Currently, several high performance liquid chromatography (HPLC) methods have been developed for measuring ROS in a variety of matrices, either alone or in conjunction with other medications. According to several studies (Lakshmana, & Suneetha, 2010; Sankar, Kumar, & Krishna, 2007; Hemant Kumar, Swathi Sri, Vara Prasada Rao, & Srinivasa Rao, 2015; Moid et al., 2018; Pimpale & Kakde, 2021; Rao &

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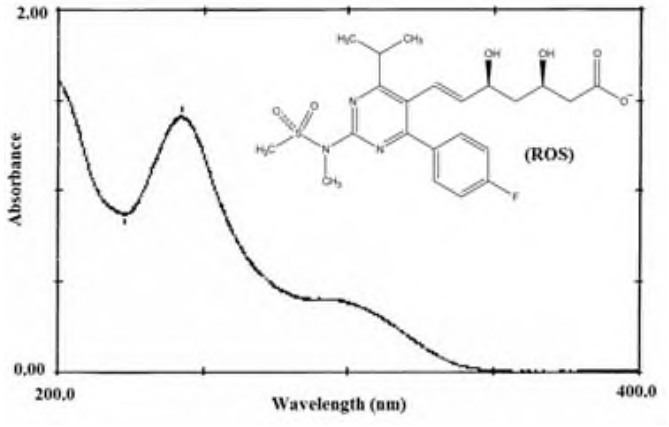

Figure 1. Chemical structure and UV spectrum of 8 µg/mL ROS in acetonitrile

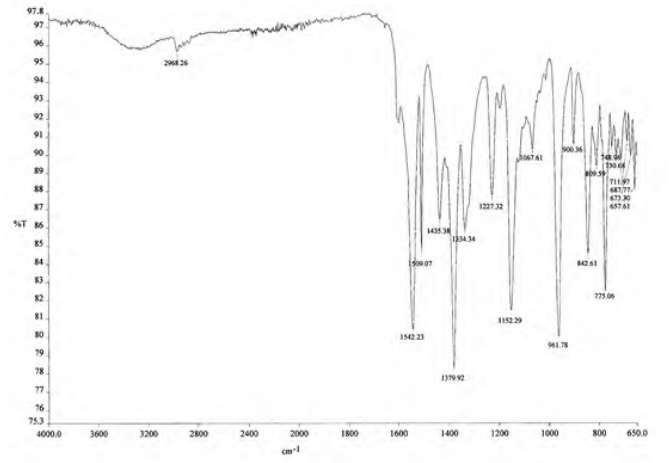

Figure 2. FTIR spectra of ROS

Suneetha, 2010), these techniques were developed for assessing ROS in bulk and pharmaceutical dosage forms. Chromatographic stability-indicating techniques have been successfully applied by Hasumati, Sadhana, Jayant, & Patel, (2009); Krishnaiah et al. (2009), Tushar, Patel, Kulkarni, & Suubbaiah, (2005); Gomes et al. (2009); Anuradha & Plaur (2016); Gholve, Pekamwar,Wadher, & Kalyankar, (2021); Hamdy, Korany, Ebied, & Haggag, (2022) for determining ROS in pure form and pharmaceutical preparations.

HPLC-UV was used to determine ROS in combined dosage forms in the following studies: Janardhanan, Manavalan, & Valliappan, (2016); Kumar, Kumar, Kumar, & Patel, (2017); Mostafa, El-Ashrey, & Mahmoud, (2023); Zuromska-Witek, Stolarczyk, Szlósarczyk, Kielar, & Hubicka, (2023); Albishri, Al-Shehri, Alshitari, & El-Hady, (2024); Alshitari, Al-Shehri, El-Hady, & Albishri, (2021); Choi, Park, & Kim, (2021); Deshpande & Gunge (2018); Hussain et al. (2022). However, the aforementioned approaches do not provide sensitive quantification because of the low concentration of the ROS active component in tablets.

The process of fluorimetric derivatization is often used to increase sensitivity. 9-anthryldiazomethane (ADAM) (Figure 3) was used as the fluorimetric label for derivatization because ROS contains a carboxylic acid group. Even in the presence of water, the carboxyl functional group reacts with ADAM at room temperature in mild circumstances without the need for an activating reagent. Reversed-phase HPLC can be used to identify these acids at picomole levels because of the derivatives generated (Toyo'oka, 1999).

In this work, ROS and ADAM were subjected to a fluorimetric derivatization reaction, and the reaction product was identified by HPLC in reference solutions. The proposed technique was effectively implemented for tablets, and the outcomes were compared with an HPLC-UV technique documented in existing literature.

This work was part of the principal author's doctoral thesis, and part of it was published in a publication. (Caglar, & Toker, 2012).

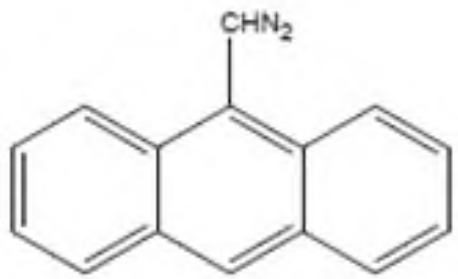

Figure 3. Chemical structure of ADAM

MATERIALS AND METHODS

Chemicals, reagents, and solutions

ROS calcium was obtained from AstraZeneca (London, UK), and lovastatin was obtained from Merck Sharp and Dohme Corp. (Whitehouse Station, NJ). Crestor 20 mg tablets were purchased from a pharmacy. ADAM was sourced from Sigma-Aldrich (Oslo, Norway). Sodium acetate, acetonitrile, ethyl acetate (EA), methyl tertiary butyl ether (MTBE), chloroform, glacial acetic acid, and anhydrous sodium sulphate were acquired from Merck (Darmstadt, Germany). Ultra-pure water was produced using the AquaMAX water purification system (Younglin Instrument (Korea). Lovastatin was used as the internal standard (IS).

A 1.0 mg/mL ROS stock solution was prepared in acetonitrile and diluted with acetonitrile to achieve a concentration of 1 mg/mL. ROS working solutions I and II were prepared at concentrations of 25 ng/mL and 1 ng/mL, respectively, in an acetonitrile–water (1:3) mixture. Solutions of 100 mg/mL ADAM and IS were prepared in acetonitrile and chloroform, respectively.

Derivatization Procedure

We prepared ADAM and IS solutions at concentrations of 100 mg/mL in acetonitrile and chloroform, respectively. Sodium acetate was dissolved in water to achieve a 0.1 M concentration for the acetate buffer solution, with the pH adjusted to 4.0 using glacial acetic acid, as per the US Pharmacopoeia. To prepare ROS base solutions and for derivatization, the same procedure was used as described in (Caglar et al. 2012). All reagent solutions were freshly prepared daily and stored away from light.

To improve the sensitivity and accuracy of ROS analysis, derivatization conditions, including the concentration of ADAM, temperature and time were optimised. Different volumes of 100 mg/mL ADAM reagent solution, ranging from 10 to 200 µL, were used to study the effects of volume and concentration. It was observed that 125 µL of 100 mg/mL ADAM was optimal for the ADAM-ROS derivative (Fig. 4).

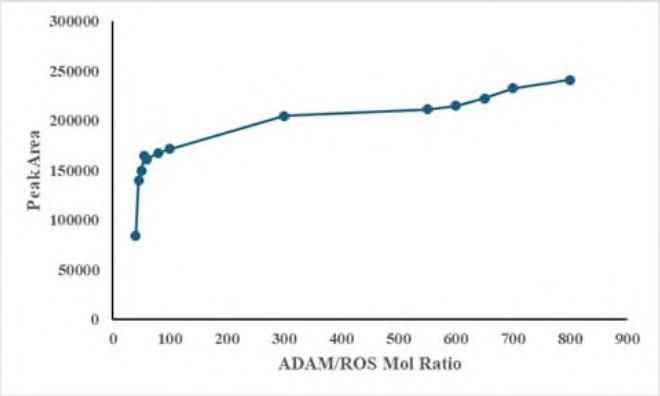

Figure 4. Effect of marker concentration on the formation of the ADAM derivative of ROS

The completeness of derivatization was investigated at different temperatures (room temperature, 30, 40, 50 and 60^0C) and reaction times. ADAM-ROS were completely derivatized at ambient temperature for 1 h. (Fig. 5)

Chromatography

A Shimadzu LC 20A liquid chromatograph equipped with an RF 10 AXL fluorescence detector and LC Solution system software was used during the study (λex=366, λem=410 nm). Separations were performed on a Phenomenex Synergi C18

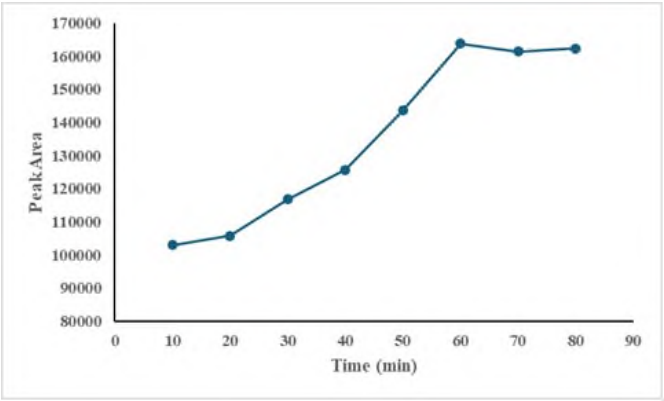

Figure 5. Effect of time on the derivatization reaction

column (4 µm, 250 x 4.6 mm) with a Phenomenex guard column.

The mobile phase, consisting of a mixture of acetonitrile and water, was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter (Waters Corporation) and sonicated for 5 min. The analysis was conducted under gradient conditions (Caglar et al. 2012) at a flow rate of 1 mL/min. Lovastatin was used as the internal standard (IS).

Application to pharmaceutical preparations

To determine the average weight of a single Crestor® 20 mg tablet, 10 tablets were weighed separately and ground into a fine powder using a porcelain mortar. A precisely weighed quantity of tablet powder equal to 20 mg of the ROS base was added to a 100 mL volumetric flask. 50 mL of the mobile phase (water: acetonitrile, 40:60) was added to this flask. After 30 min of sonication, the mixture was brought to a volume using the mobile phase and subsequently filtered through blue band filter paper, discarding the first thirty millilitres of the filtrate.

The remaining filtrate was divided into 0.5 mL aliquots and diluted with mobile phase to make 10 mL. After diluting 0.1 mL of this solution with water to make 10 mL, the final volume of the solution was examined using the developed methodology. Six repetitions of this analysis process were conducted. The equation resulting from the previously constructed calibration curve was used to determine the ROS content in the tablets.

Method Validation

For the purpose of validating the developed method, validation criteria like selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and stability, were assessed in accordance with ICH recommendations (ICH Q2 (R1), 2005).

The assessment of the linearity of ROS was conducted on a sample of six, considering the concentration of ROS in the preparation comprising the injection solution, within the range

of 0.01-20.0 ng/mL. Volumes of 0.4, 40, 200, 400, 600, and 800 µL were extracted from working solution II and subjected to derivatization to investigate linearity. After comparing the peak areas of ROS-ADAM and IS-ADAM, calibration curves were created by plotting the average peak area ratio values against the concentration data.

The formulas LOD or LOQ = SDa/b were used to calculate the values of LOD and LOQ, where a and a are 3, and 10, respectively. SD is the calibration curve intercept, and b is the slope of the calibration curve.

Three distinct concentrations were selected from the calibration curve to assess the absolute recovery. Absolute recoveries were evaluated using the standard addition method. Using the derivatization process, standard solutions of ROS at concentrations of 1.0, 5.0, and 15.0 ng/mL (n=6 each) were prepared, and the developed method was used for analysis.

Assessments of intra- and inter-day precision were part of the precision studies. On the same day or on different days, standard mobile phase solutions were prepared and evaluated at concentrations of 1.0, 5.0, and 15.0 ng/mL (n=6 each). The measured values' relative standard deviations (RSD) percentages were computed.

The ROS-ADAM derivatization solution's stability was assessed for 12, 24, 48, 72, and 96 h at 4°C in a dark environment in addition to room temperature.

RESULTS AND DISCUSSION

Method development

Biologically significant carboxylic acids can be sensitively detected at the picomole level using ADAM, a diazomethyl sensor that is frequently employed as a fluorescent label (Toyo'oka 1999). It was selected for derivatization because it can react with carboxyl groups at room temperature in mild circumstances, even in the presence of water, and it does not require an activating reagent. With reversed-phase HPLC, the products of this reaction can be found at picomole levels. The concentration, temperature, and duration of ADAM were carefully adjusted during derivatization in order to maximise the sensitivity and accuracy of ROS analysis. The ideal conditions for the ADAM-ROS derivative were found to be 125 µL of a 100 mg/mL ADAM reagent solution (Fig. 4).

Various reaction times and temperatures (room temperature, 30°C, 40°C, 50°C, and 60°C) were used to evaluate the completeness of the derivatization reaction. After 1 h at room temperature, the reaction between ROS and ADAM was determined to be complete (Fig. 5).

Figure 5 shows how temperature and reaction time affected the ROS-ADAM intensity. Furthermore, by adjusting the mole ratio of ADAM to ROS, the amount of ADAM reagent needed was determined, and it was found that a 55-fold molar excess of reagent was necessary for the entire reaction.

Selectivity

The system was injected with the mobile phase, the ROS-ADAM standard solution, and derivatization reactions (blank solution) without any other compounds to assess the method's selectivity. This made it possible to investigate interferences that might have come from the reagent, the mobile phase, or contaminants in the reaction environment. Furthermore, as shown in Figure 6, no peaks related to the solvent or reaction environment were observed during the ROS retention period, indicating that the proposed approach can isolate ROS-ADAM from any interference or background noise.

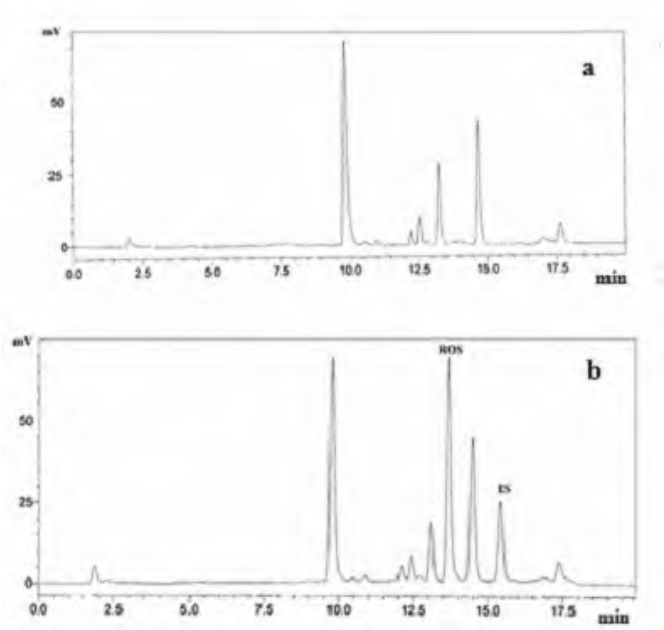

Figure 6. Chromatograms obtained from (a) the blank solution (b) the ROS-ADAM standard solution

Linearity and sensitivity

Over a concentration range of 0.01-20.0 ng/mL, the linearity of the developed approach was evaluated for pharmaceutical ROS formulations. The average regression equation, A = 0.2617±0.0005 C + 0.1566±0.0035 (R2 = 0.9975), was found, where C is the ROS concentration (ng/mL) and A is the peak area ratio. The LOQ and LOD were 0.0023 ng/mL and 0.00068 ng/mL, respectively, based on the study parameters.

Recovery

As shown in Table 1, the absolute ROS recovery values in the tablet formulation were between 98.0% and 99.9 %. The average ROS recovery was 99.2%.

Table 1. Recovery results for the assay of rosuvastatin (n=6)

Concentration (ng mL^{-1})		Recovery (%)	RSD[b] (%)
Added	**Found** (mean ± SD[a])		
1.0	0.98±0.022	98.0	1.909
5.0	4.98±0.023	99.6	0.500
15.0	14.98±0.027	99.9	0.208

[a] Standard deviation; [b] Relative standard deviation

Precision

As previously mentioned, precision evaluations were performed for both intra-day and inter-day repeatability. For intraday repeatability, the relative standard deviation (RSD%) varied from 0.24% to 3.73%, whereas for interday repeatability, it ranged from 0.24% to 3.76%. The precision values derived by the proposed method are displayed in Table 2.

The reliability of the results were demonsrated by the precisionstudy, which also meet the criteria that the RSD% should be less than 3.76%.

Table 2. Intra-day & inter-day precision and accuracy of rosuvastatin (n=6)

Concentration (ng mL^{-1})		RSD[b] (%)	RME[c] (%)
Added	**Found (mean ± SD[a])**		
Intra-day			
1.0	0.99 ± 0.037	3.73	-0.8
5.0	4.98 ± 0.049	0.98	-0.44
15.0	14.98 ± 0.036	0.24	-0.08
Inter-day			
1.0	1.01 ± 0.038	3.76	0.6
5.0	4.99 ± 0.037	0.75	-0.2
15.0	14.98 ± 0.036	0.24	-0.1

[a] Standard deviation; [b] Relative standard deviation; [c] Relative mean error

Stability

Stability study was conducted to assess the derivative by keeping it in the dark at room temperature and at +4°C for 12, 24, 48, 72, and 96 h. It was found that the derivative remained stable for up to 96 h when stored at +4°C and in the dark, as summarised in Table 3.

Table 3. Stability of rosuvastatin obtained using the proposed method

	Peak Area					
	0. hour	**12. hour**	**24. hour**	**48. hour**	**72. hour**	**96. hour**
dark at room temperature	172826	171634	170878	170176	169978	169176
the dark at +4°C	172856	172873	172435	172291	171792	171886

Determination of ROS from the tablet and comparison of the results

The tablets were also examined using the HPLC-UV technique described in the literature to compare the outcomes (Mehta, Patel, Kulkarni, & Suubbaiah, 2005). Table 4 displays the findings from both approaches, as well as the average values (Amean), standard deviation (SD), relative standard deviation (RSD), and 95% confidence level confidence interval [Amean ± (t.s/-n)], which were computed from 6 determinations.

Table 4. Statistical evaluation of the analysis results using the comparison methods

Statistical value	HPLC Metod*	Comparision Method
Mean± SD	19.902±0.048	19.942±0.06
RSD (%)	0.24	0.30
Confidence interval	0.0273	0.0336
Confidence limits	19.875-19.929	19.908-19.976
Student's t-test **	1.387	
F-test**	1.52	

**p = 0.05, t = 2.228, F = 5.05, [a*] Crestor tablet® (20 mg Rosuvastatin)

Student's t-test was used to compare the average results between the developed and reference methods, and Fisher's F-test was used to compare the standard deviation. After checking through Table 4's results, it was discovered that, at a 95% probability level and six trials, the computed t- and F-values were less than the crucial values listed in the corresponding tables. As a result, it was determined that there was no discernible variation in accuracy or precision between the reference technique and the proposed HPLC method.

CONCLUSION

In conclusion, a novel HPLC technique was developed for the sensitive, reliable, and repeatable identification of ROS in plasma and pharmaceutical formulations. By taking advantage of the simple interaction between ROS and ADAM, the approach does not require re-extraction from the reaction medium. The main benefits of this approach are its high recovery rates, good repeatability, and ease of use in terms of both the chromatographic equipment and detector setup. Therefore, it is suitable for routine pharmaceutical analyses and tracking low drug concentrations in bioavailability and bioequivalence research.

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Review Article

Translating research to clinical application: The utilization of JAK inhibitors in scleroderma

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ABSTRACT

The clinical characteristics and prognosis of scleroderma (SSc), an uncommon autoimmune disease, can vary widely. There is no specific treatment for SSc. Medications used for the treatment of SSc, such as tocilizumab, cyclophosphamide, mycophenolate mofetil, and nintedanib, have a range of potential side effects. More than 50 ligands have been shown to activate the *JAK/STAT* signalling pathway, which plays a role in cell signal transmission through an evolutionarily conserved mechanism. The pathway of *JAK/STAT* signalling contributes to autoimmune. *JAK* inhibitors are tiny compounds with various molecular configurations. Patient illness development is only slightly slowed down or stabilised when these medications are used. In animal models of SSc, *JAK* inhibitors decreased pulmonary and cutaneous fibrosis. There are only few clinical studies on the effectiveness and safety of *JAK* inhibitors in individuals with SSc. In particular, tofacitinib and baricitinib are used for treating SSc. The reduction in the modified rodnan skin score after treatment initiation was more significant in patients with previously untreated SSc. *JAK* inhibitors may be a safe and effective treatment option for skin fibrosis and interstitial lung disease in SSc. This review examines the application of JAK inhibitors in scleroderma, encompassing both fundamental research and clinical investigations. In the future, JAK inhibitors may serve as a prospective treatment for SSc; nonetheless, the paramount consideration remains the patient's well-being and quality of life. The realization of this part will be contingent upon the completion of clinical trials.

Keywords: SSc, *JAK* inhibitors, Treatment

INTRODUCTION

Scleroderma also known as systemic sclerosis (SSc), is an autoimmune, complex, heterogeneous, and rare disease that is mainly characterised by the dysregulation of the immune system, fibrosis, and vascular damage (vasculopathy), which negatively affect patients' quality of life (Perelas, Silver & Arossi, 2020; Sobanski et al., 2019). The modified Rodnan skin score (mRSS) for skin fibrosis in patients with SSc is the most widely used clinical measure. In the clinic, 30% of patients with SSc have other complications, such as internal organ fibrosis and interstitial lung disease (ILD). One of the causes of death in patients with SSc is ILD (Pokeerbux et al., 2019; Tashkin et al., 2006).

Unfortunately, the effects of treatments for SSc (e.g. nintedanib, mycophenolate mofetil, cyclophosphamide and tocilizumab) are quite limited. The use of these drugs results in only stabilising or minor reducing of disease progression in patients (Distler et al., 2019; Hu, Li & Fu, 2021; Tashkin et al., 2016).

Janus kinase inhibitors (JAKi), which have been candidates for clinical use in SSc for the past few years, represent a new and promising class of therapeutics. They are used in the therapy for other autoimmune diseases, connective tissue diseases, and cancers.

Janus kinase (JAK) / signal transducer and activator of transcription (STAT) pathway

The *JAK/STAT* signalling pathway has evolutionary conserved roles in cell signal transduction, and more than 50 ligands activating it were reported (Hu et al., 2021). The *JAK/STAT* signalling pathway (Darnell, Kerr & Stark , 1994) plays a role in autoimmunity during investigation of interferon (IFN) signalling, and IFN-specifically was implicated in the pathology of SLE and RA.

Structurally related cytokines and some hormones are the initiation factors of the *JAK/STAT* signalling pathway cascade. The extracellular binding of these ligands to their cognate transmembrane receptors initiates the *JAK/STAT* signalling cascade (Leonard & O'Shea, 1998).

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Initiation of the *JAK/STAT* signalling pathway begins with transactivation of receptors and receptor-bound *JAKs* that catalyse tyrosine phosphorylation (*p-Tyr*) of STATs, resulting in the formation of STAT heterodimers that translocate to the nucleus and direct gene transcription. Interferons induce by the *STAT1, STAT2*, and *IRF9* complex (Leonard & O'Shea, 1998).

In all mammals, there are *JAK-1, JAK-2, JAK-3*, and *Tyk-2* composed of seven homology domains (JH) organised in 4 major structural domains: the first is the FERM domain (JH5, JH6, JH7), which mediates interaction with receptors and supports kinase function; the second is the SH2-like domain (JH3, JH4), which mediates interaction with receptors; the third is the pseudokinase domain (JH2), which regulates kinase activity; and the fourth is the kinase domain (JH1) (Figure 1). *JAK-1* and *JAK-2* are activated by many cytokines and are critical for intracellular signal transduction. *JAK-3* and *TYK-2* activite relatively few cytokines (Harrison, 2012; Seif et al., 2017).

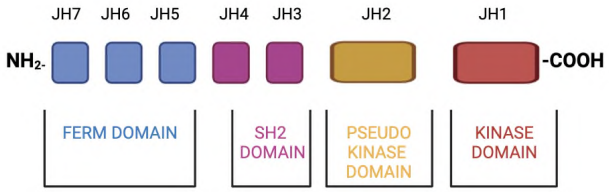

Figure 1. Domain structure of JAKs

There are also non-canonical *JAK/STAT* signalling pathways; Here, unphosphorylated *STAT* is localised on heterochromatin in association with HP1 in the nucleus. Increasing *STAT* phosphorylation significantly reduces the amount of unphosphorylated STAT localised on heterochromatin. The *JAK/STAT* signalling pathway also interferes with other signalling pathways in cells, such as *NOTC, TGF-β, NF-κB*, and PI3K/mTOR. What is important in the pathogenesis of SSc is that TGF-β profibrotic signalling regulates the JAK/STAT pathway (Aittomäki & Pesu, 2014; Mendoza, Piera-Velazquez & Jimenez, 2021). Additionally, the transcription factors *STAT* and *SMAD* from TGF-β, are sometimes included in the same transcription complex.

JAK/STAT signalling pathway negative regulators or suppressors of cytokine signalling (*SOCS*); protein tyrosine phosphatases (*PTPs*); and protein inhibitors of activated STATs (*PIASs*). These proteins are important and primary regulators of the *JAK/STAT* pathway. In fact, they act as Jack substrates that stop or block the signalling pathway (Hu et al., 2021).

JAK3 and *TYK2* germline loss-of-function (LOF) mutations are observed in autoimmune diseases and are generally considered the primary cause of disease development (Leonard & O'Shea, 1998). In addition, JAK1 somatic LOF mutations are observed in cancer cells and associated with IFN-γ resistance and cancer pathogenesis.

Gain-of-function (GOF) mutations play a role in the pathogenesis of systemic autoimmunity, particularly in *JAK-1*, polycythaemia vera (JAK2 kinase-like domain), lymphoma, leukaemia, and other cancers.

JAK inhibitors (JAKi)

JAKi are small molecules with different chemical structures. All *JAK* inhibitors exert their therapeutic effects through two distinct mechanisms. First, *JAK* inhibitors are used as immunosuppressors because the inhibition of *JAK* function reduces the levels of proinflammatory cytokines, which are increased by disease. Second, they enable the treatment of some myeloproliferative diseases and cancers in which gain-of-function JAK mutants have been identified and JAKi have successfully inhibited them (Gadina et al., 2020).

First-generation JAKis, such as baricitinib, tofacitinib, oclacitinib, ruxolitinib), are adenosine triphosphate (ATP)-competitive compounds binding to pseudokinase and kinase domains. Due to the structural similarity of these domains, these JAKi inhibit all JAK, but newer agents (such as upadacitinib) act via allosteric mechanisms (such as targeting the kinase-like domain) (Shawky, Almalki & Abdalla, 2022). Additionally, first generation of JAKi are adenosine triphosphate (ATP) competitive compounds. All of them targeted the active conformation of the tyrosine kinase domain (JH1) with a highly conserved ATP-binding pocket structure. Therefore, first-generation JAK inhibitors target multiple JAK members (Shawky et al., 2022). Therefore, they are also called pan-JAK inhibitors. Most next-generation JAKi are also ATP-competitive. However, some JAKi (e.g. Deucravacitinib) also target the JH2 domain of JAK (Shawky et al., 2022).

For the signal transduction of cytokines, *JAK-1* and *JAK-2* are critical, whereas *JAK-3* and *TYK-2* are activated by relatively few cytokines, and the selective inhibition of each might lead to fewer side effects. Thus, next-generation *JAKi* have been developed and are already used for rheumatic diseases, such as nilotinib and upadacitinib. Ritlecitinib was designed and found to be a selective *JAK-3* inhibitor (Chen et al., 2022).

AG-490i was first used in 1996 as a *JAK2* inhibitor with antileukemic activity (Shawky et al., 2022). Ruxolitinib was the first JAK inhibitor to receive FDA approval in 2011(Shawky et al., 2022). Currently, JAKs are used as important drug targets in autoimmune diseases, and JAKi has been approved for the treatment of psoriatic arthritis, rheumatoid arthritis, myeloproliferative neoplasms, vaccine-versus-host disease, and inflammatory bowel disease (Chen et al., 2022; Shawky et al., 2022). Tofacitinib and baricitinib were the first oral JAKi approved for rheumatoid arthritis (Taylor, 2019). In 2019; three JAKi were approved for clinical use. These drugs include FDA-approved lenvatinib and upadacitinib, whereas peficitinib has already been approved for rheumatoid arthritis in Japan. In

2020, delgocitinib and nilotinib were approved in Japan for the treatment of atopic dermatitis and rheumatoid arthritis, respectively (Shawky et al., 2022).

Serious adverse events are always a concern during clinical use of JAKi and require close monitoring. All JAK inhibitors have side effects such as hyperlipidaemia, cytopenia, and infection. Although tofacitinib and baricitinib have been approved for the treatment of rheumatoid arthritis after 10 and 5 years of clinical experience, respectively, they are still suspected to cause serious diseases such as malignancies and infections.

JAKi in the SSc

JAKi as a treatment for skin and musculoskeletal involvement

Dermal collagen deposition is significantly increased in bleomycin-induced SSc models. In the JAK-2 inhibitor group administered to a mouse model of SSc, a >70% reduction in dermal thickness was observed. With increasing JAK-2 inhibitor doses, dermal thickening in bleomycin-injected mice almost reached the same level as that in the control group (Dees et al., 2012).

The efficacy and effects of tofacitinib, a JAK inhibitor targeting JAK-1 and JAK-3, and ruxolitinib, a JAK inhibitor targeting JAK-1 and JAK-2, on dermal thickness were examined in an SSc model (Damsky et al., 2020). Decreased skin thickness was observed in both the tofacitinib and ruxolitinib groups (Damsky et al., 2020). Additionally, in addition to preclinical studies, tofacitinib and ruxolitinib show promise in a mouse model of interstitial lung fibrosis; This result suggests that the treatment of JAKi has a broad antifibrotic effect (Lescoat et al., 2020). According to one study, baricitinib improved the experimental SSc model lung and skin tissues in the experimental SSc model, leading to positive treatment effects. The results of immunohistochemistry demonstrated that BAR decreased the expression of *COL1A1* and *COL1A2*. BLM-induced skin and lung fibrosis improves after treatment with JAK 1-2 selective BAR at radiological, pathological, and molecular levels (Gulle et al., 2023).

As a result of the literature review, researchers performed a literature review of patients with SSc defined by the 2013 ACR/EULAR criteria and treated with JAK inhibitors by searching the Medline, Cochrane Library, and Embase databases. They included 59 (mean age 47±15 years) patients with SSc in the study. The average treatment duration of the included patients was 12 months. They were treated with JAK inhibitors (tofacitinib in 47 patients and baricitinib in 12 patients). Overall, a significant cutaneous response (>5 points on mRSS–modified Rodnan skin score and ≥25% reduction from baseline) was reported in 52 patients (88%). Additionally, among patients with ILD (n = 31), there was no disease progression. Additionally, disease progression was reported in only 2 patients during the study. Cutaneous responses were observed more frequently in treatment-naïve SSc patients. The reduction in mRSS after treatment initiation was more significant in treated patients with SSc (Moriana, Moulinet & Jaussaud et al., 2022).

Additionally, in studies on SSc, there is a role for transforming growth factor (*TGF*)-β, especially in the development of fibrosis. *TGF*-β promotes myofibroblast turnover, fibrosis, and therefore collagen deposition (Dees et al., 2012; Delany & Brinckerhoff, 1993; Xiao et al., 2008).

JAKi as a treatment for interstitial lung disease

Recently, a study demonstrated that ruxolitinib inhibited proinflammatory (M1) and profibrotic (M2) macrophages in vitro and ameliorated cutaneous and pulmonary fibrosis in a mouse model of SSc (Lescoat et al., 2020).

Aung et al. showed in a mouse model of bleomycin-induced SSc, tofacitinib, a JAK inhibitor, ameliorated cutaneous and pulmonary fibrosis with reduced Th2 and Th17 responses in the skin, IL-6-producing B lymphocytes, tissue macrophages, extracellular matrix, and profibrotic cytokine expression in the lungs (Aung et al., 2021).

Additionally, a study called SCLEROJAKI conducted in France is investigating the effect of JAK inhibitors against interstitial lung disease-related SSc (NCT05177471).

JAKi in vasculopathy treatment

SSc is a chronic and diverse connective tissue disease characterised by vascular injury (vasculopathy), immune response dysregulation linked to autoimmunity, and fibrosis development. JAKi were also proposed to have a possible function in the management of digital ulceration in a few of the examined studies, possible impact on the disease's vasculopathic component as well.

Inhibitors of JAK1 and JAK2 have been shown to alleviate skin fibrosis, and the oral JAK1/2 inhibitor baricitinib represents a potentially effective treatment for patients with diffuse cutaneous systemic sclerosis (dcSSc) exhibiting skin fibrosis and digital ulcers (DU). Baricitinib tolerated by most participants in this study. However, further large-scale clinical trials are required to validate these preliminary findings (Hou et al., 2022; Jerjen, Nikpour & Krieg, 2022).

In a study of SSc peripheral blood mononuclear cells, the pan-JAK inhibitor peficitinib was administered, and decreased *STAT-1* and *STAT-3* phosphorylation protein levels were observed (Kitanaga et al., 2020). Following favourable outcomes in patients with sclerodermatous graft-versus-host disease (GVHD) and eosinophilic fasciitis treated with ruxolitinib or tofacitinib, Deverapalli reported one of the earliest cases

of SSc treated with tofacitinib in a young patient in whom mycophenolate mofetil failed (Cao, Zhao & Hou, 2020; Hurabielle et al., 2017; Khoury et al., 2018; Kim et al., 2018; Okiyama et al., 2014).

Tofacitinib was well tolerated in the Khannas phase study; before or at week 24, no participant reported grade 3 or greater side events. The efficacy outcome measures showed a preference for tofacitinib. In subpopulations of fibroblasts and keratinocytes, baseline gene expression IFN-activated gene expression. Tofacitinib suppressed *IFN*-regulated gene expression in the basal and keratinised layers of the epidermis, as well as in *SFRP2/DPP4* fibroblasts (progenitors of myofibroblasts) and *MYOC/CCL19*, which represent adventitial fibroblasts ($p<0.05$). Tofacitinib inhibited *STAT3*, as evidenced by gene expression in DCs and macrophages ($p<0.05$). There was no clinically significant suppression of T lymphocytes and endothelial cells in skin tissue (Khanna et al., 2022).

CONCLUSION

As shown in the literature; JAK inhibitors inhibit profibrotic pathways for treating SSc and are therefore a possible treatment option. It may also show greater sensitivity and effectiveness than traditional treatments for SSc. For patients with SSc who have skin fibrosis and interstitial lung disease, particularly those who are resistant to immunosuppressive and/or immunomodulating therapy, JAK inhibitors (tofacitinib, baricitinib) may be useful. JAK inhibitors were not associated with any significant side effects in patients with SSc. Further studies are required to determine the pulmonary and long-term efficacy of JAK inhibitors in patients with SSc.

First-line therapy for refractory SSc may be successful with JAK inhibitors. The most frequent side effects that do not result in discontinuation of therapy include infections, gastrointestinal issues, elevated liver enzyme levels, and dyslipidemia. Because of their anti-inflammatory and anti-fibrotic properties, JAK inhibitors may be a potential treatment for patients with quickly progressing SSc.

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Review Article

Safety and possible risks of tea tree oil from a toxicological perspective

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ABSTRACT

Tea tree oil (TTO) is a sophisticated essential oil extracted from the *Melaleuca alternifolia* plant. It comprises around 1,000 components with a significant presence of monoterpenes and their alcohols. Terpinen-4-ol, the monoterpene that makes up 30% to 48% of TTO essential oil, is the main factor responsible for its strong antibacterial properties. TTO has been extensively used in skin care products to treat many problems, including acne, eczema, and dandruff. TTO is included in products used by children and adults. Nevertheless, the reliability of TTO in cosmetic and dermatological or derma cosmetic formulations is contingent upon numerous influential aspects, underscoring the pivotal significance of formulation and production procedures. TTO can be taken orally, topically, or ocularly. However, it is important to exercise caution, as high levels of TTO may cause phytotoxic effects and result in negative consequences such as contact allergy, inflammation, irritation, and dermatitis. Though natural, this essential oil can be harmful if not used correctly, considering factors like the route of application, exposure dose, and poor-quality contents. This review thoroughly examines the negative consequences, considerations for safety, and regulatory factors related to the usage of TTO. The study emphasizes the importance of conducting thorough research to better understand the safe use of essential oils, especially TTO. It also calls for a full assessment of the possible negative effects on vulnerable populations. Given the increasing demand for products containing TTO, it is crucial to conduct ongoing research to improve recommendations and ensure the informed and safe use of this precious essential oil.

Keywords: Tea tree oil, Risk, Safety, Adverse effects, Essential oil

INTRODUCTION

Tea tree oil (TTO) is the essential oil obtained by distilling the leaves and terminal branchlets of the narrow-leaf tea tree *Melaleuca alternifolia*, which grows in New South Wales and Queensland in Australia. It is a pale-yellow liquid with a terpenic, coniferous, and minty–camphoraceous odor. TTO is present in many cosmetics and personal care products, including ointments, skin cleansers, and shampoos (de Groot & Schmidt 2016). TTO acts as a natural bactericide against methicillin-resistant *Staphylococcus aureus* at 0.002-2% concentrations and is also suggested to have antiviral, anti-inflammatory, and analgesic effects (Vatanen et al., 2016). The European Medicines Agency (EMA) has approved TTO to treat minor superficial wounds, insect bites, tiny boils, irritation in athlete's foot cases, and minor oral mucosa inflammation. It is mainly used against skin problems such as contact allergy, irritation, eczema, dandruff, and dermatitis (de Groot & Schmidt 2016). Figure 1 summarizes the dermatological applications, biological activities, and composition of TTO.

TTO includes about 1000 ingredients, most of which are monoterpenes and their alcohols. Terpinen-4-ol is a monoterpene and the most prevalent constituent (with a minimum of 30% and a maximum of 48%) and is responsible for most of TTO's antibacterial activity (Oliva et al. 2018). TTO also contains high amounts of γ-terpinene and 1,8-cineole (eucalyptol), both of which cause skin irritation (Zeiner, Michaela & Stingeder, 2018)Sabinene, aromadendrene, δ-cadinene, ledene (viridiflorene), limonene, globulol, and viridiflorol are also present in TTO but in lower amounts. The other high ingredients are γ-terpinene, α-terpineol, p-cymene, α-pinene, and terpinolene, with maximum levels of 13%, 8%, 8%, 6%, and 5%, respectively. The minimum and maximum amounts of these chemical components are given in Table 1.

This review primarily aims to gather and summarize the findings on the safety and regulations on the use of TTO in cosmetics and dermatological pharmaceuticals. It will also provide data on accurate and relevant information regarding the toxicity of TTO.

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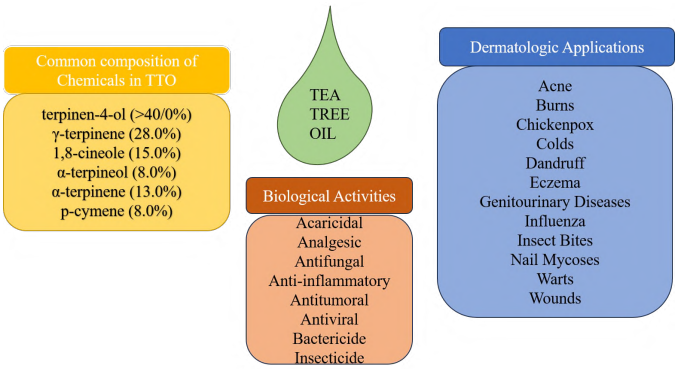
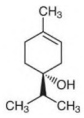
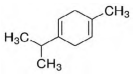
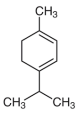
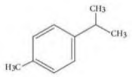
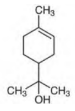
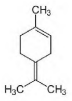


Figure 1. Summary of TTO's composition, activities, and situations where it can be applied

Table 1. *TTO's Constituents, Chemical Structures, and Percentage of Overall Content* (SCCP, 2008)

Constituent	**Content (%)**	
	Min.	**Max.**
Terpinen-4-ol 	30	48
g-terpinene 	10	28
1,8-cineole (eucalyptol) 	Trace	15
a-terpinene 	5	13
p-cymene 	0.5	8
a-terpineol 	1.5	8
α-pinene 	1	6
Terpinolene 	1.5	5

Search Strategy

The study conducts a literature search with the intent to collate, synthesize, and integrate the reports that have been published on TTO. Data have been gathered from three databases: PubMed, Web of Science, and Scopus. The searches have been restricted to papers published in English between 2015-2024, with research articles and comprehensive reviews both being chosen. The most common search terms have been chosen as "tea tree oil," safety of tea tree oil," regulations for tea tree oil," and "toxicity of tea tree oil." After searching in the databases, the exclusion criteria for the papers are articles (i) with no abstract, (ii) in languages other than English, or (iii) with no reliable data and statistics.

Safety Regarding Exposure to TTO

With regard to all the available data, TTO is considered generally safe and might help treat acne and other superficial skin infections when used topically. An earlier Scientific Committee on Consumer Products (SCCP, 2008) report calculated daily exposure to TTO for rinse-off and leave-on products. Systemic exposure levels between 1.7 and 3.33 µg/kg per day were estimated for various types of cosmetics. The SCCP report concluded that considerable systemic exposure could occur with topical application of TTO-containing products and TTO itself if used daily. The report also calculated worst-case estimations for general systemic and reproductive toxicity. However, the margin of safety (MoS) could not be calculated, as a lack of data is found regarding the bioavailability of TTO. A rate of 3% was calculated for the subcutaneous absorbance of TTO (Cross, Russell, & Roberts, 2008) . However, no data could be found on the oral bioavailability of TTO. Hence, converting between exposure routes is challenging.

Evaluating TTO Toxicity

With rising reports of TTO's therapeutic benefits, multiple TTO toxicity reviews have also been published. According to manufacturing companies, adverse effects of TTO are infrequent (less than 0.0016%) and only involve mild complaints. TTO causes numerous local adverse effects, including contact allergy (de Groot & Schmidt 2016), irritation (Zeiner et al., 2018) and dermatitis (Ambrogio et al., 2022) . However, most evidence suggests that diluting TTO can decrease these reactions.

Some components of TTO oxidize in ambient air and light, creating peroxides, epoxides, and endoperoxides, which have sensitizing properties and may cause allergic skin reactions (Ambrogio et al., 2022). These oxidation products are suggested to increase the toxicity of TTO. Although TTO is a modest skin sensitizer in susceptible individuals, oxidized TTO has stronger oxidizing effects. Manufacturers of TTO warn users against exposure to oxidized TTO (Thomas et al., 2016). According to the International Fragrance Association (IFRA), concentrated TTO is hazardous and bears the R-codes R-22 (harmful if ingested), R38 (skin irritant), and R65 (may cause lung damage if ingested), as well as the symbol Xn (harmful). These health threat indicators are also included in the safety data sheets of raw material suppliers (IFRA, 2022).

Cytotoxicity of TTO

Initially, studies were conducted to assess the cytotoxicity of TTO on cultured cells to ascertain its possible harmful effects. The toxicity of TTO was assessed over a diverse range of human cell cultures, including HeLa cervical cancer cells, MOLT-4 acute lymphoblastic leukemia cells, K562 erythromyeloblastoid leukemia cells, CTVR-1 B cells obtained from the bone marrow of a patient with acute myeloid leukemia, and fibroblast and epithelial cells. The experiments showed that TTO exhibited an inhibitory concentration 50 (IC_{50}) value for cell growth ranging from 20 to 2,700 µg/mL (Russo, Corasaniti, & Morrone, 2015). Terpinen-4-ol was shown to cause toxicity in meibomian gland epithelial cells based on dosage and exposure route (Chen, Wang & Liu, 2020) . At high concentrations, both TTO and its major ingredient terpinen-4-ol have long been known to cause cytotoxicity in human cells, including epithelial cells and fibroblasts. Moreover, TTO can exert antimicrobial activity. TTO does not directly induce cell wall alterations; however, it causes the release of autolytic enzymes associated with the cell membrane, which may induce lysis and subsequent leakage of nucleic acids across the damaged cytoplasmic membrane in bacteria (Low, Kenward & Martin, 2017).

Acute and Chronic Toxicity of TTO

TTO has an oral median lethal dose (LD_{50}) of 1,900 mg/kg in rats. According to the SCCP, undiluted TTO should not be consumed orally, as it is dangerous (Mertas et al., 2015). The LD_{50} value for γ-terpinene in orally exposed rats was found to be 5,000 mg/kg (Tabarraei, Hadi, & Mosavi, 2019). The Committee of Experts on Flavouring Substances of the Council of Europe evaluated eucalyptol as a natural flavoring content, and using a minimum lethal dose of 60 mg/kg/day with a safety factor of 300, they predicted 0.2 mg/kg/bw as tolerable daily intake (TDI) (SCCP, 2008).

No oral or dermal repeated dose toxicity studies regarding pure TTO were found in the literature. However, read-across considerations regarding the systemic toxicity of some ingredients have been performed. For terpinen-4-ol, γ-terpinene, 1,8-cineole, α-terpinene, p-cymene, α-terpineol, α-pinene, and terpinolene, the established or estimated LD_{50} and no observed adverse effect level (NOAEL) values are presented in Table 2 (European Medicines Agency, 2013).

Table 2. *Doses in Relation to TTO Constituent Toxicity and Safety* (European Medicines Agency, 2013)

TTO constituent	LD_{50} (mg/kg bw)	Animal species	Application route	NOAEL (mg/kg bw/day)	Animal species, period, toxicity
Terpinen-4-ol	1,300	rat	Oral		
	250	rabbit	dermal	400	rat, oral, 28-day study, kidney toxicity
γ-Terpinene	5,000	rat	dermal		
1,8-Cineole	430	rat	oral	300	rats and mice, subchronic toxicity study, hepatic and renal toxicity
	>2,000		dermal		
a-Terpinene	1,680	rat	oral	60	pregnant Wistar rats, oral, maternal systemic toxicity
				75	(as cumene/p-cymene) rat, oral, renal toxicity
α-Terpineol	2,900-5,170	rat	oral	500	male and female Wistar rats, oral, 28-day study
	2,000		dermal		
	2,830	mouse	oral		
	2,000		intramuscular		
α-Pinene	>2,000	rat	oral	250	weanling Osborne-Mendel male rats, oral, 28-day study, nephrotoxicity
Terpinolene	3,740	rat	oral		
	4,300	rabbit	Dermal		

TTO Toxicity Related to Exposure Routes

Essential oils enter the blood circulation in 30 seconds via mucosa and 4-12 minutes dermally. They reach internal organs and the nervous system within 20 min, resulting in systemic effects, and are excreted from the body through the kidneys (Pazyar, Yaghoobi &Kazerouni, 2013). Oral intake of the essential oil TTO lead to diarrhea, abdominal pain, rash, incoordination, and muscle weakness at relatively high doses, with these symptoms generally able to resolve within 36 hours. Oral TTO administration is not advised until more scientific investigations on its toxicity are completed (Özfenerci &Çalışkan, 2018).

Table 3 summarizes the poisoning cases in the literature. The literature does not mention cases of human death linked to TTO. A few studies have been published on accidental TTO poisoning in humans. The literature shows accidental ingestion of TTO to have varied from less than 10 mL to half a cup. Little information is found on the renal toxicity of TTO (Özfenerci &Çalışkan, 2018).

TTO is commonly administered to the skin as an essential oil. In addition, it is present in many cosmetics and personal care products, such as moisturizers, soaps, and shampoos. Therefore, dermal toxicity studies have great importance (Özfenerci &Çalışkan, 2018). After extrapolating the LD_{50} values on humans, dermally administered TTO through cosmetic products or as an essential oil can be concluded to not be assessable as harmful. Because up to 90% of TTO is a volatile liquid, it swiftly evaporates from the skin's surface. Its dermal absorption rate depends on factors such as body temperature, the integrity and age of the skin, the environment temperature and dilution rate, the amount covering the skin's surface, the chemical composition of the oil, and the application method used. TTO's lipophilic property allows it to enter the skin's surface layer; it boosts antimicrobial effects and may lead to moderate dermal toxicity (Mertas et al., 2015). However, only small amounts of the components of TTO enter the subdermal layers and into the bloodstream, with human skin not being readily able to absorb higher amounts of TTO to generate acute toxic effects (Caliskan & Karakus 2020).

In rabbits, the Draize irritation index for undiluted TTO is 5.0. This means TTO is a severe skin irritant. Human studies have presented conflicting results, such as no irritation with diluted and undiluted TTO, as well as skin irritation with undiluted TTO or cosmetic formulations containing 5% TTO. Such inconsistent results may be due to differences delivery methods, exposure routes, and exposure periods (SCCP, 2008). One Hen's Egg-Chorioallantoic Membrane Text (HET-CAM) assay found undiluted TTO and its 25% and 10% solutions in a surfactant to cause severe irritation, with TTO being a slight irritant at 5% dilution (Capasso, Abbinante & De Vernardo, 2022).

Topical administration of TTO is associated with few side effects, including irritation and allergic reactions. Irritant reactions can be reduced significantly by utilizing products with lower oil concentrations. Patch tests confirm allergic reactions

Table 3. *TTO Poisoning Case Reports in Humans*

Ingested amount	Gender and age	Clinical symptoms	Reference
½ teaspoonful	60-year-old male	a dramatic rash accompanied by leukocytosis; swollen face, hands, and feet	(Elliott, 1993)
2 teaspoons	4-year-old male	ataxia, shortly after progressed to unresponsiveness	(Morris, Donoghue, & Osterhoudt, 2003)
< 10 mL	23-mo-old male	confusion, unable to maintain balance; tripping and falling over; disorientation	(Jacobs & Hornfeldt, 1994)
< 10 mL	17-mo-old male	ataxia and drowsiness	(Del Beccaro, 1995)

to TTO can even occur at very low concentrations. Terpinen-4-ol and α-terpineol can penetrate the skin's epidermal layer and exert antibacterial, anti-inflammatory, and acaricidal effects. When testing a 20% TTO formulation in ethanol, only terpinen-4-ol (0.05% of the applied formulation) was able to completely permeate the epidermis (Thomas et al., 2016). A retrospective assessment of 41 instances of positive patch testing in Australia over 4.5 years concluded only 1.8% of the study population to be allergic to TTO (Chen et al., 2020).

Meanwhile, allergic contact dermatitis, systemic contact dermatitis, linear immunoglobulin-A disease, multiform erythema reactions, systemic hypersensitivity reactions, and idiopathic male prepubertal gynecomastia have also been observed (Mertas et al., 2015). Another study found the patch test findings of 311 volunteers to reveal an average irritancy score of 0.25. Yet another study that applied a patch test with 10% TTO to 217 people observed no irritation reaction in the volunteers, with the researchers suggesting that skin irritation could be avoided by using lower concentrations. Although many ingredients in TTO have been claimed to be able to lead to allergic reactions, the most important claim also stated that these result from oxidation products formed from outdated or badly preserved oils (Bekhof, Hunsel & Woerdenbag, 2023).

Lee et al. (2013) investigated the acute dermal toxicity of TTO using multiple dilution doses. According to their findings, skin irritation was reduced dramatically for TTO concentrations < 2.5%. They also further researched the major and minor TTO components that produce skin irritation, investigating Terpinen-4-ol, and 1,8-cineole mainly to determine whether they were the primary causes of skin irritation at a 5% concentration, TTO caused substantial skin irritation, with terpinen-4-ol comprising up to 30% of the mixture. Moreover, when examined at a concentration of 1.5%, terpinen-4-ol was determined to be non-irritating. In the Local Lymph Node Assay (LLNA), both whole TTO and its polyethylene glycol (PEG) solution (at ISO4730 quality) were found to be moderate sensitizers in mice (SCCP, 2008) .TTO is also suggested for sensitizing humans, with several patch test studies indicating an allergic contact dermatitis prevalence rate of 4.8% (European Medicines Agency, 2013) . Despite this, there is no clear data on the skin sensitization potentials of the individual constituents of TTO. Suggestions point towards the terpenoid fraction, limonene, and/or oxidative degradation products as possible culprits. Notably, oxidized TTO demonstrates three times more potent sensitization than fresh TTO. The increase in levels of p-cymene and 1,8-cineole over time may also contribute to the heightened sensitizing potency of TTO. Conversely, skin oxidative bioactivation of prohaptens to haptens is plausible. α-terpinene, a major constituent of TTO, can oxidize over time and become a hapten, potentially leading to skin sensitization (European Medicines Agency, 2013). However, further studies are necessary to determine definitively which constituent(s) of TTO are responsible for human skin sensitization.

TTO is currently being used successfully to eradicate ocular Demodex. However, an *in vitro* study showed the doses of TTO that exert demodicidal activity to be able to lead to toxic effects in human hepatic cells, cervical cells, breast epithelial cells, T cells, B cells, bone marrow cells, fibroblasts, and peripheral blood monocytes (Chen et al., 2020). A primary eye irritation study classified 1% and 5% TTO solutions as minimally irritating in rabbits. TTO concentrations less than 10% substantially reduced ocular discomfort and inflammation of the eyelids and conjunctiva (Messaoud et al., 2019) . On the other hand, TTO produces eye discomfort in certain patients when administered at high concentrations. Contact dermatitis, allergic reactions, and eye irritation are frequent consequences of TTO preparations (Ergun et al., 2020) .

Undiluted TTO was found to not cause phototoxicity in hairless mice (Infante et al., 2022). No other phototoxicity studies on TTO or its ingredients are present in the literature.

TTO's Potential for Reproductive and Developmental Toxicity

Data on the reproductive toxicity of the constituents of TTO are also limited, and the oral NOAEL values for reproductive

toxicity were found to be between 250-365 mg/kg/day. When applying α-terpinene at 30, 60, 125, and 250 mg/kg doses to female Wistar rats during their 6^{th}-15^{th} days of pregnancy, maternal toxicity was observed in both the 125 and 250 mg/kg/day dosage groups, with these two highest dosage groups showing reduced fetal body weights and increased kidney weights. Abnormal ossification of bones and minor skeletal abnormalities in fetuses were evident in the 60, 125, and 250 mg/kg dosage groups. The oral NOAEL values for embryotoxicity and fetotoxicity were suggested to be 30 mg/kg, and this value was 60 mg/kg for maternal toxicity (Cross et al., 2008; SCCP, 2008).

Genotoxicity of TTO

Testing the genotoxicity of essential oils and their components is critical for assessing their safety. The genotoxic effects of TTO and its components have been evaluated *in vitro* (Casalle & Andrade, 2020). Several mutagenicity tests in their study also examined the mutagenic potentials of TTO and its components. *Salmonella typhimurium* strains were used to test the mutagenic effects of commercially available TTOs. None of the TTO brands were found to exert a mutagenic effect on the *Salmonella* strains examined with and without metabolic activation in the Ames test. Terpinen-4-ol application also ended up with the same negative results. However, at higher doses, clear evidence of toxicity was observed in all *Salmonella* strains regarding all TTOs and terpinen-4-ol (Fletcher, Cassella & Cassella, 2005). Therefore, terpinen-4-ol was suggested as being the main constituent responsible for the significant antibacterial activity of TTO.

A genotoxicity study (Gomes-Carneiro, Felzenszwalb & Paumgartten, 1998) using *Salmonella typhimurium* strains with and without S9-mix reported no effect for TTO applications ranging from 100-1,500 µg/plate, Although TTO and most of its constituents are non-mutagenic, α-terpineol was found to cause a slight mutagenic effect related to dosage (0-2,500 µg/plate) in *Salmonella typhimurium* strain TA102 with or without metabolic activation. The other bacterial mutagenicity test strains in their study showed no mutagenic effects.

Moreover, several constituents of TTO were found to exert no mutagenic activity in various mammalian cells (SCCP, 2008). For example, Australian TTO (Batch ATTIA/0501) was tested to induce micronuclei in mouse bone marrow. Application doses were selected according to the preliminary study on mice conducted at oral doses between 500-2,000 mg/kg. All animals in the highest dose group showed wobbly gait, prostration, and labored breathing between 30 min-5 h after dosing. Polychromatic erythrocytes prepared from the bone marrow of each animal were counted for the incidence of micronucleated polychromatic erythrocytes. As a result, SCCP's study suggested Australian TTO to be non-clastogenic regarding the mouse micronucleus test.

No carcinogenicity studies have been performed with TTO or its constituents in the literature.

CONCLUSION

Many essential oils do not possess harmful effects and can be used safely via dermal application. However, concerns exist that some of these oils can be inhaled after dermal absorption, and inhalation may lead to systemic toxicity. Current scientific publications are limited, with most data having been obtained from observational *in vitro* studies. Therefore, the mechanisms underlying the toxicity of essential oils are not well documented. The past decade has seen increased interest in non-traditional and non-prescription natural medicines. Also, new approaches need to be found for treating skin diseases. When used and stored correctly (i.e., well-sealed and away from light and heat), TTO poses no danger to human health. Therefore, after reviewing the literature, this study can suggest TTO to be safe for treating dermatologic illnesses.

The stability of TTO in cosmetics and personal care products is affected by several factors. Undiluted TTO should not be used, as it clearly can cause skin reactions. Good formulation design and production techniques are critical. Moreover, users should store prepared products properly. They should be kept away from direct sunlight and avoid excessive exposure to heat and air. Antioxidants can be added to TTO formulations to prevent terpene oxidation, which causes skin sensitization. For example, one study collected storage stability data on different formulated items and monitored product stability using TTO's p-cymene content (European Medicines Agency 2013). The p-cymene content generally increased with storage duration but remained below the International Organization for Standards' upper limits.

Concluding whether or not herbal remedies pose a danger to human health is usually a complicated issue, as they are composed of various components. The effects and toxicities of herbal remedies arise as a combined effect of different chemical compounds with different characteristics. In the case of TTO, the components can make up anywhere from 1% to 48% of the total oil. Meanwhile, with their varying structures and physicochemical properties, these constituents have different kinetics and oxidation rates, thus adding another challenging and important issue regarding the further toxicological evaluation of TTO.

In conclusion, this study suggests that fresh products containing TTO are able to treat certain skin conditions at proper concentrations. As oxidation of certain constituents occurs over time, TTO should not be used after a certain date. Meanwhile, oral administration should be avoided. More in vitro and in vivo studies are needed to reveal the full safety profile of TTO and its constituents.

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Review Article

Imidazopyridine scaffold as an effective tubulin polymerization inhibitor

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ABSTRACT

Tubulin and the tubulin cycle, which have many vital cellular functions in living cells, are privileged targets for the development of anticancer drug candidates. In the processing of cellular processes, especially cell division, alpha and beta tubulin polymerize to form microtubules and continue the cycle by depolymerizing again. Disruption of the polymerization-depolymerization dynamics of microtubules by various agents causes mitotic cell arrest and subsequent cell death via apoptosis. This review summarizes the tubulin cycle, cancer, and target regions. Tubulin has three main target binding sites: taxane, vinca, and colchicine. In particular, the colchicine binding site, which is the current target for disrupting the tubulin cycle, is inhibited by various synthetic compounds, and the common properties of these compounds are emphasized. The results show that highly effective cytotoxic agents can be developed by modifying the imidazopyridine scaffold, which remains open to exploration. The remarkable antitubulin and cytotoxic effects of recently developed compounds with an imidazopyridine ring are interesting. A detailed report of anti-tubulin agents with imidazopyridine structures, among the tubulin polymerization inhibitors developed to date, and an evaluation of the structure–activity relationship is presented here. In addition, the new molecular topology established in this review based on the structure-activity relationships of imidazopyridine will inspire research groups to develop new imidazopyridine-based anti-tubulin agents with clinical anticancer potential in the near future.

Keywords: Anti-tubulin, Cytotoxicity, Imidazopyridine, Structure-Activity Relationship

INTRODUCTION

Cancer, which threatens humanity globally and ranks second among deaths with known causes, has been reported to have reached approximately 10 million deaths (or 1 in every 6 deaths) per year since 2020, according to WHO data, and even cancer-related deaths are increasing every year (Sung et al. 2021). Cancer, a heterogeneous and multifactorial disease, occurs with the uncontrolled growth and proliferation of cells with a series of molecular or genomic alterations in the cells (Brown et al. 2023). Under normal circumstances, after a cell completes its task, it receives a message that it has died and is replaced by a new, healthy cell (Galluzzi et al. 2007). However, cancer cells can continue to live and proliferate by exploiting the microenvironment around them to their advantage and begin to prevent the survival of other healthy cells (Aponte & Caicedo, 2017).

Chemotherapeutic agents continue to be an important key point in the treatment of cancer at the cellular level (Tilsed, Fisher, Nowak, Lake, & Lesterhuis, 2022). In general, classical chemotherapeutics can be classified as alkylating agents that cause DNA damage (Chu & Rubin, 2018), antimetabolites that inhibit DNA or RNA synthesis (Devita, Lawrence, & Rosenberg, 2008), topoisomerase inhibitors that disrupt DNA topology (Tewey, Rowe, Yang, Halligan, & Liu, 1984), and tubulin inhibitors that disrupt cellular functions (Schiff, Fant, & Horwitz, 1979). However, countless studies have been conducted on the agents that cause DNA-induced cellular death, which also cause serious side effects in healthy cells, and efforts are still being made to develop agents with low side effects (Swift & Golsteyn, 2014). Therefore, the development of targeted and minimally adverse chemotherapeutic agents is of vital importance in modern cancer treatment. Based on these findings, in this review, we focused on the treatment of cancer at the cellular level, specifically tubulin-targeted inhibitors.

Tubulin and microtubular cycle

Tubulin is a vital component of the eukaryotic cytoskeleton and microtubules in living cells. Alpha and beta tubulin, the main structures of tubulin proteins, polymerize and build microtubules that are involved in many cellular functions (Moore & Sarah, 2020). A single microtubule with a diameter of 25 nm is formed by the lateral combination of 13 end-to-end protofilaments, and when it reaches a specific concentration,

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the heterodimerization and subsequent polymerization process begins (Kaur, Kaur, Gill, Soni, & Bariwal, 2014). In addition, microtubules exhibit dynamic stability, constantly transitioning between periods of growth and shrinkage. The polymerization process of α and β-tubulins in microtubules, which hydrolyse GTP to GDP, undergoes depolymerization with GDP, causing the cycle to repeat (Figure 1) (Sontag, Staley, & Ericson, 2005; Akhmanova & Steinmetz, 2008). In addition to its important role in many cellular processes, such as protection of the cellular structure, intracellular transport, and signal transduction, it plays an important role in cell division. Disruption of this cycle makes microtubules an important target in anticancer drug research (Howard & Hyman, 2003).

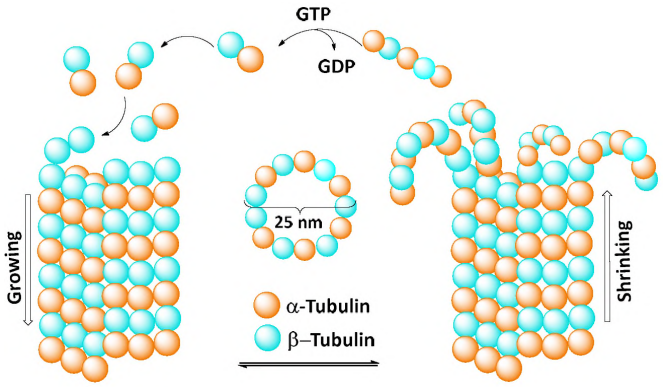

Figure 1. Cycle of microtubule; polymerization and depolymerization

Disruption of the polymerization-depolymerization dynamics of microtubules by various agents causes mitotic cell arrest and subsequent apoptosis (Jordan & Wilson, 2004). These agents target three binding sites in tubulin: taxane, vinca, and colchicine (Nogales, Whittaker, Milligan, & Downing, 1999; Ravelli et al., 2004). Among these agents, agents targeting the taxane binding site inhibit depolymerization, while agents targeting the vinca or colchicine binding site inhibit polymerization (Dumontet & Jordan, 2010). Although taxane and vinca alkaloids are highly effective, they have a complex structure that is difficult to synthesize, generally have poor bioavailability, and resistance to such ligands in cancer cells has focused research on the colchicine binding site (Li et al., 2015).

Colchicine binding site for inhibition of microtubule polymerization

The colchicine binding site consisted of a deep hydrophobic pocket located at the dimerized internal interface between α-tubulin and β-tubulin (Figure 2) (Gou, Li, Guo, & Zhen, 2019). The hydrophobic nature of the colchicine binding site has enabled the development of new tubulin inhibitors with colchicine-like hydrophobic properties.

The structure of colchicine (**1**) is less complex in terms of synthesis, allowing colchicine and its derived ligands to advance to clinical trials for the discovery of antitumor agents (Jordan, 2002). However, although the narrow therapeutic window of colchicine towards this target has enabled the development of some colchicine prodrugs, the short half-life of colchicine-based prodrugs and serious side effects, such as cardiotoxicity, have caused them to be withdrawn from clinical use (Lu, Chen, Xiao, Li, & Miller, 2012; Field, Kanakkanthara, & Miller, 2014; Lippert, 2007).

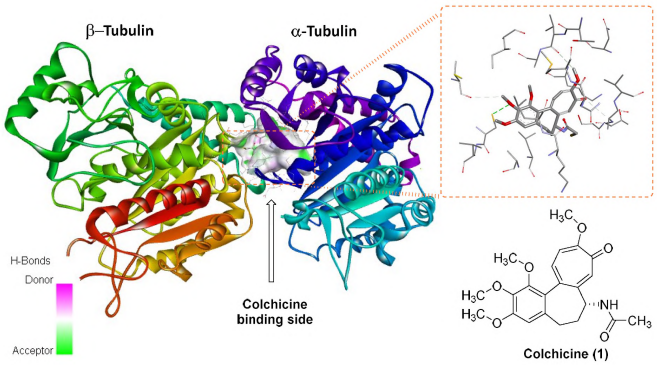

Figure 2. Colchicine binding site and colchicine structure located between α-tubulin and β-tubulin (Pdb code:1SA0)

Natural compounds targeting the colchicine binding site include steganacin (**2**), podophyllotoxin (**3**), and combretastatin (**CA-1** and **CA-4**), which are important antitumor compounds bearing bi-aryl groups like colchicine (Figure 3). Among these, the simple chemical structure and strong anti-tubulin cytotoxic effects of combretastatin derivatives are particularly interesting. However, the poor aqueous solubility, low bioavailability, and short biological life of combretastatin derivatives have led to studies on the development of new and more stable anti-tubulin ligands (Ohsumi et al., 1998).

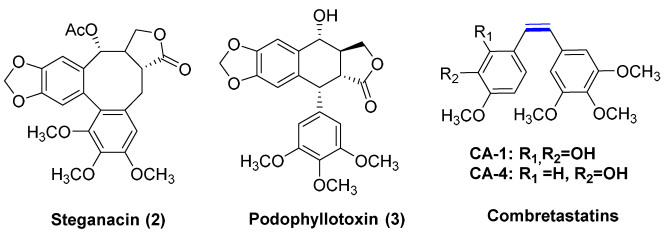

Figure 3. Natural compounds with antitubulin effects that target the binding site of colchicine

The simplicity of its structure and important pharmacological properties makes combretastatin a leading compound in the development of new anti-tubulin inhibitors. In structure-activity studies of combretastatin derivatives, it was determined that the bi-aryl group connected by an ethylenic bridge with a cis configuration provides optimum tubulin inhibition and cytotoxicity (Nam, 2003). This perspective has, over time, allowed the

development of heterocyclic structures such as imidazole (**4**), isoxazole (**5**), oxadiazole (**6**), triazole (**7,8**), and imidazopyridine (**9**) to lock the ethylenic cis configuration between the two aryl rings and to develop additional groups that can interact in the colchicine binding site (Figure 4) (Shan, Zhang, Liu, Wang, & Dong, 2011; Stroylov et al., 2020; Thammathong et al., 2023; Zhang et al., 2007).

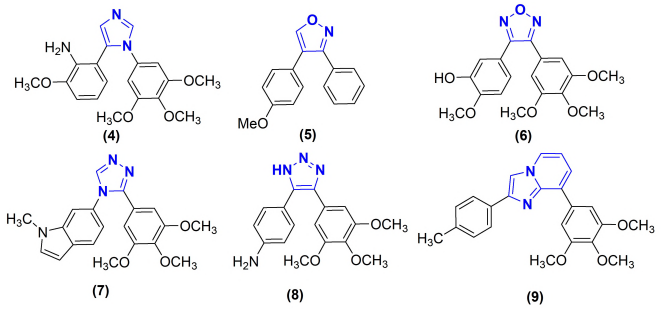

Figure 4. Tubulin polymerization inhibitors with a heterocyclic core bridge

The antitubulin effects of heterocyclic structures other than imidazopyridine on tubulin polymerization has been the subject of many reviews. The remarkable antitubulin and cytotoxic effects of recently developed compounds with an imidazopyridine ring are interesting. Therefore, this review focused on compounds with imidazopyridine rings that target the colchicine binding site for tubulin inhibition. The data to be presented will shed light on various research groups investigating the development of promising imidazopyridine-based anti-tubulin agents.

The privileged structure of imidazo[1,2-a]pyridines

The N-containing heterocyclic imidazopyridine structure, formed by the fusion of imidazole and pyridine rings, has attracted great attention in the field of medicinal chemistry due to its unique pharmacological properties. It is known that the imidazopyridine ring has major antiprotozoal, antibacterial, antifungal, antiviral, anti-inflammatory, hypnoselective, antipyretic, anxioselective, antiapoptotic, and anticancer activities. In fact, it has the main structure of imidazopyridine, which is in many clinical uses. For example, zolpidem (**10**) in the treatment of insomnia, alpidem (**11**), necopidem (**12**), and saripidem (**13**) as anxiolytics, zolimidine (**14**) in the treatment of peptic ulcers, rifaximin (**15**) in the treatment of traveller's diarrhoea, olprinone (**16**) as cardiotonic, miroprofen (**17**) as an analgesic, GSK812396 (**18**) in HIV detection, ND-09759 (**19**), and Q203 (**20**) in antituberculosis treatment have the imidazopyridine pharmacophore structure used in clinical and preclinical trials (Figure 5) (Khatun, Singh, Bader, & Sofi, 2022).

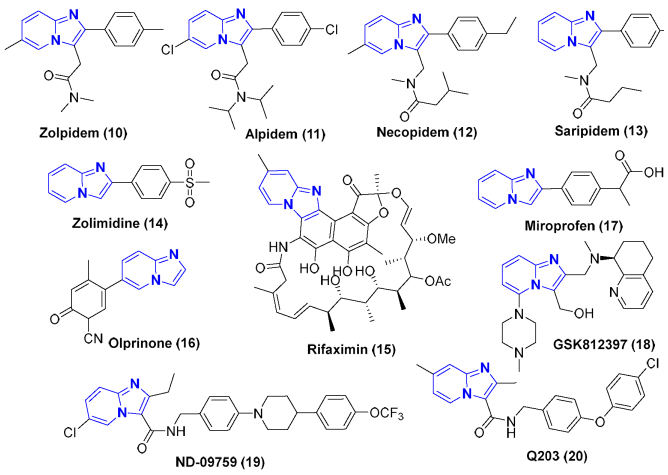

Figure 5. Structures of imidazopyridine derivatives and their candidate compounds

Recent studies have shown that compounds with an imidazopyridine structure can be used as anticancer agents because of their selective effects on various cancer pathways. These imidazopyridine-containing compounds, such as compounds **21** (Kim, Jeong, Lee, Hong, & Hong, 2011) and **22** (Kendall et al., 2007) as angiogenesis and PI3K inhibitors, compounds **23** (Kamal et al., 2010) and **24** (Martínez-Urbina et al., 2010) as CDK inhibitors, compound **25** as a promising agent for glioblastoma (Güçlü et al., 2018), and compound **26** (Xi et al., 2017) as a Nek2 inhibitor, have been reported to act as apoptosis-inducing agents (Figure 6).

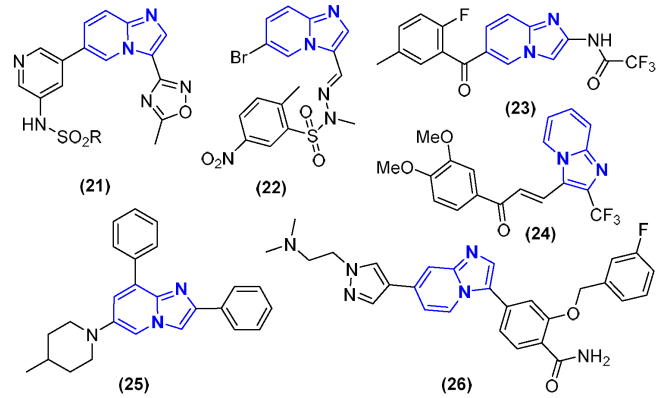

Figure 6. Imidazopyridine derivatives exert antiproliferative effects through various pathways

Efforts to develop different synthetic strategies for this privileged structure of imidazo[1,2-a]pyridines have been investigated, and various approaches have been adopted for this purpose. These can be classified into some subcategories, such as condensation, multicomponent, oxidative coupling, tandem reaction, and hydroamination reaction, and Figure 7 provides a

summary of the synthesis of imidazopyridine (Bagdi, Santra, Monir, & Hajra, 2015).

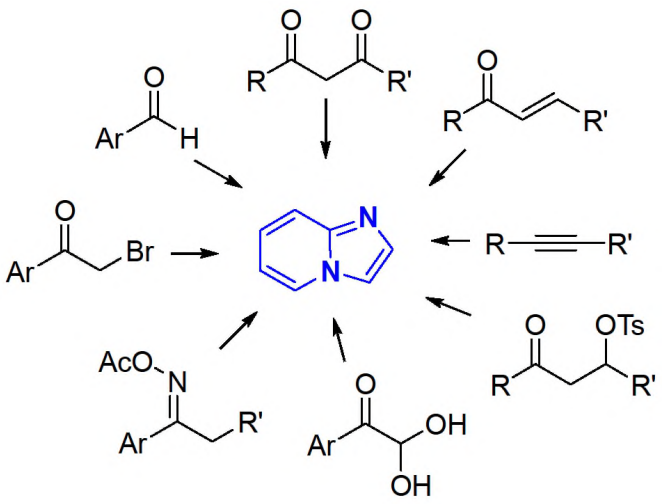

Figure 7. Synthesis of imidazo[1,2-a]pyridines from basic chemicals.

The unique pharmacological properties of imidazopyridine and their facile synthesis using easily accessible starting reagents intensify research on this ring. In this review, studies on the anticancer potential of imidazopyridine structure through tubulin polymerization inhibition, particularly in recent years, are summarized.

Scaffolds of imidazopyridine as tubulin polymerization inhibitors

In 2007, isoquinoline-linked imidazopyridine derivatives were synthesized using a three-component method, and their cytotoxic effects were examined using the A549 cancer cell line. These derivatives (especially compound **27**) exerted antitumor effects of up to 65% on A549 cancer cells at a concentration of 12.5 µM (Meng et al., 2007). Later, a new type of isoquinoline-fused imidazopyridine derivative was developed in 2011, with the idea that this compound could exert antitumor effects by inhibiting tubulin polymerization. It has been reported that compound **28**, developed as a microtubule-targeting agent, causes conformational changes in tubulin in cancer cells by targeting the colchicine binding site and can inhibit it by binding to tubulin at a concentration of 10.6 µM in a cell-free environment (Zhang et al., 2011). Subsequently, in 2016, a structurally simpler group was developed again based on the imidazopyridine structure, and it was found that compound **29** had higher cytotoxic activity in HeLa cancer cells and higher tubulin polymerization inhibition (IC_{50}: 3.41 µM) than colchicine (IC_{50}: 3.79 µM) (An et al., 2016) (Figure 8).

In another study in 2013, oxindole-linked imidazopyridine derivatives were designed and synthesized to investigate their

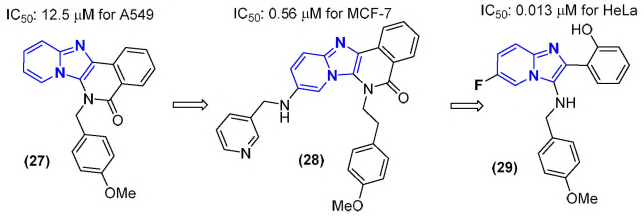

Figure 8. Development of tubulin polymerization inhibitors based on isoquinoline-fused imidazopyridine

cytotoxic effects on breast cancer cell lines. Among the synthesized series, compound **30** was observed to have a potential anticancer effect at an IC_{50}: 0.6 µM concentration and it arrested MCF-7 cells in the G2/M phase. Additionally, compound **30** inhibited tubulin polymerization comparable to that of colchicine, and SAR and molecular docking studies also showed that compound **30** targets the colchicine binding site (Kamal et al., 2013) (Figure 9).

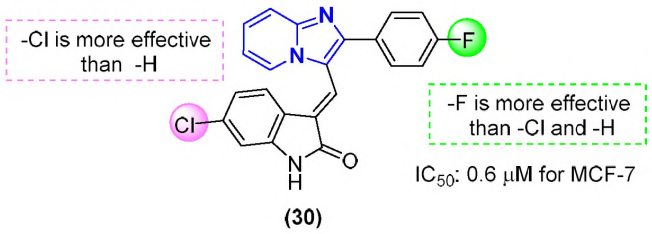

Figure 9. Oxindole-linked imidazopyridine derivatives for tubulin polymerization inhibition

In 2014, imidazopyridine-benzimidazole hybrid compounds were designed and synthesized for tubulin polymerization. The synthesized compounds were found to be effective in large-scale cancer cell line studies, especially in leukaemia, lung, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast cancer cells. Among the molecules, compound **31** was found to have the highest antiproliferative effect with IC_{50}: 0.43-7.73 µM concentration in the tested cell lines. Additionally, compound **31**, which inhibits tubulin polymerization at IC_{50}: 1.75 µM concentration, was found to arrest MCF-7 cells in the G2/M phase, inducing apoptosis. It has been reported that **31**, which targets the colchicine binding site in molecular docking studies, can also inhibit the PI3K/Akt pathway (Kamal et al., 2014) (Figure 10).

In another study in 2014, 2-aryl-3-arylamino-imidazopyridine derivatives were designed as tubulin polymerization inhibitors based on the CA-4 structure, and their effects on ex vivo tubulin polymerization were examined. Among the compounds understood to disrupt tubulin microtubule dynamics, compound **32** inhibited tubulin polymerization with IC_{50}: 12 µM concentration (IC_{50} for CA-4: 9 µM). It was determined that its cytotoxic effect on kidney cancer (HEK 293T) caused 50% cell death at 10 µM

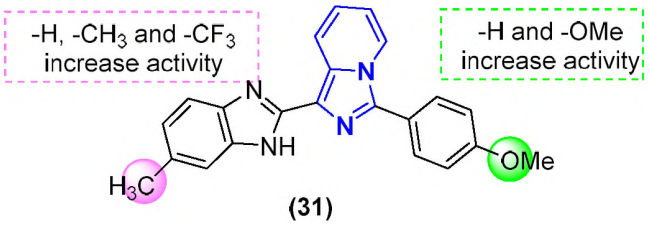

Figure 10. Benzimidazole-linked imidazopyridine derivatives for tubulin inhibition

concentration and had a lower cytotoxic effect on healthy Vero cells. In cell cycle analysis, compound **32**, which arrested HEK-93T cells in the G2/M phase, triggered apoptotic cell death and targeted the colchicine binding site via molecular docking studies (Figure 11) (Sanghai et al., 2014).

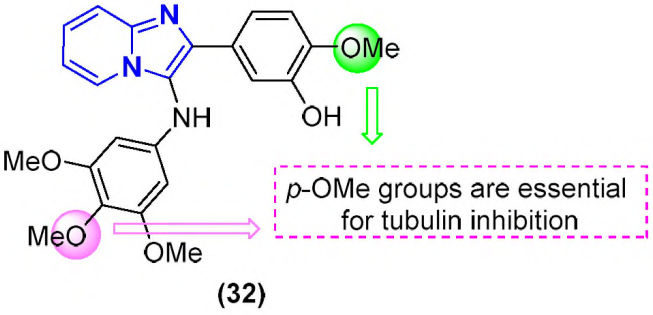

Figure 11. CA-4-inspired imidazopyridine derivatives inhibit tubulin growth

In a study conducted in 2015, a series of imidazopyridine-benzimidazole hybrid compounds were designed and synthesized, based on nocodazole with a benzimidazole structure, which is a colchicine binding site-targeted tubulin polymerization inhibitor. In the results of the cytotoxic effect of the synthesized compounds on the A-549 (lung), Hela (cervical), DU-145 (prostate), and B-16 (melanoma) cancer cell lines, compound **33** targeted the A549 cancer cell line with an IC_{50}: 1.48 µM concentration. In the cell cycle analysis of the A549 cell line, compound **33** arrested the cells in the G2/M phase. Compound **33**, which was found to trigger apoptotic cell death in advanced cell culture applications, inhibited tubulin polymerization at a concentration of 2.06 µM. In molecular modelling studies, it has been emphasized that compound **33**, which exhibits high binding affinity to the colchicine binding site, may be a potential anticancer agent (Kamal et al., 2015) (Figure 12).

A series of imidazopyridine-propenone conjugates for tubulin polymerization inhibition were designed and synthesized in 2017. The cytotoxic effects of the synthesized compounds on prostate (DU-145), lung (A549), cervical (Hela), and breast (MCF-7) cancer cell lines were examined, and compound **34** was found to have a significant cytotoxic effect on the A549 cancer cell line (IC_{50}: 0.86 µM). Flow cytometry analysis showed

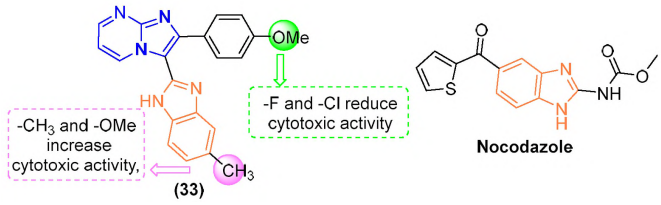

Figure 12. Nocodazol-inspired imidazopyridine/pyrimidine derivatives inhibit tubulin growth

that compound **34** arrested cancer cells in the G2/M phase by inhibiting tubulin polymerization (IC_{50}: 1.82 µM). Additionally, compound **34** was found to induce apoptosis in Hoechst staining and Annexin V-FITC assays and was also found to target the colchicine binding site via molecular dynamics studies. Therefore, it has been reported that the developed imidazopyridine derivatives may be new anticancer agents that inhibit microtubules (Sayeed, Nayak, Shareef, Chouhan, & Kamal, 2017) (Figure 13).

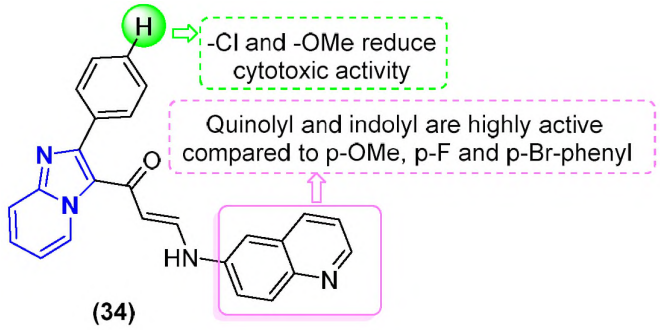

Figure 13. imidazopyridine-propenone conjugates for tubulin inhibition

In 2017, carbonitrile-substituted imidazopyridine derivatives targeting the colchicine binding site were designed based on the CA-4 structure. First, cytotoxic activity studies were conducted by synthesizing derivatives with 2,3,4-trimethoxyphenyl groups, and compound **35** was determined to be the most potent compound in terms of structure–activity relationship. Based on the structure–activity relationship results, a series of indolyl-containing imidazopyridine derivatives were synthesized using the biosterism approach. The cytotoxic effects of the compounds on five different cancer cell lines (HT-29, H460, A549, MKN-45, and SMMC-7721) were examined. The most potent compound **36** exhibited remarkable cytotoxic effects with IC_{50} values of 0.01 µM, 0.04 µM, 0.54 µM, 2.4 µM, and 5.6 µM, respectively. Immunofluorescence studies showed that the compound competitively targets the colchicine-binding site, and the compound was found to indeed target the colchicine-binding site in *in silico* studies (Liu et al., 2017) (Figure 14).

In another study, a series of triazole-linked imidazopyridine derivatives were designed and synthesized in 2018. The cytotoxic effects of the compounds on prostate (DU-145), lung

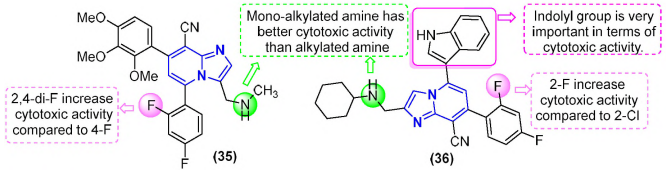

Figure 14. Carbonitrile-substituted imidazopyridine derivatives for tubulin polymerization inhibition

(A549), HCT-116 (Colon), and breast (MCF-7) cancer cell lines were examined, and compound **37** was found to have an IC_{50}: 0.51 µM value in the A549 cancer cell line. In flow cytometry analysis, it was emphasized that it causes cell death through apoptosis by arresting cells in the G2/M phase. Additionally, in immunocytochemistry studies, compound **37** was found to inhibit the polymerization of microtubules with a nocodazole-like effect (Sayeed, Vishnuvardhan, Nagarajan, Kantevari, & Kamal, 2018) (Figure 15).

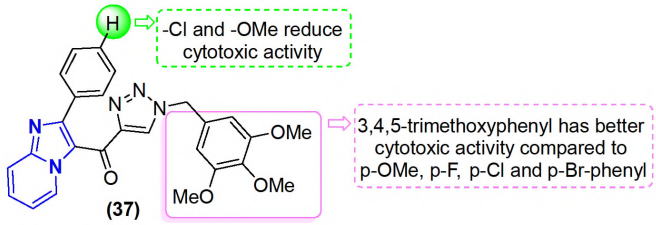

Figure 15. Imidazopyridine-linked triazoles as tubulin inhibitors

In 2018, new compounds with curcumin-inspired imidazopyridine structures were reported to be potential anticancer agents as tubulin polymerization inhibitors. Their antiproliferative effects on cancer cell lines were tested using the MTT assay. Compounds are effective against cervical cancer (HeLa), gastric cancer (HGC-27), lung cancer (NCI-H460), prostate cancer (DU-145 and PC-3), and breast cancer (4T1) compared with normal human prostate (RWPE-1) cells. The results showed that compound 38 was the highest antiproliferative agent in PC-3, HGC-27, and HeLa (IC_{50}: 2.11 ± 0.27 µM, 2.21 ± 0.25 µM, 2.53 ± 0.01 µM respectively). Additionally, compound **38** was found to be effective in the G2/M phase in PC3 cells and inhibited tubulin polymerization with IC_{50}: 8.44 µM. Additionally, molecular docking results confirmed that compound **38** targets the colchicine binding site (Ramya et al., 2018) (Figure 16).

In a study conducted in 2021, oxadiazole-linked imidazopyridine derivatives were designed as tubulin polymerization inhibitors, and their antiproliferative activities were examined in lung cancer (A549) and prostate cancer (PC-3, DU-145) cell lines. Among the compounds, compound **39** showed very high cytotoxicity with an IC_{50} value of 2.8 µM in the A549 cell line and arrested A549 cancer cells in the G2/M phase. Additionally, compound **39** was observed to inhibit tubulin polymerization (IC_{50} 3.45 µM). Molecular modelling studies have determined

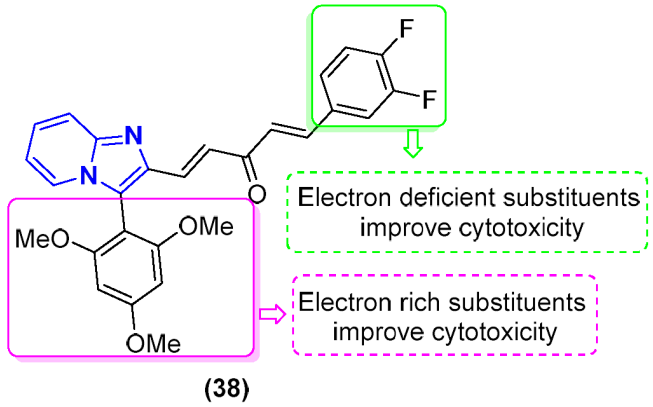

Figure 16. Curcumin-inspired imidazopyridine derivatives inhibit tubulin growth

that the compound has a high binding affinity in the α/β-tubulin active site (Sigalapalli et al., 2021) (Figure 17).

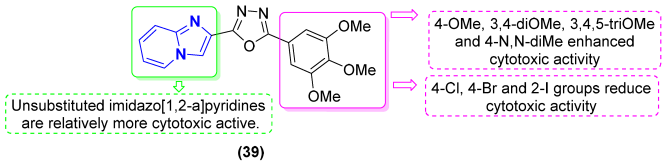

Figure 17. Oxadiazole-linked imidazopyridine derivatives for tubulin inhibition

In a study conducted in 2022, by performing structure-based virtual screening for microtubule-targeted ligands, it was determined that 1000 ligand molecules matched according to colchicine binding site-targeted pharmacophore groups. 2746 of these compounds were eliminated by the docking method, 99 were determined to be antitubulin targeted, and 13 were able to exceed toxicological risks. The cytotoxic effects of 13 related compounds (MCF-7, MDA-231 and A549) on cancer cells were examined, and compound **40** was determined to have very high cytotoxicity ($IC_{50} \leq 20$ µM). Interestingly, among these compounds, only the imidazopyridine-containing compound was determined to be the highest tubulin polymerization inhibitor (IC_{50}: 6.1 uM). It was determined that compound 40, which was determined to arrest MCF-7 cells in the G2/M phase in cell cycle analysis, actually targets the colchicine binding site in molecular docking and dynamic studies. The synthesizability of the target compounds was also investigated. Based on the SAR analysis results of compound **40**, a new tubulin polymerization inhibitor compound **41** containing pyrimidine was synthesized and found to be a tubulin polymerization inhibitor from the molecular dynamics results (Elseginy, Oliveira, Shoemark, & Sessions, 2022) (Figure 18).

In a study conducted in 2023, novel N-imidazopyridine-noscapine derivative anti-tubulin agents with high affinity for the colchicine binding site were designed using *in silico* meth-

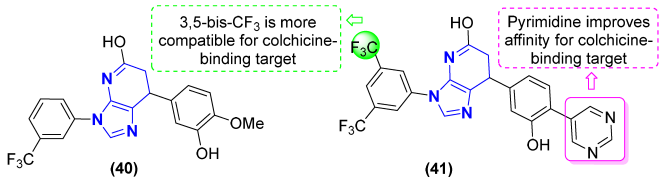

Figure 18. Imidazopyridine scaffolds were prepared via virtual screening and docking for tubulin inhibition

ods. Among the designed and synthesized compounds, compound **42** was found to have very high antiproliferative activity in MCF-7 and MDA-MD-231 breast cancer cell lines (IC_{50} value is 5.26 µM against MCF-7 and 7.78 µM against MDA-MB-231) and had no cytotoxic effect on healthy cells (IC_{50}: 1510.4 µM for HEK cell line). It was determined that compound **42** arrested the MDA-MD-231 breast cancer cell line in the G2/M phase and dramatically reduced the solid tumour without damaging any organs in the *in vivo* model (Pragyandipta et al., 2023) (Figure 19).

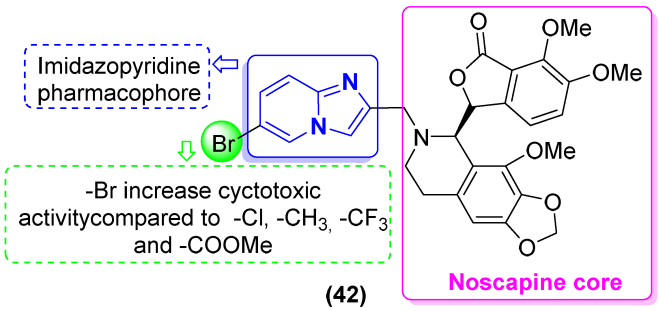

Figure 19. Imidazopyridine-noscapinoids for tubulin inhibition

CONCLUSION

Microtubules are a promising target for developing potent anticancer drug candidates. Inhibition of the tubulin cycle, which is involved in many cellular processes, enables the development of safe drug candidates with lower toxicological risks. Targeting the colchicine-binding site in tubulin targets and even developing simpler chemical structures than colchicine and combretastatin for this target could create potential anticancer drug candidates. Based on the crystal structure of tubulin, it is now easier to design selective synthetic compounds for tubulin inhibition. Highly effective antiproliferative agents have been developed with various cyclic and heterocyclic molecular modifications, such as substituted phenyl, indole, quinoline, and benzimidazole, which have been developed for the colchicine binding site. However, due to the unique pharmacophore feature of the imidazopyridine structure for the colchicine binding site, there is no doubt that even more effective tubulin polymerization inhibitors can be achieved with various molecular modifications of this compound.

Based on the imidazopyridine structure we present here, various tubulin polymerization inhibitors developed to date have been investigated in detail in terms of structure–activity relationship and summarized under four main headings. Imidazopyridine ring; 1st: The unsubstituted form of positions 5, 6, 7, and 8 is an effective pharmacophore group; 2nd: the 2-position should be phenyl or phenyl with electron-donating groups; 3rd: The 3-position must contain a sp^2 hybridized atom or a directly bonded cyclic or heterocyclic group; 4th: It can be said that heteroaryl groups, especially heterobicyclic groups, are more effective (Figure 20).

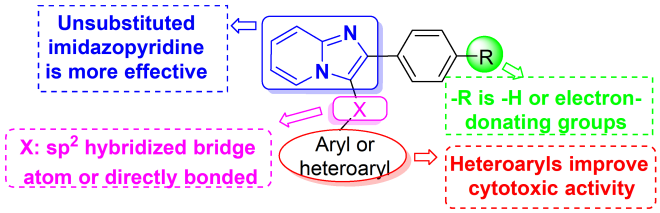

Figure 20. Structure–activity relationship of the imidazopyridine scaffold as a tubulin inhibitor

Together with all these suggestions, this article provides insights for the innovative design of new imidazopyridine-based antitubulin agents with increased efficacy, specificity, safety, and openness to discovery. Moreover, the imidazopyridine structure is a promising pharmacophore for tubulin polymerization and may serve as a potential lead for the synthesis of clinically important candidates in the near future.

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Case Report

Hypersensitive reactions to platelet transfusion: A case report of urticarial hives and pre-septal cellulitis in the context of a patient with Pre-B acute lymphoblastic leukaemia patient

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ABSTRACT

Background and Aims: Adverse transfusion reactions (ADR), such as fever, chills, and urticarial rashes, are significant in clinical practice, particularly in patients with complex histories. Urticarial reactions, characterised by red, itchy welts, are hypersensitivity reactions, whereas cellulitis, a bacterial infection, differs in aetiology and treatment. This study contributes to a better understanding of hypersensitivity in platelet transfusion and improves clinical management strategies for similar cases in the future. In a contemporary case, a 13-year-old man with pre-B ALL receiving chemotherapy developed widespread urticarial rashes, eye redness, and swelling after receiving four units of random donor platelets. Symptoms were successfully managed with symptomatic treatment after consultations with an ophthalmologist and dermatologist.

Materials and Methods: The methodology involves a systematic approach using the Naranjo ADR Probability Scale, Hartwig Severity Scale, and Shumock and Thornton Preventability Scale. These scales comprehensively evaluate different aspects of the case, including the likelihood, severity, and preventability of adverse drug reactions.

Results: Platelet transfusion causes urticarial hives and pre-septal cellulitis. The Naranjo ADR Causality Assessment scored it as 8 ('probable'). The Hartwig Severity Scale classified it as Level 3 ('Moderate'), and the Shumock and Thornton Scale deemed it 'Probably Preventable', emphasising the need for preventive measures.

Conclusion: This case underscores the complexities of managing transfusion responses in pre-B ALL patients, emphasising the need for close monitoring, timely intervention, and the use of structured adverse drug reaction (ADR) evaluation tools to effectively minimise the risks associated with blood transfusions.

Keywords: Urticarial, Hives, Cellulitis, Transfusion reaction, Platelet transfusion, Leukaemia

INTRODUCTION

The act of transfusing blood has evolved since the first effort in the 17th century, beginning with the use of whole blood and its components for specific uses, such as Red blood cells, platelets, White blood cells, frozen plasma, and plasma derivative products. Platelets are essential for haemostasis because they respond to vascular injury. When the serious and deadly haemorrhagic side effects of chemotherapy in leukaemia were studied in the 1950s and 1960s, the necessity of platelet component therapy was widely recognised (Freireich, 2011). Blood was first collected in glass bottles in the mid-twentieth century, causing platelets to degrade over time. The invention of plastic bags altered blood storage at the same time. To store their short shelf life of 5 days (Askari, Nollet, Debol, Brunstein, & Eastlund, 2002).

Platelet transfusions can cause allergic and anaphylactic reactions, with between 0.09% and 21% of individuals at risk. The severity of these reactions varies greatly, with isolated pruritus and urticarial infection being the only cutaneous manifestations (Behnke, 1970). Systemic effects include bronchitis, hypotensive response, and shock. Only 5% of allergic reactions are linked to temperature increases of one degree or higher.(Behnke, 1970).

Transfusion reactions are adverse reactions to the transfusion of complete blood or one of its components and can be minor to life-threatening in intensity (Behnke, 1970). Acute reactions

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can occur immediately after transfusion, whereas delayed reactions may occur from days to weeks later. Pathophysiology varies according to the transfusion reaction (Jacquot & Delaney, 2018)

Acute reactions include mild allergic, febrile non-haemolytic, septic, acute haemolytic transfusion reactions, transfusion-related acute lung injury (TRALI), transfusion-associated circulatory overload and anaphylactic reactions (TACO). Anaphylactic reactions have more serious outcomes, such as the development of antibodies against IgA in patients with IgA deficiency. Delayed reactions and transfusion-associated graft-versus-host disease are also possible (Suddock & Crookston, 2024).

In cases of suspicious reactions, transfusions should be immediately withdrawn, and the blood bank and treating physician should be informed (Siddon, Kenney, Hendrickson, & Tormey, 2018). This report serves as a reminder to physicians of the therapeutic consequences of transfused blood products in such patients and the possibility of urticarial hives and cellulitis in patients allergic to blood products (Tariket et al., 2018; Margo, 1999).

MATERIALS AND METHODS

Study design

A detailed case study involving a patient with pre-B acute lymphoblastic leukaemia who experienced hypersensitive reactions, including urticarial hives and pre-septal cellulitis, following platelet transfusion was conducted. The patient's comprehensive medical history, presenting signs and symptoms, and treatment regimen were meticulously reviewed and documented.

Ethical clearance for this study was obtained from the Institutional Ethics Committee of KLE College of Pharmacy. Approval was granted on February 23, 2023, with reference number KLE/COP/EC/135/2023-2024, under the study title "Hypersensitive Reactions to Platelet Transfusion: A Case Report on Urticarial Hives and Pre-Septal Cellulitis in the Context of a Pre-B Acute Lymphoblastic Leukaemia Patient."

Following ethical approval, further actions included detailed monitoring of the patient's response to treatment, comprehensive data collection, analysis of incidence using ADR scales, and management of hypersensitive reactions.

Adverse drug reaction (ADR) scales such as the Naranjo Algorithm adverse drug reaction assessment tool, Hartwig's Severity Assessment Scale, and Schumock & Thronton Preventable Scale are employed to systematically assess the likelihood that a drug or transfusion reaction is due to a specific medication or component. These scales utilise a series of questions to evaluate various factors, including the temporal relationship between drug administration and adverse events, alternative causes, drug levels, patient history, and outcomes following de-challenge and re-challenge. Utilising these scales helps ensure a standardised approach to identify and manage ADRs, enhance patient safety, and guide clinical decision-making. In the context of this study, applying ADR scales allows for a rigorous evaluation of the hypersensitive reactions observed, ensuring that the findings are robust and actionable (Figure 1).

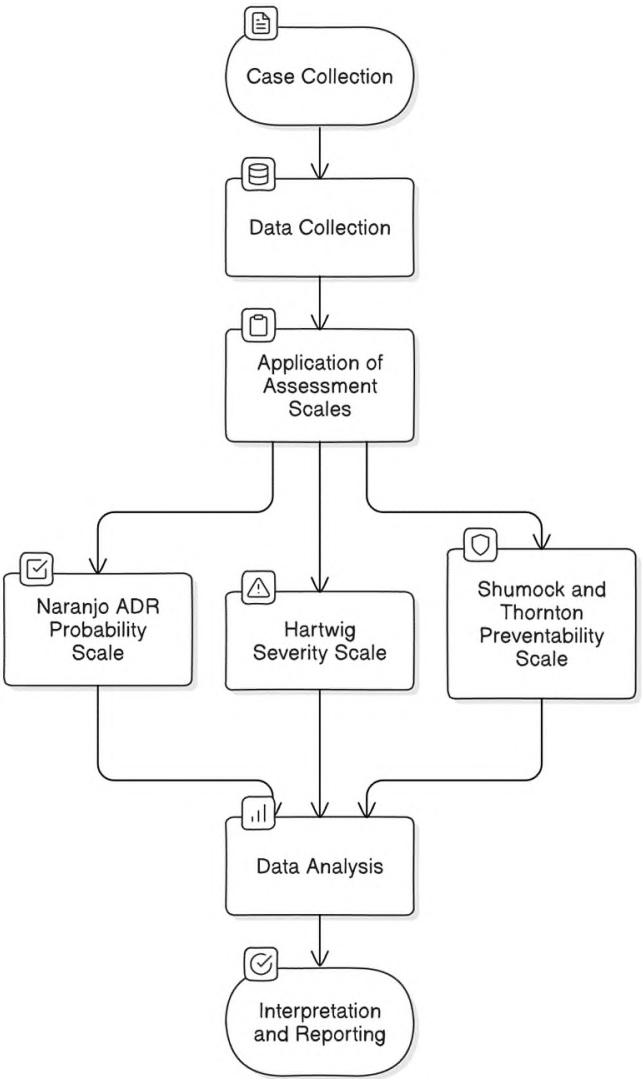

Figure 1. Methodology

Patient Characteristics

A 13-year-old boy who had previously experienced pre-ball came to the emergency room complaining of three episodes of loose stools with a yellowish consistency, as well as a cough that began slowly but was not associated with expectoration. In addition, there had been one episode of vomiting food particles, followed by nausea and facial petechiae for a day. All these signs were subtle. Laboratory results revealed abnormalities, including thrombocytopenia (96 x 103/μL), decreased lymphocyte counts (14%), elevated neutrophil counts (76% of the

manual leukocyte differential count), and no eosinophil counts. The red cell count, packed cell volume, reticulocyte count, and haemoglobin levels were 3.38 x 106/cm3, 10.5% g/dL, and 32.1%, respectively. Bilateral crept into the infra-capsular and infra-axillary regions, according to a systemic review.

The child was admitted to a paediatric emergency ward with a history of lower respiratory tract infection and Pre-B-ALL for 10 years. No family history of Pre-B-ALL was found. The patient was subsequently directed to an ENT specialist for treating laryngitis (lower respiratory tract infection). He complained of a foreign body sensation and a dull, throbbing pain in his throat that had been there for five days. The pain was subtle at first but gradually worsened and was accompanied by trouble swallowing foods and drinks. Due to a history of nasal obstruction for the past 3 days, the patient was advised to take medication in the form of nasal drops, i.e., Otrivin (xylomethazoline) nasal spray. Local examination of the Eyes, Nose &Throat indicated the following:

1. Oral throat cavity is normal.
2. Oropharynx: Grade 3 tonsil hypertrophy (+); posterior pharyngeal wall: postnasal dry (+).
3. Nose: ala vestibule (+), crusting (+), bilateral nasal cavity crusting (+), no para-nasal sinus tenderness.

The final diagnosis of LRTI was grade 3 tonsillitis. This was managed using the following treatments: saline nasal drops (2 drops thrice a day), Otrivin-P (Xylometazoline Hydrochloride and Sorbitol) nasal spray (2 drops thrice a day), Syrup Mucolite (Ambroxol) 5 ml BD, Alex lozenges (Dextromethorphan Hydrobromide) BD, Tab. Paracetamol (Acetaminophen) to treat pain and betadine (Povidone iodine) gargle. The patient was further managed with antibiotics (Piperacillin/Tazobactam, Amikacin, Azithromycin, Ciprofloxacin/Tinidazole) and started on i.v. fluids (Sodium chloride), and shifted to the paediatric haematology-oncology ward for further management of Pre-B-All. On the 14th day of hospitalisation, after the LRTI symptoms had been suppressed, he was transfused with 2 points of packed cell volume due to a considerable reduction in haemoglobin to 6.4%, which was 9.5% after receiving 2 pints of PCV. On the 16th day of his stay, he received a 4-pint random donor platelet (RDP) transfusion due to thrombocytopenia (28x103/µL) (see Table 1). His grandfather's records revealed that he developed rashes all over his body with itching immediately after receiving a platelet transfusion in less than ten minutes—and that within 2 days that is 48 hrs, he noticed redness in his eyes with swelling and pus production. These symptoms were initially insignificant but were eventually intensified. He had received an Avil injection (Pheniramine Maleate) and a Cetirizine tablet (Cetirizine Hydrochloride) after consultation with dermatologists and ophthalmologists. Subsequently, ophthalmologists discussed local examinations and noted conditions such as erythema (+), lid oedema (+), inflammation symptoms (+), conjunctional congestion (+), and watery discharge linked to pus (+), which led to the diagnosis of pseudo-septal cellulitis. To address this, he was prescribed an eye drop called moxiflox (moxifloxacin) and was advised to take antibiotics. The dermatologist reported an itchy rash with hypopigmentation all over the body. After performing local examinations, the condition was ultimately determined to be urticarial hives, for which the patient was prescribed cetirizine tablets to be taken orally and Calamine lotion to be administered topically. The patient's rashes were improving, and the puffiness in his eye decreased after receiving anaphylactic treatment. As indicated in Table 2, the Naranjo Score of "08" so the adverse medication reaction can be categorised as probable. (causality assessment). The ADR was categorised as moderate in severity by Hartwig's severity evaluation scale (Table 3) and probably preventable by Schumock and Thornton's Preventability Scale (Table 4). All the evaluation findings were combined into an ADR analysis, which is described in Table 5. The results of all evaluations were compiled into an ADR analysis, as shown in Table 2.

- **Annotation:** According to **Naranjo's Adverse Drug Reaction Causality Assessment**, a score of 8 indicates that an adverse drug reaction (ADR) is probable, indicating a strong likelihood that the drug caused the reaction, although other factors might be involved. This score reflects a high level of evidence supporting the drug's role in the reaction, based on criteria such as timing, de-challenge, and re-challenge.
- Hartwig's Severity Assessment Scale assigns a severity level of 3, indicating moderate severity. This rating indicates that ADR had a significant impact on patient health but did not present an immediate threat to life or require urgent care.
- The **Schumock and Thornton Preventability Scale** classifies ADR as probably preventable. This suggests that proper premedication, monitoring, and alternative transfusion strategies could potentially prevent hypersensitivity reactions—urticarial hives and pre-septal cellulitis—could have potentially been avoided, thus enhancing patient safety (Table 2).

DISCUSSION

Urticarial hives, one of the most common ADRs, can cause severe hypersensitivity reactions in people with low immunoglobulin A if therapy is delayed and is considered a Type I (immediate) hypersensitivity reaction, typically triggered by allergens, medications, or infections, leading to the release of histamine and other inflammatory mediators (Freireich, 2011; Behnke, 1970). Hypersensitivity reactions are defined as an exaggerated or inappropriate immune response to a substance that is harmless to most people (Freireich, 2011; Tariket et al., 2018). Cellulites are likely to cause a strong urticarial hypersensitivity reaction to abnormal stimuli. Studies have indicated that cellulitis can develop within 24 hours of a tender rash on the body (Choi et al., 2021; Ramanathan, Triulzi,

Table 1. Complete blood count (CBC) laboratory investigations before and after platelet transfusion

	30/01	**03/02**	**11/02**	**18/02**	**24/02**	**01/03**
HAEMOGLOBIN (13-16 %)	10.9	8.3	7.1	8.3	10.1	10.2
RED BLOOD CELL (4.5-6.5 million/cmm)	3.38	2.81	2.44	2.84	4.17	4.07
PLATELETS (1.5-4.5lakhs/cmm)	96000	1.14	22000	34000	34000	2.02
WHITE BLOOD CELL (4000-11000)	5.5	11.3	1200	1000	2100	4900
NEUTROPHILS (40-70%)	76	76	65	44	46	56
LYMPHOCYTES (20-40%)	14	15	21	34	20	20
EOSINOPHILS (1-8%)	0	0	2	15	26	22
MONOCYTES (2-10%)	10	9	12	7	9	1
BASOPHILS (1-10%)		0	0	0		
ABSOLUTE NEUTROPHIL COUNT (2500-6000 /µL of blood)			0.8	400	900	1000
ABSOLUTE LYMPHOCYTE COUNT (1000-4800 /µL of blood)			0.3	400	400	2700

Table 2. Analysis of platelet transfusion-related adverse reactions using different ADR assessment scales.

Adverse Drug Reaction Assessment Scale						
Drug	**Adverse drug Reaction caused**	**Naranjo 's ADR casuality assessment**		**Hartwig severity assessment scale**		**Schumock–Thronton preventable scale**
		Score	**The type of ADR**	**Level**	**The type of ADR**	
Platelet transfusion	Urticarial hives and pre-septal cellulitis	8	Probable	3	Moderate	Probably preventable

& Logan, 1997). Most cases in the study included subjects of all sexes and ages with clinical symptoms of hypotension, chest pain, dyspnoea, severe cyanosis, and transient diffused pulmonary infiltrates, with a history of Hodgkin's lymphoma, acute melody leukaemia, Chronic Obstructive Pulmonary Disease and Diabetes, wherein the condition was managed by either withdrawing platelet transfusion or by administering corticosteroids and histamines (Nevala-Plagemann, Powers, Mir-Kasimov, & Rose, 2019). This study presents a 13-year-old male patient with a complaint of loose stools and a cough yellow in colour. To treat thrombocytopenia, platelet transfusion was routinely performed on examination of unusual reports along with other medications, such as antibiotics (Injection Pipzo (Piperacillin/Tazobactum) 2.5 mg IV TID, Injection Akmin (Amikacin) 375 mg IV OD, Injection Targocid (Teicoplanin) 250 mg IV BD, Tablet Azee (Azithromycin) 250 mg OD), Injection Paracetamol 375 mg IV SoS in the event of a fever spike, and supportive treatment for tonsillitis and LRTI. Numerous case studies and reviews have demonstrated that hypersensitive reactions are more frequently linked to platelet transfusion (Zaki, 2011). Although platelet transfusion is effective in treating urticarial hives in several studies and publications, this incidence continues to occur, most likely because of a lack of awareness among some populations regarding the possibility of adverse drug reactions. If such reactions are to be prevented in the future, concerns regarding targeted therapy and drug reaction monitoring during transfusions in a specific population must be addressed. In the present study, the assessment of adverse reactions was performed using the ADR causality assessment scale, the Naranjo scale, where the total score was calculated as 8 (Zaik, 2011), the Hartwig severity assessment scale (Askari et al., 2002), and the Schumock and Thornton preventability scale (Blieden et al., 2014).

The Naranjo ADR Causality Assessment scored the reaction as 8 ('probable'). The Hartwig Severity Scale classified it as Level 3 ('Moderate'), and the Shumock and Thornton Scale deemed it 'Probably Preventable,', emphasising the need for preventive measures (Nevala-Plagemann et al., 2019)

Table 3. Comparison of pertinent articles focusing the shared and contrasting aspects of case reports.

References (Author names)	Age/ gender	Clinical features	Onset of symptoms	Laboratory investigation	Patient history	Treatment
Eche et al., 2019	43-year-old male	Right scrotal mass and elevated human chorionic gonadotropin and lactate dehydrogenase.	20 min	Chest tightness followed by mild dyspnea and dry cough.	Cell tumour, comprised of seminoma (40%), choriocarcinoma (20%)	IV 100 mg of hydrocortisone, 25 mg of diphenhydramine, and 20 mg of famotidine
Margo, 1999	A 67-year-old woman	----------	immediately	bilateral turgescence and redness of the conjunctiva and eyelids	----------	----------
Blieden et al., 2014	A 59 year-old male	hypotension (blood pressure 77/40mmHg), diaphoresis, respiratory distress, and atrial fibrillation with rapid ventricular response (heart rate 200 beats per minute)	10 min after blood transfusion	elevated white blood count ($37\times10^3/\mu L$) with markedly increased eosinophilia (46% of manual leukocyte differential cell count) and thrombocytopenia ($17\times10^3/\mu L$). Hemoglobin was 11.5g/dL and hematocrit was 34.3%	atrial fibrillation	methylprednisolone sodium (for 3 days) and immunoglobulin (400 mg/day for 5 days), followed by oral PSL (50 mg/day).
Freireich, 2011	An 88-year-old female	red, swollen, and intense, macular erythema of the palms and soles	immediately after transfusion	fever, acral tingling, pruritis, burning sensation, and pain followed by a rash in the acral area, pancytopenia with a platelet count of 13,000, and neutropenia with a white blood cell count of 1,200	Myelodysplastic syndrome (MDS)	cool water soaks and 1% hydrocortisone cream
Ling, Shi, & Chen, 2017	73-year-old female	abdominal pain that had persisted for 1 week and melena for 5 days Furthermore, diagnosed as antral gastric mucosa exhibited chronic active inflammation	immediately	----------	----------	amoxicillin, clarithromycin, pantoprazole, and colloidal bismuth pectin
The current case study	13-year-old boy	rashes all over his body, redness in his eyes, and swelling and pus secretion	immediately after transfusion	decrease in haemoglobin to 6.4% and 9.5%	LRTI and PRE-B -ALL	Inj. Avil (Pheniramine Maleate) stat, Moxifloxacin, Tab Cetirizine to be taken orally, and calamine lotion

CONCLUSION

Severe reactions, such as urticarial hives and pre-septal cellulitis, can be caused by commonly performed platelet transfusions. To avoid the development of such events in the future, a proper risk assessment using a history-taking process, previous transfusion reactions, and concurrent medications that may interact with blood transfusion is necessary. Pharmacists play a key role in reporting adverse events correlated with platelet transfusions to hemovigilance systems.

Healthcare providers play a multifaceted role in hemovigilance activities related to blood transfusions, including education, risk assessment, individualised treatment planning, monitoring, adverse event reporting, and quality improvement initiatives. By actively engaging in these activities, pharmacists, as healthcare provider team members, can contribute to enhancing the safety and quality of blood transfusion practises and minimising the risk of adverse reactions for patients. Preventive measures include pre-transfusion medication protocols for patients with known allergies, strict adherence to aseptic techniques to prevent infections, close monitoring during and after transfusions, and ensuring that patients are well hydrated to mitigate the risk of transfusion-associated circulatory overload (TACO). Additionally, thorough donor screening and matching, along with robust training for caregivers and healthcare staff, are crucial steps in safeguarding paediatric patients undergoing platelet transfusions.

Ethics Committee Approval: Ethical clearance for this study was obtained from the Institutional Ethics Committee of KLE College of Pharmacy. Approval was granted on February 23, 2023, with reference number KLE/COP/EC/135/2023-2024

Informed Consent: Informed consent was obtained from the participants

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Obituary

Biography of Dr. Özlem Akbal Dağıstan

Dr. Özlem Akbal Dağıstan was born on January 1, 1985. She began her undergraduate studies at Istanbul University Faculty of Pharmacy in 2004 and completed her doctorate in the Department of Pharmaceutical Technology. Between 2010 and 2024, she worked as a research assistant in the same department.

Starting her academic journey at a young age, Dr. Özlem Akbal Dağıstan studied at Freie Universitat Berlin as an ERASMUS student during her undergraduate program in the 2006-2007 academic year. In 2016-2017, she conducted her doctoral research at Trinity College Dublin. She also spent part of her academic career at Van Yüzüncü Yıl University. Dr. Özlem Akbal Dağıstan was pursuing a second doctorate as a PhD candiadate in Pharmacy Management at Ankara University and had earned specialized certifications in this field from Utrecht University.

Dr. Özlem Akbal Dağıstan's research focused on developing innovative pharmaceutical dosage forms for inhalation and targeted lung delivery. During the COVID-19 pandemic, she dedicated her efforts to antiviral inhalation therapies, achieving significant success with one of her projects, which successfully completed Phase 2 clinical trials.

Throughout her career, Dr. Özlem Akbal Dağıstan mentored numerous students, authored 41 scientific publications, and played a pivotal role in various research projects. She was known as a visionary, determined, and selfless academician. Her untimely passing was deeply felt across both national and international academic communities.

Admired for her problem-solving skills and quick, practical intelligence, Dr. Özlem Akbal Dağıstan was deeply empathetic and socially adept. She was known for fostering sincere connections with individuals from diverse backgrounds, her strong sense of human values, and her vibrant, optimistic spirit. With a profound sense of duty, coupled with her cheerful and compassionate approach to life and work, she left an inspiring legacy behind.

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