



Istanbul Journal of Pharmacy

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Istanbul Journal of Pharmacy (Istanbul J Pharm) aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of pharmaceutical sciences. The journal publishes original articles, short reports, letters to the editor and reviews.

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The editorial and publication processes of the journal are shaped in accordance with the guidelines of the International Committee of Medical Journal Editors (ICMJE), World Association of Medical Editors (WAME), Council of Science Editors (CSE), Committee on Publication Ethics (COPE), European Association of Science Editors (EASE), and National Information Standards Organization (NISO). The journal is in conformity with the Principles of Transparency and Best Practice in Scholarly Publishing (<https://publicationethics.org/resources/guidelines-new/principles-transparency-and-best-practice-scholarly-publishing>).

Istanbul Journal of Pharmacy is currently indexed in Web of Science-Emerging Sources Citation Index, TÜBİTAK ULAKBİM TR Index and CAS database.

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The subjects covered in the manuscripts submitted to the Journal for publication must be in accordance with the aim and scope of the Journal.

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After plagiarism check, the eligible ones are evaluated by the editors-in-chief for their originality, methodology, the importance of the subject covered and compliance with the journal scope. The editor provides a fair double-blind peer review of the submitted articles and hands over the papers matching the formal rules to at least two national/international referees for evaluation and gives green light for publication upon modification by the authors in accordance with the referees' claims.

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Only those manuscripts approved by its every individual author and that were not published before in or sent to another journal, are accepted for evaluation.

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Editor-in-Chief does not allow any conflicts of interest between the authors, editors and reviewers. Only he has the full authority to assign a reviewer and is responsible for final decision for publication of the manuscripts in the Journal.

Reviewers must have no conflict of interest with respect to the research, the authors and/or the research funders. Their judgments must be objective.

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Only those manuscripts approved by its every individual author and that were not published before in or sent to another journal, are accepted for evaluation.

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An approval of research protocols by the Ethics Committee in accordance with international agreements (World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects," amended in October 2013, www.wma.net) is required for experimental, clinical, and drug studies. If required, ethics committee reports or an equivalent official document will be requested from the authors. For manuscripts concerning experimental research on humans, a statement should be included that shows that written informed consent of patients and volunteers was obtained following a detailed explanation of the procedures that they may undergo. For studies carried out on animals, the measures taken to prevent pain and suffering of the animals should be stated clearly. Information on patient consent, the name of the ethics committee, and the ethics committee approval number should also be stated in the Materials and Methods section of the manuscript. It is the authors' responsibility to carefully protect the patients' anonymity. For photographs that may reveal the identity of the patients, signed releases of the patient or of their legal representative should be enclosed.

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All acronyms, abbreviations, and symbols used in the manuscript must follow international rules and should be defined at first use, both in the abstract and in the

Table 1. Limitations for each manuscript type

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Original Article	3500	250 (Structured)	6	7 or total of 15 images
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Short Paper	1000	200	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	No tables	No media



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For plant materials, herbarium name (or acronym), number, name and surname of the person who identified the plant materials should be indicated in the Materials and Methods section of the manuscript.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text. Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

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Citations must be indicated with the author surname and publication year within the parenthesis.

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(Esin et al., 2002; Karasar, 1995)

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(Sayiner & Demirci, 2007)

Citation with three, four, five authors;

First citation in the text: (Ailen, Ciambune, & Welch, 2000) Subsequent citations in the text: (Ailen et al., 2000)

Citations with more than six authors;

(Çavdar et al., 2003)

Citations in the Reference

All the citations done in the text should be listed in the References section in alphabetical order of author surname without numbering. Below given examples should be considered in citing the references.

Basic Reference Types

Book

a) Turkish Book

Karasar, N. (1995). *Araştırmalarda rapor hazırlama* (8th ed.) [Preparing research reports]. Ankara, Turkey: 3A Eğitim Danışmanlık Ltd.

b) Book Translated into Turkish

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c) Edited Book

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d) Turkish Book with Multiple Authors

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e) Book in English

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f) Chapter in an Edited Book

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g) Chapter in an Edited Book in Turkish

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h) Book with the same organization as author and publisher

American Psychological Association. (2009). *Publication manual of the American psychological association* (6th ed.). Washington, DC: Author.

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Sidani, S. (2003). Enhancing the evaluation of nursing care effectiveness. *Canadian Journal of Nursing Research*, 35(3), 26–38. Retrieved from <http://cjnr.mcgill.ca>

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Fuchs, G. (2004). Light the menorah. On *Eight nights of Hanukkah* [CD]. Brick, NJ: Kid Kosher.

REVISIONS

When submitting a revised version of a paper, the author must submit a detailed “Response to the reviewers” that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer’s comment, followed by the author’s reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be cancelled. If the submitting author(s) believe that additional time is required, they should request this extension before the initial 30-day period is over.

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The comparison of losartan and ramipril on the incidence of hyperkalaemia among hypertensive patients with chronic kidney disease

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ABSTRACT

Background and Aims: The Joint National Committee 8th guideline recommends the initiation of renin angiotensin aldosterone system (RAAS)-linked drugs for hypertensive patients with chronic kidney disease (CKD). Losartan, an angiotensin receptor blocker (ARB), and ramipril, angiotensin converting enzyme (ACE) inhibitors, are widely utilized antihypertensive agents with sound efficacy and safety. The study compared the hyperkalemia incidences in CKD patients exposed to losartan and ramipril.

Methods: A prospective observational study was conducted for 12 months in the nephrology outpatient setting of a private tertiary care referral hospital in the Malabar region of Kerala. CKD patients with hypertension who were on ACE inhibitors or ARB therapy constituted our study population. Their demographic details, serum creatinine and potassium and urine protein were documented for three consecutive patient consultations.

Results: There were an equal number of samples (n=186) in each of the losartan and ramipril administered groups. Losartan and ramipril doses preferred by the nephrologist were 1.25, 25, 50 & 100 mg and 0.625, 1.2, 2.5, 5 and 10 mg, respectively. The Mann-Whitney U test showed statistical significance (p>0.05) between RAAS-related drugs and patients' total daily doses. We noticed more frequency of hyperkalemia in the losartan group (n=11, 5.9%) than in the ramipril group (n=4, 2.2%). Initially, the mean serum potassium was low in the ACE inhibitor (4.35±0.55) subset, and then there was augmentation in the second (4.46±0.52) and third (4.52±0.55) patient consultations. The repeated measures ANOVA tests depicted the samples to be different (p<0.05) in the serum potassium measurements within the losartan group (p= 0.018) and ramipril group (p<0.001).

Conclusion: Losartan gave favorable clinical effects in CKD patients with regards to serum creatinine and potassium. However, the frequency in reduction of proteinuria was profound in ramipril understudies.

Keywords: Ramipril, Losartan, Hyperkalemia, Serum creatinine, Urine Protein

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INTRODUCTION

The prevalence of chronic kidney disease (CKD) is increasing enormously and the impairment is responsible for pathophysiological changes in the serum electrolyte levels (Coresh et al., 2007). The incidence of hyperkalemia in such patients can lead to cardiovascular complications (Esposito et al., 2004; Mandal, 1997). The distorted glomerular filtration rate (GFR) with lack of dietary control can aggravate dyselectrolytemia. The extracellular potassium shift is further sensitized by antihypertensive agents, mainly, renin angiotensin aldosterone system (RAAS) blockers (Palmer, 2004; Reardon & Macpherson, 1998; Weir, 1999).

Angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) are commonly administered renoprotective agents. The persistent increment in the serum potassium observed in patients can be an impact of CKD progression. End stage renal disease (ESRD) without serum potassium homeostasis, even after optimal pharmacotherapeutic management and dietary restriction, warrants renal transplantations. It is the responsibility of the clinical pharmacist to prevent and treat at the earliest any drug-induced dyselectrolytemia. Thus, we assessed the impact of losartan and ramipril, the commonly prescribed ACE inhibitor and ARBs, on the patients' serum potassium in CKD with co-existing hypertension.

MATERIAL AND METHODS

Ethical approval

The prospective observational study was conducted in KIMS Al Shifa tertiary care referral Hospital, Kerala, India, from 2018 to 2019. The study was approved by the ethics committee of KIMS Al Shifa Hospital as per the letter KAS/EC/2018-29 (dated 30-04-2018), and written informed consent was obtained from each patient.

Patient selection and study design

The sample size was statistically determined using the equation of estimation of prevalence.

$$N = (Z_{\alpha/2}^2 \times p \times (1-p) \times D) / E^2$$

Where, $Z_{\alpha/2}$ = normal deviate for two tailed hypothesis, P = proportion from previous studies, D = design effect, E = margin of error.

A total of 375 subjects were enrolled in the study based on the selection criteria. All the patients who consulted the nephrologist during the research period, and those on ramipril or losartan therapy and diagnosed with CKD and hypertension were included. Patients who were hypertensive but did not have CKD, unconscious and mentally ill patients, pregnant patients, and those with combination antihypertensive therapy were excluded from the study. However, we could not properly follow up with a few patients and there were 3 dropouts. Thus, 372 patients were finally considered for results analysis.

The patient's demographic details, such as age, gender, CKD stages, and duration of CKD were retrieved from their medical case file and direct patient interview, along with information

regarding the nephrologist's clinical decision. Serum creatinine and serum potassium levels were noted on three consecutive specialist consultations. The adverse drug reactions pointed out were reported, and their causality assessment was performed with the Naranjo scale.

Statistical analysis

The data was put into Microsoft Excel 2007 version and exported into SPSS version 26. The chi-square test was employed to associate the variables. Mann-Whitney U and repeated ANOVA tests were computed to identify the difference between urine protein values and serum creatinine at different consultations. Spearman's ratio was analyzed to correlate the serum creatinine with the total daily dose and duration of CKD. The Bonferroni test was instituted for the pair wise comparison of serum potassium across consultations. The significance level (p) was fixed at 0.05 with a confidence interval of 95%.

RESULTS

We obtained 372 samples according to our inclusion criteria. Among them, the losartan group ($n=186$) and ramipril group ($n=186$) constituted equal proportions. Patients aged between 60 and 70 years were more common among both the ramipril and the losartan-administered groups (Table 1). CKD patients in stages 3 and 4 were significant in our study. Losartan prescribing predominated in stage 3 but less so in stage 4 when compared with the ramipril group.

The losartan doses preferred by the nephrologist were 12.5 mg, 25 mg, 50 mg, and 100 mg (Table 2). There were more patients who were administered with the 50 mg tablet ($n=84$). Few patients were prescribed 12.5 mg ($n=1$) and 100 mg ($n=3$). On the other hand, the chosen ramipril doses were 0.625 mg, 1.2 mg, 2.5 mg, 5 mg, and 10 mg. Limited participants had 0.625 mg ($n=2$) and 10 mg ($n=2$). The abundance of the 1.25

Table 1. The frequency distribution of patient's age and CKD stages (years) observed in ramipril and losartan groups.

Age of the patients	Ramipril (n=186)	Losartan (n=186)
less than 50 years	29 (15.6%)	46 (24.7%)
50 to 60 (years)	34 (18.3%)	42 (22.6%)
60 to 70 (years)	62 (33.3%)	53 (28.5%)
70 above (years)	61 (32.8%)	45 (24.2%)
Total	186 (100%)	186 (100%)
CKD	Ramipril (n=186)	Losartan (n=186)
Stage 1	7 (3.7%)	8 (4.3%)
Stage 2	10 (5.4%)	21 (11.3%)
Stage 3	85 (45.7%)	93 (50%)
Stage 4	83 (44.6%)	63 (33.9%)
Stage 5	1 (0.5%)	1 (0.5%)
Total	186 (100%)	186 (100%)

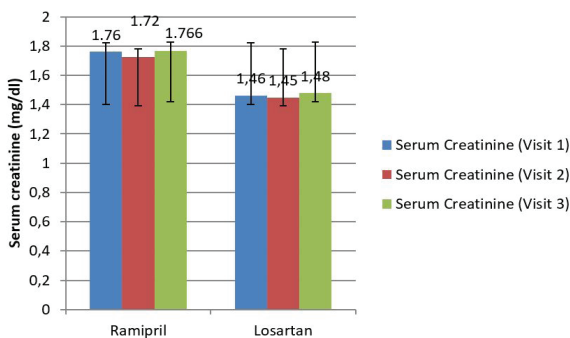
Table 2. The frequency of the distribution of doses of losartan and ramipril groups.					
Losartan (n=186)					
Dose (mg)	Once daily	Twice daily	Thrice daily	Total	
12.5	1 (0.5%)	0 (0%)	0 (0%)	1 (0.5%)	
25	60 (32.3%)	38 (20.4%)	0 (0%)	98 (52.7%)	
50	40 (21.5%)	43 (23.1%)	1 (0.5%)	84 (45.2%)	
100	1 (0.5%)	2 (1%)	0 (0%)	3 (1.6%)	
Total	102 (54.8%)	83 (44.7%)	1 (0.5%)	186 (100%)	
Ramipril (n=186)					
Dose (mg)	Once daily	Twice daily	Total		
0.625	2 (1%)	0 (0%)	2 (1%)		
1.25	88 (47.3%)	15 (8.1%)	103 (55.4%)		
2.5	38 (20.4%)	8 (4.3%)	46 (24.7%)		
5	22 (11.8%)	11 (5.9%)	33 (17.7%)		
10	1 (0.5%)	1 (0.5%)	2 (1%)		
Total	151 (81.2%)	35 (18.8%)	186 (100%)		
(n = 372)		Median	IQR	"Z"	P value
Total daily dose Losartan	Ramipril	2.50	1.25 to 2.50	-16.951	< 0.001*
	50	25 to 100			

mg prescriptions was noticed. The Mann-Whitney U test was used to compare the patients' total daily dose according to the RAAS drug groups. There was a difference in the samples ($p < 0.05$) concerning the two variables.

We found that ramipril ($n=86$) decreased proteinuria better than losartan ($n=61$). However, the losartan group ($n=74$) dominated in the patient set with same proteinuria throughout the three consultations (Table 3). The chi-square test was used to compare the change in patients' urine protein across the two drug groups and observed to have a relation with a p value < 0.05 . Similarly, the Mann-Whitney U test found a difference ($p < 0.05$) in urine protein between the losartan and ramipril groups for the first, second, and third consultations. There was one substantial patient with abnormal serum creatinine in both the ramipril and losartan populations observed in their

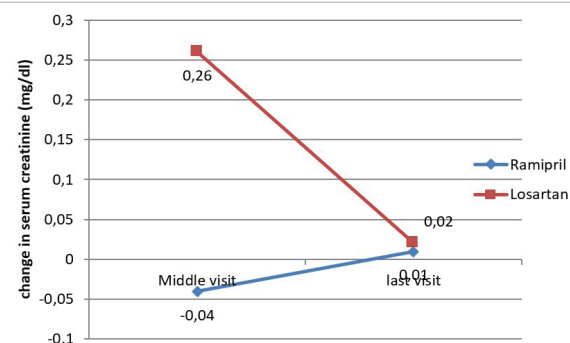
first consultations. The serum creatinine of most CKD patients in the ramipril (first visit mean=1.76) and losartan (first visit mean=1.46) groups decreased initially (second visit: ramipril, mean=1.72; second visit: losartan, mean=1.45) and then increased slightly (third visit: ramipril, mean=1.77; second visit: losartan, mean=1.48) (Figure 1). The independent sample "t" test was employed for the patient's serum creatinine in each of the three consultations and each sample was identified to be different ($p < 0.05$). The changes in mean serum creatinine between the first and second consultations and the second and third showed that the ramipril group had a decrease followed by a mild increase. But the losartan group showed a drastic increase in the serum creatinine observed in the middle and then sloped down in the end (Figure 2). The Spearman's ratio was calculated and there was a negative correlation (Ramipril group: Spearman's ratio=-0.154, $p=0.036$; Losartan: Spearman's

Table 3. Change in proteinuria preceding 3 consultations in ramipril and losartan groups.						
Proteinuria					Chi square	P value
	Ramipril (n=186)		Losartan (n=186)			
Same	74 (39.8%)		99 (53.2%)		7.864	0.020*
Decreased	86 (46.2%)		61 (32.8%)			
Increased	26 (14%)		26 (14%)			
Consultations	Ramipril		Losartan		"Z"	P value
	Median	IQR	Median	IQR		
1 st	3	2 to 3	2	2 to 3	-4.166	<0.001*
2 nd	2	2 to 3	2	2 to 2	-4.033	<0.001*
3 rd	2	2 to 3	2	2 to 2	-2.307	0.021*



Independent t test, $p < 0.05$

Figure 1. The bar diagram shows the mean and standard deviation of serum creatinine on 1st, 2nd and 3rd consultation, administered with ramipril and losartan.

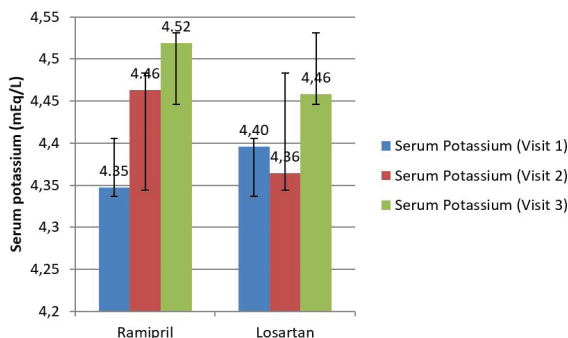


Independent t test, $p < 0.05$

Figure 2. The line diagram plotted against the change in mean serum creatinine level of patients on 2nd and 3rd consultation with respect to the 1st.

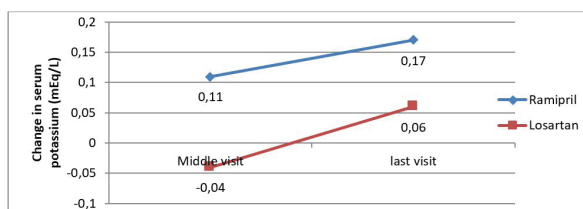
ratio=-207, p value=0.005) between serum creatinine with the total daily doses of RAAS drugs. A positive correlation (Spearman's ratio=0.182, $p=0.013$) was noted among the patients treated with losartan and the duration of their CKD.

From our study, the initial mean serum potassium (4.35) was low in the ACE inhibitor group. However, a drastic shift was observed in the second (4.46) and third (4.52) consultations. However, the losartan group had a lesser increase in mean serum potassium (Figure 3). The repeated measures ANOVA test portrayed difference ($p < 0.05$) in the measurements of serum potassium within losartan group ($p=0.018$) and ramipril group ($p < 0.001$). When we closely examined the change in mean serum potassium of each group, the ramipril group had a further increase in value. In the losartan group, even though the second consultation showed a decrease, there was a slight increase in later consultation (Figure 4). The Bonferroni test was computed for the pair wise comparison of serum potassium across consultations. There was a difference in serum potassium between the measurements of first and second consultations (mean difference=-0.116, $p=0.001$) and first and third consultations (mean difference=-0.172, $p < 0.001$) among the ramipril group, and second and third consultations (mean difference=-0.094, $p=0.014$) in the losartan group.



ANOVA, Losartan: $p < 0.05$, Ramipril: $p < 0.01$

Figure 3. The bar diagram shows the mean and standard deviation of serum potassium on 1st, 2nd and 3rd consultation, administered with ramipril and losartan.



Bonferronic test, Ramipril: Middle visit- $p < 0.05$, Last visit- $p < 0.01$, Losartan: $p < 0.05$

Figure 4. The line diagram plotted against the change in mean serum potassium level of patients on 2nd and 3rd consultation with respect to the 1st.

Table 4. The frequency distribution of serum creatinine and potassium of losartan group and ramipril group.

Serum creatinine		
Losartan (n=186)	Male	Female
Normal	23 (12.4%)	38 (20.4%)
Abnormal	87 (46.8%)	38 (20.4%)
Total	110 (59.1%)	76 (40.9%)
Ramipril (n=186)	Male	Female
Normal	17 (9.1%)	24 (12.9%)
Abnormal	101 (54.3%)	44 (23.7%)
Total	118 (63.4%)	68 (36.6%)
Serum Potassium		
	Ramipril (n=186)	Losartan (n=186)
Normal	182 (97.8%)	175 (94.1%)
Abnormal	4 (2.15%)	11 (5.9%)
Total	186 (100%)	186 (100%)

The reported ADRs were classified as probable according to Naranjo's scale and are represented in Table 5. Ramipril had more cases of hypercreatinemia ($n=93$) and lesser patients with hyperkalemia ($n=4$). The Likelihood Ratio test inferred the adverse events can occur equally among ACE inhibitor or ARBs-administered patients ($p > 0.05$).

Table 5. Frequency of the distribution of adverse drug reactions encountered with ramipril and losartan.

	(n=412)	Ramipril		Losartan		Likelihood Ratio	P value
		Frequency	%	Frequency	%		
Adverse Events	Cough	9	4.33	9	4.41	11.884	0.104
	Dizziness	2	0.96	4	1.96		
	Fatigue	18	8.65	17	8.33		
	Headache	7	3.37	4	1.96		
	Hypotension	1	0.48	0	0.00		
	Hypercreatinemia	93	44.71	65	31.86		
	Hyperkalemia	4	1.92	5	2.45		
	No Adverse Event	74	35.58	100	49.02		

DISCUSSION

ACE inhibitor or ARBs are the best antihypertensive agents preferred for CKD patients (Zhang et al., 2020). The study had a higher geriatric population, constituted to be significant in the ramipril group. The epidemiology status of CKD has changed and expanded to the adult population (Hallan et al., 2006). The misuse of medicines, genetic susceptibilities, and sedentary lifestyle influence patients, aggravating CKD's prevalence (Baker & Perazella, 2020). Poly-pharmacy and the irrational administration of medicine during pregnancy can bring insult to kidney for both mother and offspring.

The proportion of end stage renal disease (ESRD) patients who were on ramipril or losartan was negligible. This is because preferred antihypertensive agents may not be a RAAS-related agent in advanced kidney disease. On the other hand, a study of patients in Karnataka district showed that the results of a huge number of patients in stage 3 coincided with our results (Duan et al., 2019).

The oral bioavailability of losartan is 33%, with significant first-pass metabolism utilizing cytochrome P450 system. The CYP2C9 and CYP3A4 are specifically involved in the biotransformation, which is 10 to 40 times more potent by weight than the parent molecule (Ripley & Hirsch, 2010). The L losartan dose ranged from 1.25 to 100 mg in our samples. This variation was intended to normalize the blood pressure (BP) and reduce proteinuria (Maione, Annemans, & Strippoli, 2009). A study that compared the efficacy of losartan with amlodipine concluded that there was considerable decrease in urine protein excretion after a year of therapy (Praga et al., 2003). Thus, we can label the drug to be an excellent renoprotective agent among ARBs (Ono, Sanai, Miyahara, & Noda, 2013).

The initial dose for ramipril among renal impaired patients is 1.25mg and can be titrated to an effective dose not exceeding 5 mg/day. For a patient without CKD, the antihypertensive dose can be increased up to 20mg per day in divided doses (James et al., 2014). The drug generates action for 24 hours, and as the creatinine clearance decreases less than 40 ml/minute/1.73m², the peak levels of its metabolite are approximately doubled. This is the reason behind restricting the maximum

dose to 5 mg/day. Our study depicted prescription pattern of ramipril to be between 0.625 and 10 mg for CKD patients. The clinical trial conducted to assess the efficacy of ramipril in kidney disease patients showed that even though a small dose of ramipril (1.25 mg daily therapy) had a limited effect on improving hypertensive symptoms, it could preserve the kidney function (Doggrell, 2001). It's the pharmacist's duty to select a brand that imparts the dose according to the prescription and if not possible, breaking the tablets into halves should be their duty.

The main advantage of ACE inhibitors and ARBs is their ability to decrease the proteinuria in CKD patients. This is also a determinant that quantifies kidney function improvement. Here, we found that ramipril decreased the proteinuria among CKD patients more than the losartan group. This was supported by Kahvecioglu et al. where they conducted a 12-month follow up and observed 1% more reduction in the former group (Kahvecioglu et al., 2007).

Reno-protection happens with adequate BP control, which slows progression of CKD. Alone and in combination with other antihypertensive agents, losartan tends to normalize blood pressure (Chan et al., 1995; Tikkanen, Omvik, & Jensen, 1995). Researchers claimed that the antihypertensive and renoprotective effects of ARBs are similar to ACE inhibitors. The Optimal Anti-proteinuric Doses (ROAD) trial concluded that losartan can reduce the risk of doubling serum creatinine concentration and ESRD after titrating the dose beyond usual antihypertensive ranges (Hou et al., 2007).

The normal values of serum creatinine are slightly different for males and females. It is approximately 0.6 to 1.2 mg/dl in males whereas it is 0.5 to 1.1 mg/dl in females. CKD patients should monitor serum creatinine and serum potassium from 3 days to a week after initiation of the RAAS-related agent. Hypercreatinemia is observed within a few days after beginning the therapy, as angiotensin II levels are rapidly reduced or blocked from binding. This would result in efferent arteriolar dilatation and a decline in effective glomerular filtration rate (GFR). Our patients reported with abnormal serum creatinine in both the ramipril- and losartan-administered groups. However, an increase up to 20 to 30 percent is acceptable (Council on Credentialing in

Pharmacy, Albanese, & Rouse, 2010; Whelton et al., 2018), but the therapy should be withdrawn if creatinine levels rise above 30% after 6 to 8 weeks (Bakris & Weir, 2000). A cohort study in a primary care population showed an increased adverse incidence of cardio-renal outcomes even with a small creatinine increase after initiating the ACE inhibitor or ARB (Schmidt et al., 2017). The decrease in renal functions other than introduction of the drug was more likely to lead to serum creatinine elevation at higher systolic blood pressure targets (Collard, Brouwer, Peters, Vogt, & van den Born, 2018).

The normal serum potassium is 3.5 to 5.5 mEq/L in healthy adults. A fully functioning kidney would not abnormalize serum potassium level after a RAAS drug. The decline in GFR to 20 ml/min/1.73m² would sensitize the body to hyperkalemia, as ACE inhibitors or ARBs would reduce serum aldosterone (Palmer, 2004). Moreover, the prevalence of hyperkalemia is approximately 10 percent in outpatients within a year of ACE inhibitor consumption (Bakris & Weir, 2000; Reardon & Macpherson, 1998). This increase in serum potassium would lead to discontinuation of the antihypertensive therapy in CKD patients. The study found that more patients encountered hyperkalemia in the losartan group compared to the ramipril group (Sadjadi, McMillan, Jaipaul, Blakely, & Hline, 2009).

The hypercreatinemia was significant in the ramipril group and was reported as ADR. Few patients had RAAS drug induced hyperkalemia. When considering the normal physiology, angiotensin II and increased serum potassium can elevate aldosterone secretion. Further, they can lead to the elimination of the excess potassium from the body. In a normal person, studies pointed out that RAAS-related medicine only increases the blood potassium level less than 0.5 mEq/L 37. However, there would be more prominent increase in this electrolyte level in CKD patients (Magvanjav et al., 2019). The risk is greater in ACE inhibitors or ARBs-treated patients who are on dialysis (Knoll et al., 2008). In ESRD patients, the inefficiency of the kidney and colon in pushing potassium out of the body can be the prime reason for hyperkalemia.

The American Heart Association (AHA) also recommends the initiation of ACE inhibitors or ARBs to patients in early stages and in advanced CKD. This is based on the fact that the reduction in proteinuria can slow the progression of kidney damage. Thus, we should focus on the two major factors that slow kidney damage when treating CKD patients. The first one is treatment of the underlying disease, and the second is treatment that is predictive of progression, such as elevated blood pressure. We observed that ARBs influence the renal function more than ACE inhibitors concerning serum creatinine, urine protein and serum potassium.

CONCLUSION

Losartan was found to impart display favorable clinical effects in CKD patients. This was evident from their its lesser incidence of hyperkalemia and abnormal serum creatinine. However, reduction in proteinuria reflects the efficacy of antihypertensive agents in CKD patients, which was observed with ramipril.

Abbreviations

ACE – Angiotensin Converting Enzyme
 AHA- American Heart Association
 ARB – Angiotensin Receptor Blocker
 ESRD- End Stage Renal Disease
 CKD – Chronic Kidney Disease
 RAAS- Renin Angiotensin Aldosterone System

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

Ethics Committee Approval: This study was approved by the Kims Al Shifa Ethics Committee (Date: 30.04.2018 No: KAS/EC/2018-29).

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

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Cardiac effects of dapagliflozin in diabetic rats with subacute exposure

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ABSTRACT

Background and Aims: Dapagliflozin (DAPA) is a sodium-glucose co-transporter 2 (SGLT2) inhibitor used for the treatment of type 2 diabetes mellitus (T2DM) as a monotherapy or combination therapy with other antidiabetic medicines. The Food and Drug Administration (FDA) recently approved DAPA to minimize the risk of hospitalization due to heart failure in patients with T2DM because of its antihypertensive and antihyperglycemic activities. However, further study of DAPA is necessary to ensure the safety of patients.

Methods: T2DM was induced by streptozotocin (STZ) injection (35 mg/kg b.w. i.p.) in male rats that were fed a high-fat diet for two weeks before STZ injection. The diabetic rats were exposed to 10 mg/kg DAPA by oral gavage during sub-acute treatment. Total cholesterol levels and oxidative stress parameters were evaluated. Heart tissues were histologically examined, and cardiac troponin T (cTnT) levels were measured.

Results: DAPA has the potential to inhibit diabetes-induced oxidative stress and morphologic damage to heart tissue, and increased cTnT levels of the heart, which is important for cardiac contractility.

Conclusion: DAPA might have a protective effect on the heart at a 10 mg/kg oral dose; however, further experimental and clinical studies are required to clarify the cardio-protective potential of DAPA.

Keywords: Dapagliflozin, SGLT2 inhibitor, Type 2 diabetes mellitus, Cardio-protection

INTRODUCTION

T2DM is one of the risk factors for the development of cardiovascular diseases (CVD) such as heart failure, atherosclerotic disease, and myocardial infarction. The risk is 2 to 4-fold higher in patients with diabetes (Fox et al., 2004; Ptaszynska, Hardy, Johnsson, Parikh, & List, 2013). It is well known that managing blood glucose levels is very important for preventing cardiovascular complications in diabetic patients (ESC, 2020). DAPA is one of the SGLT 2 inhibitors that inhibits glucose absorption from the kidney, and was approved in 2014 for the treatment of T2DM as a monotherapy or in combination with other antidiabetic medications (FDA, 2014; FDA, 2019). DAPA leads to natriuresis by decreasing sodium reabsorption concomitantly with glucose reabsorption from the kidney, thereby reducing systolic and diastolic blood pressure without affecting heart rate, which is different from standard antihypertensive agents (Reed, 2016).

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A large phase III multicenter clinical study (DECLARE-TIMI-58, NCT011730534) was designed to investigate the cardiovascular safety of 10 mg of DAPA daily, administered T2DM by health professionals in patients with or without T2DM. In that study, it was reported that DAPA lowered the rates of cardiovascular disease-related death and hospitalization for heart failure in patients who had or were at risk for atherosclerotic disease (Wiviott et al., 2019). Similarly, DAPA has been reported to reduce hospitalization for heart failure in patients with or without heart failure with reduced left ventricular ejection fraction, as well as the rate of cardiovascular disease-related death in patients with heart failure with reduced left ventricular ejection fraction (Kato et al., 2019). The FDA has also approved DAPA to reduce hospitalization for heart failure in patients who have CVD or CVD risk factors (Kelly, 2019).

The cardiovascular safety of DAPA has been evaluated by several studies in recent years (Ptaszynska et al., 2013; Wiviott et al., 2019; Kato et al., 2019). The effects of DAPA on the heart must be explicitly investigated due to the potential for DAPA usage rates to increase in cardiovascular patients following FDA approval. More research is needed to clarify the effects of DAPA on the heart with regard to patient safety. Therefore, we aimed to investigate the effects of DAPA on the heart after sub-acute DAPA exposure at 10 mg/kg b.w. dose. The effects of 10 mg/kg of DAPA on the levels of plasma cholesterol, glutathione (GSH), malondialdehyde (MDA), and the selective cardiotoxicity marker cTnT in the heart were evaluated.

MATERIAL AND METHODS

Animals and study design

Male Sprague-Dawley (SD) rats aged 10-12 weeks were obtained from Acibadem University Laboratory Animal Application and Research Center. The rats were housed in polystyrene cages (up to five animals per room) at 21-23°C and humidity (55 ±5%). The rats were fed ad libitum throughout the experiments at Istanbul University Faculty of Pharmacy Animal Faculty Unit (EDEHAB). The study was approved by Istanbul University Local Ethics Committee of Experimental Animals (IUHADYEK; 2018/24). The rats were fed a high-fat diet for 2 weeks and then randomly divided into 3 groups as shown in Table 1. Diabetes was induced by a single i.p. injection of STZ (35 mg/kg in citrate buffer solution) in Groups II and III. Group I animals received a single i.p. injection of citrate buffer solution (Skovsø, 2014). One week after STZ treatment, the blood glucose levels were measured with a glucose analyzer device (Vivachek, Biotech Inc., China) from the tail blood of the animals. The animals with blood glucose levels >270 mg/dL were included in the study (Furman, 2015).

No observed adverse effect level (NOAEL) of DAPA is 50 mg/kg/day in rats (EMA, 2012). One study showed a maximum glucose-lowering effect of DAPA at 10 mg/kg b.w. dose in SD rats (Han et al., 2008). Additionally, another study reported that the area under the plasma drug concentration-time curve (AUC) at 10 mg/kg DAPA in rats is 130 times higher than in humans at the same dose, which means plasma concentration in SD rats is 130 fold that of human (Reilly et al., 2014). Therefore, we used a 10 mg/kg dose of DAPA, a relatively high dose compared to

humans, suspended in 0.5% methyl cellulose, to evaluate the effects of DAPA in the present study. And, the rats in Group III were treated with 10 mg/kg b.w. DAPA (Table 1).

Table 1. Experimental groups in the study.

Groups	Sub-acute (28 days) treatment
Group I (n:5)	35 mg/kg citrate buffer injection (i.p.) 1 mL/kg water (p.o.)
Group II (n:8)	35 mg/kg STZ injection (i.p.) 1 mL/kg methyl cellulose (p.o.)
Group III (n:8)	35 mg/kg STZ injection (i.p.) 1 mL/kg DAPA (p.o.)

Plasma cholesterol level determination

The rats were euthanized by taking a large volume of blood under inhalation anesthesia on the 28th day of exposure. The blood samples were centrifugated at 3000 rpm at 4°C for 20 min (Hettich Universal 32R Germany); the supernatant was collected and used for cholesterol level determination.

The rats were weighed three times a week (Sartorius, Mettler H20, Germany) and observed for clinical signs. After sacrifice, the hearts were surgically dissected and weighed (Precisa XB220A, Switzerland), and the relative heart weights (%) were calculated (Liro, 1985). The plasma cholesterol levels were determined using a commercial kit according to the manufacturer's protocol (Sunred, 201-11-0785, China). The MDA and GSH levels in the tissue samples were evaluated using commercial kits following the manufacturer's guideline (Elabscience, E-EL-0060; E-EL-0026, USA). The tissues were homogenized in phosphate-buffered saline (PBS) (1:10, w/v) and kept at -80°C (Daihan-Scientific Wisecry, South Korea).

Hematoxylin and eosin (H&E) staining

For histological examination, the heart samples were fixed in 10% neutral buffered formalin and processed for routine paraffin embedding. The paraffin sections approximately 4 µm-thick were stained with Hematoxylin and Eosin (H&E). The sections were examined and photographed using a light microscope (BX51; Olympus Corp., Tokyo, Japan) attached to a digital camera (DP72; Olympus Corp., Tokyo, Japan). The cardiomyocyte and vascular morphology were evaluated in each section.

Determination of cTnT expression level

For immunohistochemistry, formalin-fixed paraffin tissue blocks were cut into 4-5 µm thick transversal sections using a Leica 1212 rotary-microtome (Germany). The paraffin sections were placed on poly-L-lysine coated microscope slides. Then, the sections were deparaffinized, rehydrated, and rinsed with distilled water. For antigen retrieval, the sections were heated in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave oven. The endogenous peroxidase activity was suppressed with 3% (v/v) H₂O₂ for 15 min. After the sections were blocked to avoid non-specific binding, the mouse monoclonal anti-cTnT antibody (1:100, Thermo, USA) was applied to the sections and stored overnight at +4°C. The peroxidase activity was visualized with 3-amino-9-ethyl carbazole (AEC). The sections were counterstained with Mayer's

Haematoxylin, mounted in glycerol vinyl alcohol (GVA), and observed with an Olympus BX53 microscope. For negative control staining, the sections were incubated with PBS.

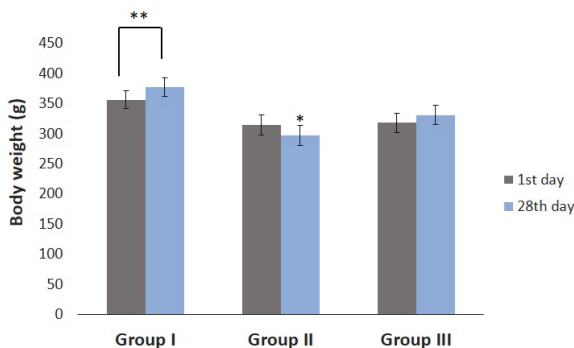
The statistical differences between the experimental groups were analysed by one-way analysis of variances (ANOVA) using SPSS v.20 (Chicago, IL). Significant differences were determined by Tukey post-hoc test. The results are given as values \pm standard error of the means (SEM). All biochemical experiments were performed in triplicate for each animal. H-score of cTnT immune-reactivity was calculated using the formula: $[1 \times (\text{weak cells } \%) + 2 \times (\text{moderate cells } \%) + (\text{dense cells } \%)]$. The intensity of staining was graded as 1, weak; 2, moderate; or 3, dense; plus, the percentage of positive cells.

RESULTS

Changes in body weights and relative heart weights

In diabetic groups (Group II and III), an increase in water consumption, diuresis, and fatigue were observed. At the end of the 28-day exposure period, the body weight was significantly lower in Group II compared with Group I, which observed a significant increase in the body weight, whereas the body weight of Group II decreased insignificantly ($p > 0.05$) in comparison with the beginning of treatment (Figure 1).

The relative heart weights did not show significant changes in diabetic groups (Figure 2).



* $p < 0.05$ vs Group I, ** $p < 0.05$ vs 1st day of the treatment.

Figure 1. Effects of DAPA on the body weights. Data were shown as mean value \pm SEM.

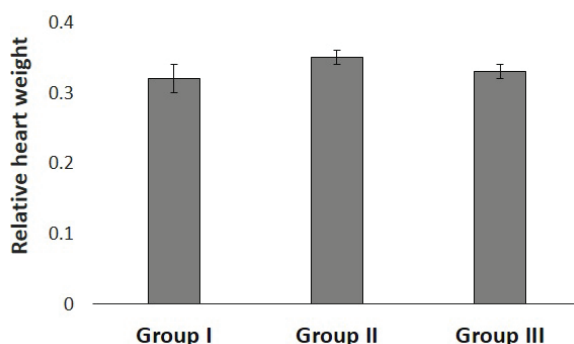


Figure 2. Effects of DAPA on relative heart weights. Data were shown as mean \pm SEM.

Effects of DAPA on the plasma cholesterol level

As shown in Figure 3, the plasma cholesterol levels increased slightly in Group II, which was not found statistically significant ($p > 0.05$).

Changes in MDA and GSH levels in the heart tissue

In Group II, the GSH levels in heart tissues decreased significantly, whereas the MDA levels increased significantly compared with Group I. DAPA treatment distinctively ameliorated reduced GSH levels in Group II (Figure 4).

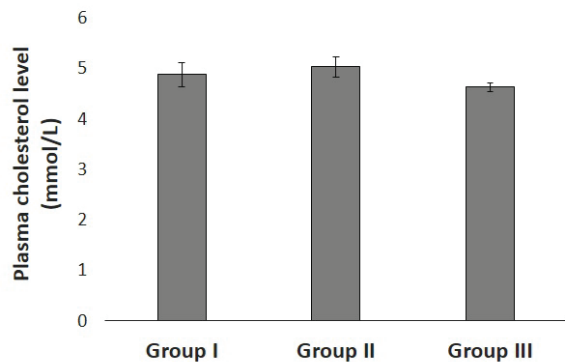
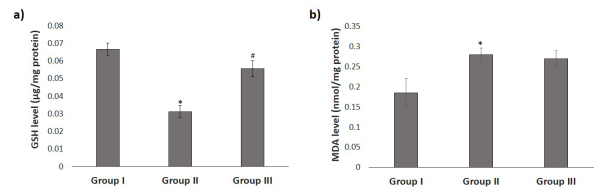


Figure 3. Effects of DAPA on plasma cholesterol levels. Data were shown as mean \pm SEM.



* $p < 0.05$ vs Group I, and # $p < 0.05$ vs Group II, GSH: Reduced glutathione, MDA: Malondialdehyde

Figure 4. Effects of DAPA on (a) GSH levels and (b) MDA levels in heart tissue. Data were shown as mean \pm SEM.

Histological changes in the heart tissue

According to the histological examination, cardiomyocytes with regular morphology and typical vascular morphology were seen in Group I. Severe vascular congestion and disorganized myofibrils in cardiomyocytes were detected in Group II. Moderate vascular congestion and a decrease in myofibril disorganization in cardiomyocytes were observed in Group III (Figure 5).

Changes in cTnT expression levels

As shown in Figure 6, cTnT levels in heart tissue were significantly lower (20%, $p \leq 0.01$) in Group II than in Group I. DAPA treatment ameliorated 17% of this decrease in Group III as compared with the diabetic groups.

DISCUSSION

DAPA is a novel therapeutic option for the treatment of T2DM. It is known that DAPA reduces blood pressure without affecting heart rate (Inzucchi et al., 2015), and this can be a major advantage over standard antihypertensive drugs used for

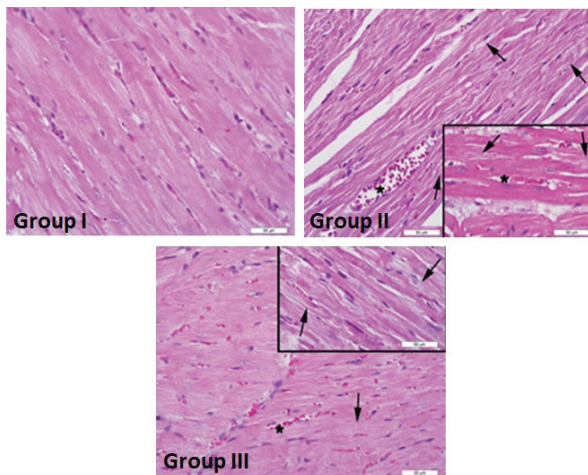
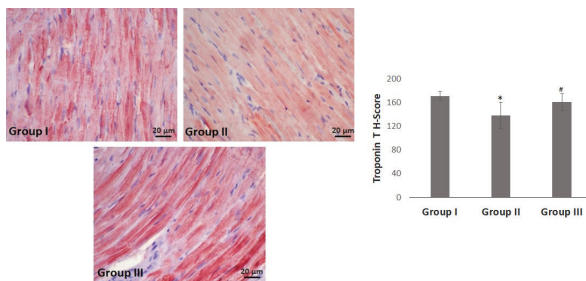


Figure 5. Representative photomicrographs of heart tissue in the experimental groups. Regular cardiomyocyte and vascular morphology are seen in Group I. Vascular congestion (asterisk) and disorganization of myofibrils (arrow) of cardiomyocytes are seen in Group II. Mild vascular congestion (asterisk) and decreased disorganized myofibrils in cardiomyocyte (arrow) are seen in Group III. H&E staining scale bars: 50 µm.



* $p < 0.05$ vs Group I, and # $p < 0.05$ vs Group II.

Figure 6. Changes in cTnT expression in the heart in different experimental groups. Histological sections representing cTnT immunopositivity in the heart of different experimental groups. Graph representing H-score of cTnT immunoreactivity in the heart. Data were expressed as mean \pm SEM.

diabetic patients with hypertension. Following FDA approval, DAPA may increase in usage for patients with a risk of heart failure, making it necessary to identify its cardiotoxic potential. For this reason, we investigated the biochemical and histological effects of DAPA on heart tissue at a 10 mg/kg dose.

Our results showed that body weight decreased significantly in Group II and III compared with Group I as a result of STZ injection-induced diabetes. Diabetes has been shown to reduce body weight in many experimental studies (Thomson et al., 2012; Rebolledo-Solleiro & Fernández-Guasti, 2018). At the end of the experiment, the body weight of Group III, which was treated with 10 mg/kg b.w. DAPA slightly increased compared with the beginning. This could be associated with an ameliorative effect of DAPA on diabetes by reducing blood glucose levels and inducing hyperphagia (Devenny, Godonis, Harvey, Rooney, Cullen & Pelleymounter, 2012). It is known that relative heart weight increased in diabetic cardiomyopathy, and this may result from cardiac hypertrophy (Zhang et al., 2018). In our study, relative

heart weights did not show significant change among the groups because exposure time may not be enough to increase the relative heart index. Similarly, Singh et al. did not show a difference in the relative heart index one week after diabetes induction (Singh, Le, Khode, Baker, & Kumar, 2008).

It has been reported that DAPA leads to an increase in cholesterol levels in patients with T2DM (Gallwitz, 2018). However, plasma cholesterol levels did not show a significant change after sub-acute DAPA exposure in the present study. This might be associated with the duration of exposure, which was not sufficient to detect changes in plasma cholesterol levels.

Oxidative stress is one of the underlying mechanisms of cardiac dysfunction and diabetic complications. Oxidative stress may lead to lipid peroxidation and membrane damage (Dhalla, Temsah & Netticadan, 2000; Liu, Wang & Cai, 2014). MDA, an oxidized lipid, indicates lipid peroxidation and oxidative stress (Gawel, Wardas, Niedworok & Wardas, 2004). Reduced GSH levels are very important for antioxidant defense and protect cells from reactive species (Forman, Zhang & Rinna, 2009). An increase in MDA and depletion of GSH levels have been seen in the plasma or some tissues of diabetic patients (Wei, Liu, Tan, Liu, Li & Cai, 2009; Tiwari, Pandey, Abidi & Rizvi, 2013). DAPA has been demonstrated to elevate GSH levels and reduce MDA levels in heart tissue at 1 mg/kg concentration after 4 weeks of exposure (Hussein, Eid, Taha, Elshazli, Bedir & Lashin, 2020). Similarly, we observed that GSH levels of heart tissue decreased in diabetic Group II, and this decrease was significantly ameliorated by DAPA treatment in Group III. The MDA levels in heart tissue were elevated in Group II, and DAPA treatment slightly inhibited the elevation of MDA levels in Group III. Some studies also showed that DAPA reduced oxidative stress markers in rats at 1 mg/kg and 10 mg/kg doses (Tanajak et al., 2018; Kingir et al., 2019). Our histological results parallel the increase in oxidant damage parameters in Groups II. DAPA might have ameliorated morphological damage via the regulating of the oxidant/antioxidant balance in STZ-induced cardiotoxicity.

Troponin is important for cardiac contractility, and it plays a structural and modulator role in the heart (Gomes, Barnes, Harada & Potter, 2004). cTnT, one of the cardiac-specific troponin isoforms and a sensitive marker for cardiotoxicity, is widely used in the clinic (Lorenzo-Almoros, Tuñón, Orejas, Cortés, Egido & Lorenzo, 2017). An increase in serum/plasma cTnT levels indicates myocardial damage (Wallace et al., 2004). However, decreased cTnT levels in heart tissue have been observed in cardiac damage in experimental models (Sehnert et al., 2002; Jankowski et al., 2010). Similarly, our study showed that DAPA treatment significantly raised cTnT levels in heart tissue as compared with diabetic groups.

In conclusion, DAPA showed a protective effect on the heart by reducing oxidative stress and inflammation at a 10 mg/kg dose by sub-acute exposure. Still, further studies are needed to confirm our results by identifying the long-term effects of DAPA on the heart.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: The study was approved by Istanbul University Local Ethics Committee of Experimental Animals (IUHADYK; 2018/24).

Author Contributions: Conception/Design of Study- T.B., B.U.K., G.Ö.; Data Acquisition- T.B., B.U.K., A.K.K., E.K., F.E., G.Ö.; Data Analysis/Interpretation- T.B., B.U.K., A.K.K., E.K., F.E., G.Ö.; Drafting Manuscript- T.B., B.U.K., A.K.K.; Critical Revision of Manuscript- E.K., F.E., G.Ö.; Final Approval and Accountability- T.B., B.U.K., A.K.K., E.K., F.E., G.Ö.

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


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Cytotoxicity and genotoxicity evaluations of oleic acid and conjugated linoleic acid

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ABSTRACT

Background and Aims: Oleic acid (OLA) and conjugated linoleic acid (CLA) occur in dairy products and meats and are also widespread at lower levels in many other foodstuffs. It is known that OLA and CLA are very bioactive compounds with substantially anti-carcinogenic effects. The objective of this study was to evaluate the cytotoxic potentials of OLA and CLA which were tested against cancerous and non-cancerous cell lines and to determine their genotoxicity.

Methods: The cytotoxic activities of OLA and CLA against to cancer cell lines (U-87-MG, A549, MCF-7, CaCo-2, HeLa and PC-3) and a control cell line (HEK293) were assessed by MTT assay. Ames MPF™ mutagenicity assay on 4 strains (TA98, TA100, TA 1535 and TA 1537) of *Salmonella typhimurium* was used for genotoxicity determination.

Results: CLA showed cytotoxic activity on PC-3 cells, while OLA was created on A549 and PC-3 cell lines with the IC₅₀ of 20 nM and 15 nM, respectively. No cytotoxic activity was observed on MCF-7, HeLa, U-87-MG, and CaCo-2 cells with the administered doses of OLA and CLA. It has been proved that OLA and CLA are characterized by a high cytotoxic activity towards cancer cells, as observed in the cell line test. There was no evidence for a mutagenic effect of OLA and CLA in the Ames test, with or without metabolic activation (S9) against *Salmonella typhimurium* strains.

Conclusion: These *in vitro* test results indicate that these fatty acids can be considered a beneficial dietary supplement for enhancing anti-cancer therapy.

Keywords: Fatty acid, Conjugated linoleic acid, Oleic acid, Cytotoxicity, Mutagenicity

INTRODUCTION

Oilseed crops that contain fat, protein, carbohydrates, minerals and vitamins have an important place in human and animal health (Liu, Johnson, Blacksaw, Hossoin, & Gan, 2019). Safflower, which is a popular medicinal oilseed plant, belongs to the family of *Asteraceae* (Zhang et al., 2019). Recently, the importance and beneficial health effects of safflower have been shown in various studies (Martinez, Sosa, Higa, Fornes, & Capobianco, 2012; Khalid et al., 2017). It is a rich source of unsaturated fatty acids, including monounsaturated and polyunsaturated fats. It is a famous and widely used traditional Chinese medicine that has been verified to have anti-inflammatory, antioxidant, and cytotoxic activities (Yanli, Jian, Zhenyun, Peipei, & Kan, 2018). Safflower plants are used in feed, biodiesel industry, in various fields such as phytoremediation and it are able to grow under different climatic conditions

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(Madan, Mudgal, Mishra, Srivastava, & Singh, 2011). Plant oils are generally composed of unsaturated fatty acids, oleic, linoleic and mainly saturated fatty acids, palmitic and stearic acids (Aydeniz, Güneser, & Yılmaz, 2014). However, the distribution of these fatty acids varies based on the type of plant species. Safflower oil is composed of 6–8% palmitic, 2–3% stearic, 16–20% oleic and 71–75% linoleic acids and exhibits the highest linoleic acid content among all the commercial oils (Aydeniz et al., 2014). The quality of oil is determined by the ratio of fatty acids content. The total unsaturated fatty acid level is very high in plant oil, which is very important for human health. Linoleic acid (Omega-6) is a fatty acid with 18 carbons and contains a double bond at 10th and 12th positions in the cis configuration (Woodcock, Salvatore, Freeman, & Schopfer, 2021). Dairy and meat products contain linoleic acid, and it is often used in chemical and animal feed industries. As a result of microbial biohydrogenation, the digestive tract of ruminant animals naturally produces Conjugated linoleic acid (CLA).

It was indicated that CLA daily supplementation after carcinogen exposure affected linoleic acid metabolites content in rats' serum. Also, a decrease in cancer incidence was shown in CLA supplemented groups (Małgorzata, Agnieszka, Iwona, Hanna, & Andrzej, 2017). Oleic acid (OLA, Omega-9) is a monounsaturated cis-9-octadecenoic acid (Engelbrecht, Schroeter, Haus, & Neubert, 2011). OLA treatment against the starvation might be important to confirm the effect of lipophagy cells (Lee, Ahna, Jang, Ha, & Jung, 2019). In addition, many studies have shown that OLA acted synergistically with cytotoxic drugs by enhancing their antitumor effect (Menendez et al., 200; Carrillo, Cavia, & Alonso-Torre, 2012). In recent years, studies have increased on the type of plants with high ratio of oleic acid and linoleic acid (Cao et al., 2013). Experimental studies of CLA on animals have been shown CLA to be protective against breast cancer (Białek, Zagrodzki, & Tokarz, 2016; Zhang et al., 2018). Studies on rats have shown that CLA has antidiabetic effects by increasing insulin sensitivity (Guiberna et al., 2019). The findings in most studies show that OLA reduces the risk of cancer, especially breast cancer (Simonsen et al., 1998). Therefore, plant derived compounds have been an important source for medicines that treat various diseases. Safflower oil derivatives are also understood to be an important source for cancer studies and phytotherapy (Yang et al., 2018). In this study, we aimed to evaluate the cytotoxic potentials of OLA and CLA, which were tested against cancerous and non-cancerous cell lines, and to determine their genotoxicity.

MATERIAL AND METHODS

Preparation of oleic acid and conjugated linoleic acid stock solutions

Test substance both 10t-12c CLA (Cas No: 2420-56-6) (Fig.1a.) and OLA (Cas No: 112-80-1) (Fig.1b.) were obtained from Sigma-Aldrich Chemical in liquid form to be restructured with DMSO (Sigma-Aldrich Chemical, CAS No: 67-68-5) as 45 μ M CLA, 30 μ M as a stock solution and kept at -20 °C.

Cell culture and cytotoxicity test

In this study, human cervix adenocarcinoma (HeLa, ATCC[®]CCL-2[™]), human colon colorectal adenocarcinoma

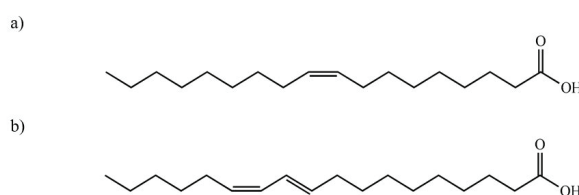


Figure 1. Structures of OLA (a) and CLA (b).

(CaCo-2, ATCC-HTB-37), human glioblastoma-astrocytoma (U-87 MG, ATCC[®]HTB-14[™]), human breast adenocarcinoma (MCF7, ATCC[®]HTB-22[™]), human lung adenocarcinoma (A549, ATCC[®]CCL-185[™]) and human prostate cancer (PC3, ATCC CRL-1435[™]) were used as cancer cell lines while human embryonic kidney cells (HEK293, ATCC-CRL-1573) were used as a non-cancerous cell line. Cells were maintained in DMEM/F12-Dulbecco's Modified Eagle's Medium with FBS (%), L-glutamine (2 mM), Pen-strep at 37 °C in a CO₂ incubator. ATCC-formulated Eagle's Minimum Essential Medium was used for the HEK293 (Embryonic kidney, ATCC[®]CRL-1573[™]) cell line. A U.V/ visible spectrophotometer (Thermo Scientific, Germany) was used to measure the optical density. The cultured cells (1x 10⁵ cells/mL) were treated with different doses (0.3 nM-30 nM dose range for OLA and 0.45 μ M- 45 μ M dose range for CLA) and incubated for 48 hours at 37 °C. After the incubation period, the viability percentage of the test substance was calculated using equation

$$\% \text{ Viable cells} = \left[\frac{\text{(The treated cells absorbance)} - \text{(The blank absorbance)}}{\text{(The blank absorbance)}} \right] \times 100$$

Cytotoxicity of OLA and CLA were fitted to a sigmoidal curve and a 4-parameter logistic model was used to calculate the inhibition of the cells (Mosmann, 1983).

In morphological investigations, the cells which were treated with different concentrations of agents and control cells were examined for morphological changes by inverted microscope. All experiments were done in triplicate.

Bacterial reverse mutation test (Ames test)

An Ames MPF[™] mutagenicity assay (Xenometrix Inc. Switzerland) was conducted on 4 strains of *Salmonella typhimurium*, tester strains TA98, TA100, TA 1535 and TA 1537 according to OECD Guideline 471. TA98 and TA 1537 strains are used for the detection of frameshift mutations and TA100 and TA1535 for base pair substitutions. Mice liver post-mitochondrial (S9) fraction was used for metabolic activation. The 30% S9 mix contained cofactors such as phosphate buffer pH 7.4, MgC₂, KCl, Glucose-6-phosphate, NADP and NaH₂PO₄ buffer. Reference negative control (0.5% DMSO) and strain-specific positive control were tested in all *Salmonella* strains (Maron & Ames, 1983). In the study, a 96-well dilution microplate was used for dilution of the test substances (6 doses) and 24-well microplates were used for bacterial exposure to the test substance during the process. For the detection of mutagenic activity of exposure, 384-well microplates were used. The first dilutions were performed in 96-well microplates for the test substance and were transferred to 24-well exposure microplates. The bacteria culture was incubated at 37°C, 250 rpm. After the incubation period, the cytotoxic dose of the compounds was evaluated. The lowest dose showing cy-

toxicity was chosen as the highest concentration in the test. According to the manufacturer's instructions, appropriate solutions for OLA and CLA were prepared (0.8, 4, 20, 100, 500, 2000 µg/mL). After 90 minutes incubation, 2.8 mL of indicator broth was added to each well of a 24-well exposure microplate. The exposure culture was transferred from the 24-well microplate to 384-well microplates. These plates were incubated at 37°C for 2 days. Then the microplate was removed from the incubator for counting. Raw data interpretation and calculation were performed according to manufacturer's instructions.

An Ames MPF™ Mutagenicity Assay kit is available for strain-specific positive control chemicals. The following positive controls were used in assessing the performance of the Ames assays (Table 1).

Strains	without S9		with S9	
		Concentration		Concentration
TA 98	2-Nitrofluorene	2 µg/mL	2-Aminoanthracene	25 µg/mL
TA 100	4-Nitroquinoline-N-oxide	0.1 µg/mL	2-Aminoanthracene	62.5 µg/mL
TA 1535	N ⁴ -aminocytidine	100 µg/mL	2-Aminoanthracene	125 µg/mL
TA 1537	9-aminoacridine	15 µg/mL	2-Aminoanthracene	125 µg/mL

Statistical analysis

Statistical analyses were performed by using SPSS for Windows 10.0 and GraphPad Prism 7 statistical analysis programs. The results were compared according to the control group using the Student's-t Test. Values were expressed as mean ± SD. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Cell culture and cytotoxicity test

Depending on the exposure to compounds, each cell line showed differences in IC₅₀ values (Table 2). No cytotoxic activity on MCF-7, HeLa, U-87-MG, and CaCo-2 cells was observed with the administered doses of OLA and CLA. The OLA IC₅₀ values were found to be 20 nM and 15 nM for the 48 h treatment on A549 and PC3 cells, respectively. The IC₅₀ values for CLA were determined to be 27 µM and 38 µM on PC3 and HEK-293 cells (Fig. 2,3).

After the post-treatment of the agents, the morphological changes were observed on PC3, A549 and HEK293 cells (Fig 4). OLA was shown to have a cytotoxic effect at 30 nM doses

Table 2. IC₅₀ values of OLA and CLA.

Cell Lines	IC ₅₀ Values	
	OLA	CLA
A549	20 nM	> 45 µM
U-87-MG	> 30 nM	> 45 µM
MCF-7	> 30 nM	> 45 µM
CaCo-2	> 30 nM	> 45 µM
HeLa	> 30 nM	> 45 µM
PC-3	15 nM	27 µM
HEK-293	> 30 nM	38 µM

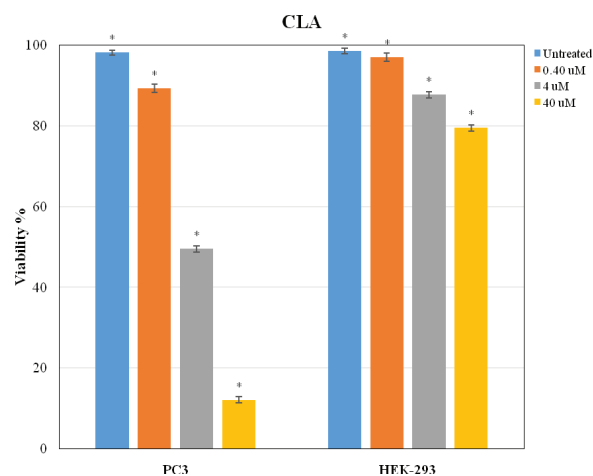


Figure 2. Cytotoxic effects of CLA on PC-3 and HEK-293 cells after 48 h exposure. All test substances were reconstituted with DMSO, which was evaluated as a control. Control was exposed only to vehicles which were 100% viable. Data are expressed as mean ± SD.

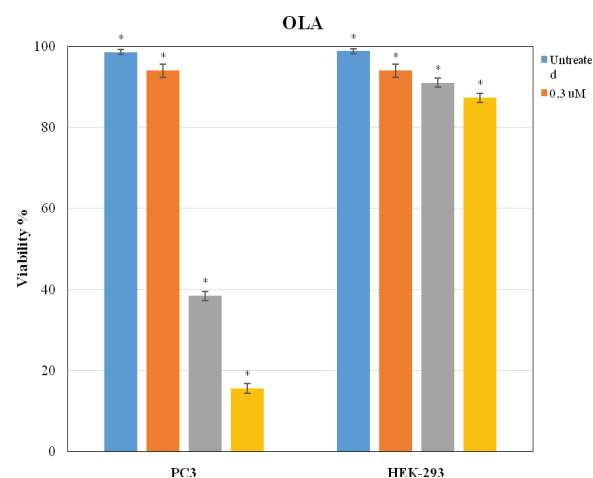


Figure 3. Cytotoxic effects of OLA on PC-3 and HEK-293 cells after 48 h exposure. All test substances were reconstituted with DMSO, which was evaluated as a control. Control was exposed only to vehicles which were 100% viable. Data are expressed as mean ± SD.

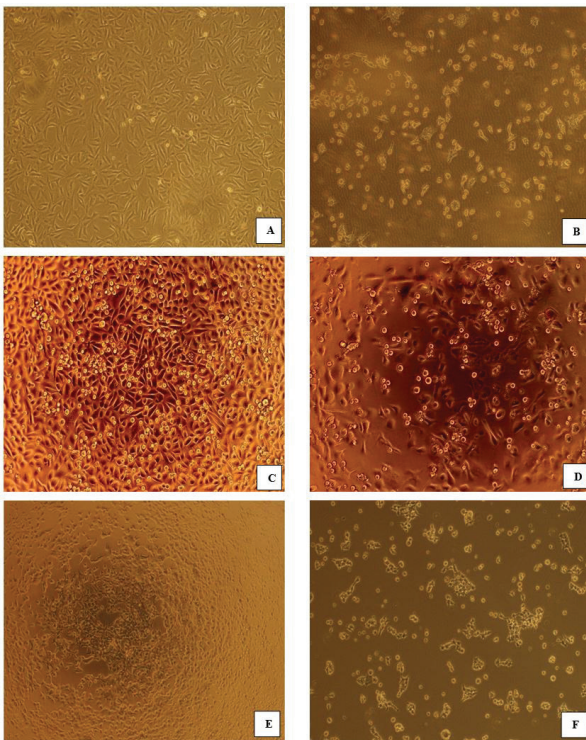


Figure 4. Morphological characterization cell lines. A. A549 untreated control cells; B. OLA treated A549 cells (30 nM); C. PC3 untreated control cells; D. OLA treated PC3 cells (30 nM); E. HEK-293 untreated control cells; F. CLA treated HEK-293 cells (45 μ M).

on PC3 and A549 cancer cell lines. CLA was effective at a 38 μ M dose on HEK293 cells. There was a clear difference, such as various morphological abnormalities, between the untreated control cells and treated cells. The cells lost their normal appearance when compared with untreated control cells.

Bacterial reverse mutation test

In the Ames assay, the mean number of positive yellow wells per 6 doses was calculated from the triplicates and the fold increases above the baseline were determined for each dose of OLA and CLA. According to the results no mutagenic evidence was determined for the maximum dose of 2000 μ g/mL of OLA and CLA (Fig 5-6).

DISCUSSION

Safflower plants contain medical and biologically essential flavonoids, alkaloids, steroids, and polysaccharide compounds such as fatty acids. These compounds form the basis of the therapeutic efficacy of the plant (Khalid et al., 2017). An increasing trend for safflower and its production has been observed over the previous few years, as evident from the increase of crop land at the rate of 4.9% per annum. Due to the importance of alternative and complementary medicine in the treatment of many diseases, safflower and its products, which are traditional herbal products, have started to be studied intensively (Bae, Kim, Lee, Kim, & Son, 2015; Guner, Kizilsahin, Nalbantsoy & Karabay Yavasoglu, 2020). Therefore, in this study, the anti-cancer potential of OLA and CLA, which are bioactive

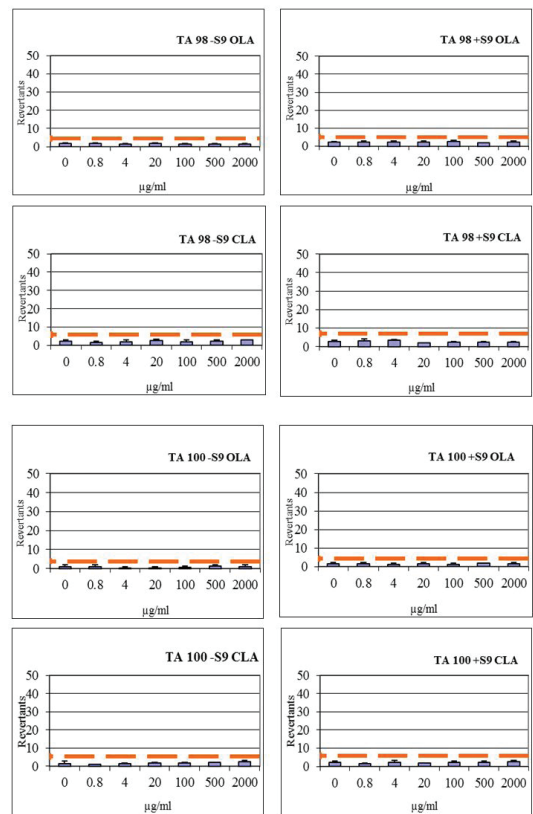


Figure 5. Mutagenicity test results of OLA and CLA with *Salmonella typhimurium* TA 98 and TA 100 strains.

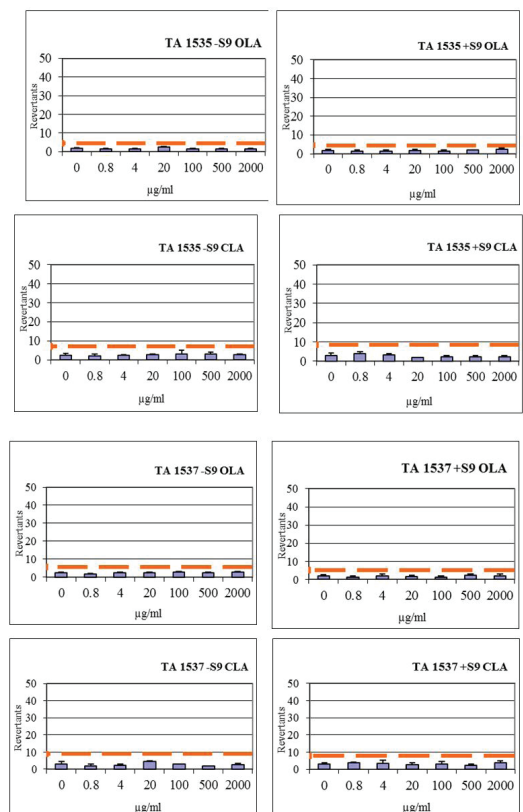


Figure 6. Mutagenicity test results of OLA and CLA with *Salmonella typhimurium* TA 1535 and TA 1537 strains.

components of the safflower plant, was investigated. Investigating of biological effects of these bioactive components will contribute to the conversion into pharmaceutical forms of these structures for therapeutic purposes in the future.

In the *in vitro* anticancer efficacy study, cells exposed to the compounds showed different LD₅₀ values. These results thought that different cell lines are affected by different doses of the compounds. In addition to this, another reason for the difference in results was considered to be use of different isomers of the compounds. Moreover, differences in the proliferation activities of cancer cell lines have led to change in the results. According to the results of MTT assay on the U-87 MG cell line, OLA and CLA have not shown any cytotoxic effect. Also, CLA at 45 µM dose was also exhibited similar effects against A549, MCF-7, and CaCo-2 HeLa cell lines. Nevertheless, in a study evaluating the effects of 5 different purified isoforms of CLA and a mixture of CLA isoforms on estrogen receptor positive (ER+) breast cancer cells (MCF-7), it was revealed that CLA (t10, c12) isomer showed dose dependent inhibition on the MCF-7 cell line (Tanmahasamut, Hendry, & Sidell, 2004). The observed effect is thought to be related to administration as a mixture and the application dose. In our study, CLA showed cytotoxic activity on PC-3 prostate cancer cells after 48 hours of exposure with an IC₅₀ value of 27 µM. These findings were similar to other studies on this subject (Cohen, Zhao, Pittman, & Scimeca, 2003; Palombo, Ganguly, Bistriani, Menard, 2002). Also our results exhibited that OLA was found effective on A549 and PC-3 cancer cell lines with the IC₅₀ of 20 nM and 15 µM, respectively. Although anti-proliferative activities using different doses of OLA were determined on mouse lung carcinoma, LLC cells and human prostate cancer cells (PC-3) in many *in vitro* studies (Hughes-Fulford, Chen, & Tjandrawianata, 2004; Kritchevsky, 2002), there was no data on its anti-proliferative activity on A549 cells in the literature. In this study, we determined the cytotoxicity of OLA on A549 cells for the first time. However, the administered OLA and CLA concentrations on HeLa, CaCo-2, MCF-7, and HEK293 cell lines also did not present any anti-proliferative activity. In the literature, it was confirmed that there was no toxic effect of the compounds on control cells HEK293. In this study, cytotoxic effects of OLA and CLA were screened on many cancer cell lines for the first time.

In genotoxicity evaluation of OLA and CLA, according to Ames MPFTm assay *S.typhimurium* TA 98, TA 100, TA 1535, and TA 1537 strains and S9 fraction were used. According to the assay, a substance with mutagenic effect must be show a statistically significant difference for at least 1 dose. The substance should be compared with the control group and it should increase the number of revertant colonies more than 2 times. In this study, safflower oil derivatives at even a 2000 mg/mL concentration, which is the maximum dose, according to OECD, did not cause mutation effects on TA 98, TA 100, TA 1535, and TA 1537 strains with/without S9 fraction. Similarly our findings, in the anti-mutagenic effect studies conducted with many isomers of CLA originating in different foods, no mutagenic effect was observed at the application doses of the isomers (Kritchevsky, 2002). However, there was no study of mutagenic activity related to OLA in the literature. Thus, this is the first genotoxic potential evaluation of oleic acid.

CONCLUSION

In conclusion, OLA and CLA, which are derivatives of safflower, are important oil components for biologic activity. According to our results, these components can be considered as beneficial dietary supplement for supporting anti-cancer therapy without side effects.

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






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Sex-dependent effects of resveratrol and regular exercise on markers related to cellular stress in the hearts and kidneys of rats

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ABSTRACT

Background and Aims: Regular exercise and several food supplements are recommended for a healthy lifestyle. In this study, the sex-dependent effects of resveratrol and regular exercise training on cellular stress response were investigated in the hearts and kidneys of rats.

Methods: Male and female Wistar rats (3-month-old) were used. Resveratrol (7.5 mg/kg, in the drinking water) and exercise training (for 40 minutes, three days per week) were applied for 6 weeks. The markers related to cellular stress were measured in left ventricle and kidney tissues.

Results: Cardiac total antioxidant capacity (TAC) levels were greater in males than females, but no difference was observed in kidney tissue. Exercise training increased both TAC and nitrite levels in the heart in females but not in males. Even cardiac nitrite levels were decreased in males by exercise. Cardiac phosphorylated-protein kinase RNA-like endoplasmic reticulum kinase (p-PERK) expression and renal glucose-regulated protein-78 (GRP78) expression were higher in the control male group compared to the female group. Cardiac tumor necrosis factor- α (TNF- α) expression was higher in females than males but not in kidneys. However, matrix metalloproteinase-2 (MMP-2) expression was higher in males than females in both kidney and cardiac tissues. The phospho-inhibitor κ B- α (p-I κ B- α) did not change between gender in cardiac tissues but had a decreased level in male kidneys compared to the female control group. Expression of phospho-extracellular signal-regulated kinase (p-ERK) in females and phospho-protein kinase B (p-Akt) in males were lower in kidney tissue. Resveratrol and exercise treatments markedly decreased Akt protein expression in male kidney tissue.

Conclusion: Our findings indicate that the markers involved in cellular stress response were affected differently by the resveratrol and exercise treatments considering sex and tissue dependencies.

Keywords: Cellular stress, Sex, Resveratrol, Exercise, Heart, Kidney

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INTRODUCTION

Sex is a serious determinant of life span. Biological differences between males and females resulting from genetic, epigenetic, and hormonal factors are known as sex differences. Sex differences are implicated as a crucial factor in the biology and pathophysiology of various disorders as well as outcomes of treatments (EUGenMed Cardiovascular Clinical Study Group et al., 2016; Campesi et al., 2017; Bairey Merz et al., 2019). However, little is known about the sex differences in physiological and pathophysiological processes, and the information about the effect of sex in these processes is not sufficient, either.

Epidemiological studies suggest that non-pharmacological approaches, such as dietary intervention and regular physical activity, can prevent or delay the progression of diseases. Physical activity is associated with better health and recent research has reported that regular physical activities can protect body organs against several acute and chronic diseases (Chen et al., 2019, Zhang et al., 2019). Regular exercise training has been shown to improve total antioxidant capacity (TAC), increase endothelial nitric oxide (NO) production, re-establish impaired endoplasmic reticulum (ER) homeostasis and calcium cycling, alleviate inflammation and fibrosis to protect many organs, including the heart, vessels, and kidney (Rolim et al., 2007; Tung et al., 2015; Han et al., 2018; Kosaki et al., 2019). Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin found in grapes, berries, peanuts and wines. Resveratrol has been reported to exert antioxidant, anti-inflammatory, anti-fibrotic and anti-apoptotic effects by affecting different signaling pathways (Han et al., 2018, Singh et al., 2019; Cheng et al., 2020). There are many studies examining the beneficial effects of orally administered resveratrol in the range of 3-22 mg/kg (Soylemez, Gurdal, Sepici & Akar, 2008; Soylemez, Sepici & Akar, 2009, Han et al., 2018; Cheng et al., 2020). Importantly, resveratrol has been shown to display hormetic actions, being protective at lower doses and detrimental at higher doses in both humans and animals (Juhász, Mukherjee, & Das, 2010; Singh et al., 2019; Cheng et al., 2020). In our previous study, it has been shown that resveratrol intake and regular exercise training have beneficial effects on vessel functions and the vascular expression of mRNA of inflammation and oxidative stress-related molecules in an age- and sex-dependent manner (Han et al., 2018). Although there is growing evidence suggesting that resveratrol and regular exercise have beneficial effects on heart and kidney, sex differentiation of the effects and exact mechanisms of their action have not been clarified yet. Considering that gender difference affects many physiological/pathological processes and is even organ tissue-specific, this issue becomes more important.

Therefore, in the current study, the sex-dependent effects of resveratrol intake and regular exercise training on the NO and TAC levels and the expressions of some proteins related to cellular stress response, which play a role in the pathogenesis of cardiovascular and renal diseases, were investigated in the heart and kidney tissue of rats.

MATERIAL AND METHODS

Animal care and experimental protocol

The experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The research protocols were approved by the Local Ethics Committee for Animal Experiments of Ankara University.

Wistar male and female rats (n:8 rats per group, total 48 rats) were taken from The Laboratory Animal Service of the University of Ankara and maintained under standard conditions (24±1°C room with a 12:12 hour light/dark cycle) for the duration of the experiment. The ages of the rats were adjusted to be 3 months-old at the time of sacrifice. Water and standard rat chow were provided *ad libitum* during the experimental period. The rats were randomly divided into six groups: Control male group (n=8), Control female group (n=8), Resveratrol male group (n=8), Resveratrol female group (n=8), Exercise male group (n=8) and Exercise female group (n=8).

The rats in the control groups were given tap water (without resveratrol) and the exercise training was not performed. All rats in the exercise groups were familiarized with a horizontal rodent treadmill (May Tme 0804 Animal Treadmill, Turkey) for one week (with a speed of 20 m/min at 0° incline, for 15 min/day, 3 days a week). After the one-week acclimatization period, the animals underwent a 40-min running session on a horizontal treadmill at 20 m/min (0° incline), 3 days per week, for 6 weeks. In the resveratrol group, resveratrol was dissolved in absolute ethanol and diluted with drinking water to a concentration of 50 mg/L, at a level sufficient to provide the appropriate dose (7.5 mg/kg) based on the consumption (bottles were protected from light). The concentration and administration route of resveratrol were chosen from previous *in vivo* studies (Soylemez et al. 2008; Soylemez et al. 2009). At the end of the study, the rats were anesthetized with 40 mg/kg i.p. sodium pentobarbital. The left ventricle and kidney tissues were quickly removed and frozen in liquid nitrogen after washing with phosphate-buffered saline (PBS) and stored at -80°C.

Biochemical measurements

The left ventricle and kidney tissues were homogenized in PBS (approximately 1g tissue/10 mL PBS) on ice. The homogenates were centrifuged at 4°C and 10000 rpm for 30 minutes. Then, the supernatants were carefully collected for biochemical measurements. The protein concentration of the supernatants was evaluated using the Bradford method. The tissue nitrite level as an indicator of nitric oxide (NO) production was measured spectrophotometrically by the method based on the Griess reaction. TAC levels of the tissue homogenates were measured by the method based on the reduction of Cu⁺² to Cu⁺¹ by the antioxidants (Usanmaz & Demirel-Yilmaz, 2008).

Tissue homogenization and Western Blot experiments

The total protein was extracted from left ventricle and kidney tissue via homogenization in homogenization buffer (approximately 50 mg tissue/1mL buffer) (including 50 mM Tris pH: 8.8, 1% NP40, 1 mM PMSF, 2 mM disodium-EDTA, 10% Sucrose and protease-phosphatase inhibitor) with a homogenizer. The ho-

mogenate was centrifuged at 1000 g for 15 min at 4°C, and then the supernatant was collected. The protein concentration of the supernatants was evaluated using the Bradford method. Equal amounts (50 µg *per lane*) of proteins from different groups were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 4 % stacking gel and 10 % separating gel with a Mini Protean Tetra electrophoresis apparatus (Bio-Rad Laboratories) and transferred onto PVDF membranes using a semi-dry electro-blotting apparatus (TransBlot Turbo, BioRad, Puchheim, Germany) (Towbin, Staehelin & Gordon, 1979; Bal et al., 2020). After the transfer period, the membranes were incubated for 1-1.5 h in blocking solution containing 5% non-fat dried milk or 3% BSA (for phospho proteins) in Tris-buffered saline (TBS). Then, the membranes were incubated overnight at 4 °C with one of the following antibodies: glucose-regulated protein-78 (GRP78), phosphorylated-protein kinase RNA-like endoplasmic reticulum kinase (p-PERK), sarco/endoplasmic reticulum Ca²⁺-ATPase2 (SERCA2), inhibitor κB-α (IκB-α), p-IκB-α, matrix metalloproteinase-2 (MMP-2), Tumor necrosis factor-α (TNF-α), extracellular signal-regulated kinase (ERK), protein kinase B (Akt), p-ERK and p-Akt. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Then, the membranes were incubated in chemiluminescence substrate solution (Clarity™ Western ECL solution, Bio-Rad). Images of the blots were taken with the chemiluminescence detection system (Bio-Rad). GAPDH was used as the loading control protein. The relative expression of proteins with respect to GAPDH was calculated using the Image Lab 4.1 software. The results of protein expression demonstrate the average of all rats used in the experiment (n= 5-8), and the best image representing the result has been used.

Statistical analysis

The data were expressed as means±SEM. Statistical analyses were performed using GraphPad Prism Instat 5.01 (GraphPad Software, Inc., La Jolla, USA). Results were analyzed using Student's t-test (for comparison of sex differences) or One-way ANOVA followed by post hoc Dunnett's test (for inter-group comparisons). P-values ≤0.05 were considered statistically significant.

RESULTS

In heart tissue, TAC levels of the female control and resveratrol groups were lower than those of the male groups (Fig. 1A). Exercise training significantly increased the cardiac TAC levels only in female animals. In heart and kidney tissue, the TAC levels of female exercise group were higher than those of the female resveratrol group (Fig. 1A and B). The cardiac TAC levels were lower than the renal TAC levels in female animals (Fig. 1C).

Exercise training significantly alleviated the cardiac nitrite levels in the male rats (Fig 2A). The nitrite levels of heart tissue were higher in female resveratrol and exercise groups compared to those of the male groups (Fig. 2A). In kidney tissue, nitrite levels were similar in all groups (Fig. 2B). The cardiac nitrite levels of female resveratrol and exercise groups were greater than the renal nitrite levels (Fig. 2C).

GRP78 expression, which is an important marker of endoplasmic reticulum stress (ERS), was similar in the hearts of male and female

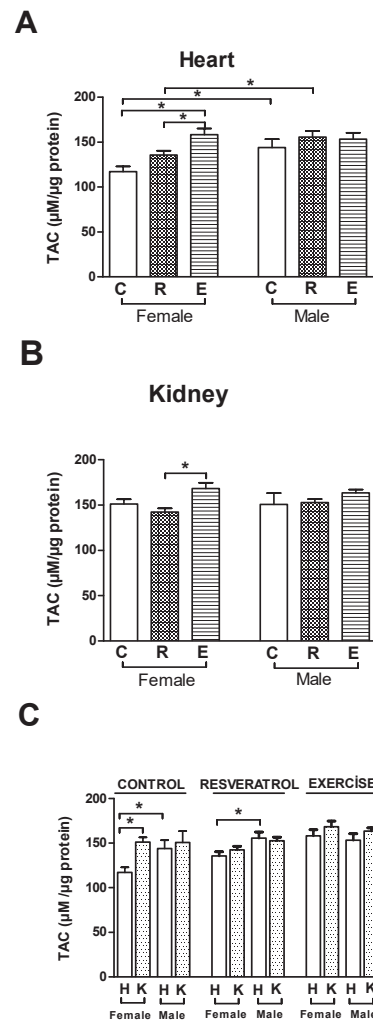


Figure 1. Cardiac and renal TAC levels in control, resveratrol, and exercise training rats. Cardiac TAC levels were higher in males than in females (A). While exercise only increased the cardiac TAC levels in females, resveratrol did not affect the TAC levels in both tissues (B). Renal TAC levels are greater than cardiac TAC levels in control female rats (C). * $p < 0.05$. Values are expressed as mean \pm SEM. H: Heart, K: Kidney.

rats. In male resveratrol and exercise-trained groups, GRP78 expression was higher than that of females (Fig 3A and B). The PERK pathway is one of the unfolded protein responses (UPR) arms that is active during ERS. The p-PERK expression of the male rats was higher than that of females, while resveratrol and exercise did not affect p-PERK expression in both sexes (Fig 3A and C). SERCA2 is an important influx pump in the storage of Ca²⁺ into the ER. The expression of SERCA2 was similar in all groups (Fig 3A and D).

In the kidneys, the GRP78 expression of female rats was lower than that of male rats, but resveratrol consumption and exercise training did not alter the GRP78 expression in both sexes (Fig. 4A and B). Resveratrol significantly increased the p-PERK expression in females. In the female resveratrol and exercise groups, the p-PERK expression was markedly greater than that of male groups (Fig 4A and C).

TNF-α is an important proinflammatory cytokine, and cardiac TNF-α protein levels were higher in the female group. On the

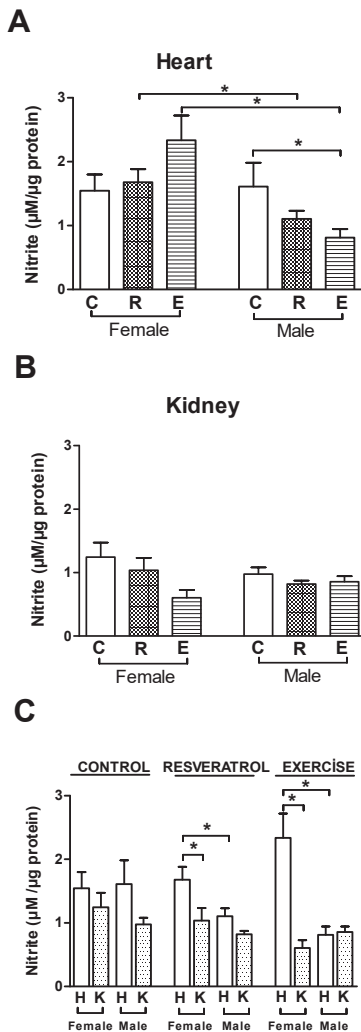


Figure 2. Cardiac and renal nitrite levels in control, resveratrol, and exercise training rats. The cardiac nitrite levels of female exercise and resveratrol groups were higher than those of males. The exercise reduced the cardiac nitrite levels in male rats (A). The renal nitrite levels were similar in all groups (B). Cardiac nitrite levels are higher than renal nitrite levels in resveratrol and exercise-treated female rats (C). * $p < 0.05$. Values are expressed as mean \pm SEM. H: Heart, K: Kidney.

other hand, profibrotic marker MMP-2 expression was lower in the heart of the female group compared to that of the male group (Fig. 5 A, B and E). Resveratrol treatment and exercise training did not affect TNF- α , I κ B- α , p-I κ B- α , and MMP-2 protein levels in the heart tissue of both sexes (Fig 5).

In the female resveratrol group, renal TNF- α expression was markedly greater than that of the male group (Fig 6A and B). While p-I κ B- α protein levels were higher (Fig 6A and C), MMP-2 expression was lower in female rats compared to male rats (Fig. 6A and D). Exercise training did not alter renal TNF- α , p-I κ B- α , and MMP-2 protein expressions in both sexes (Fig. 6).

Akt, p-Akt, ERK, and p-ERK protein levels were similar in the heart tissue of female and male rats. Resveratrol intake did not change these protein levels in both sexes, but exercise training significantly decreased the ERK expression only in male group (Fig 7).

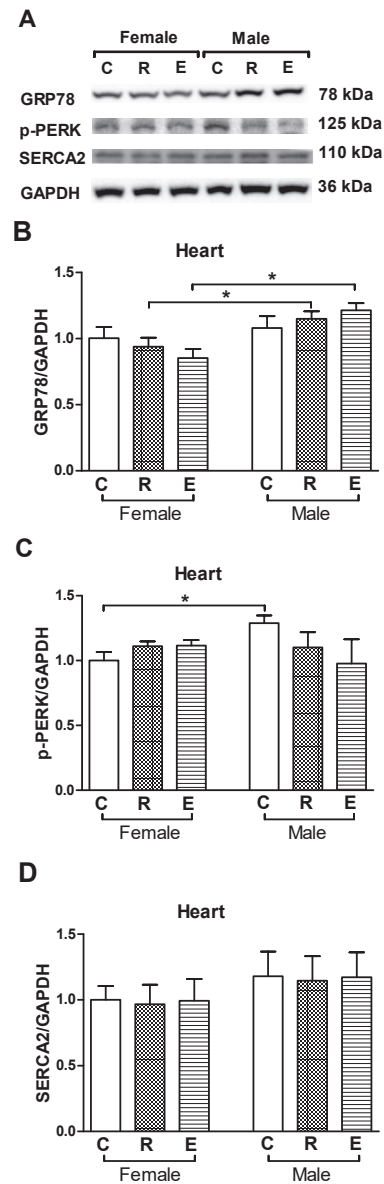


Figure 3. Sex-dependent effects of resveratrol treatment and exercise training on cardiac GRP78, p-PERK, and SERCA2 expression. In male resveratrol and exercise groups, the cardiac GRP78 expression was higher than that of female groups (A and B). In female rats, cardiac p-PERK expression was lower than that of males (A and C). The SERCA2 expression was similar in all groups (A and D). * $p < 0.05$. Values are expressed as mean \pm SEM.

In the kidneys, resveratrol intake and exercise training significantly decreased the Akt expression in males (Fig 8A and B). p-Akt levels of the female control and resveratrol groups were greater than those of the male groups (Fig 8A and C). Similarly, the ERK expression of the female resveratrol group was higher than that of the male resveratrol group. The resveratrol treatment markedly reduced ERK expression in male rats (Fig 8A and D). Unlike p-Akt expression, p-ERK expression was lower in the female rats compared to the male rats (Fig 8A and E).

In this study, SERCA2 and I κ B- α expressions were also examined in kidney tissue isolated from all groups. However, since the expres-

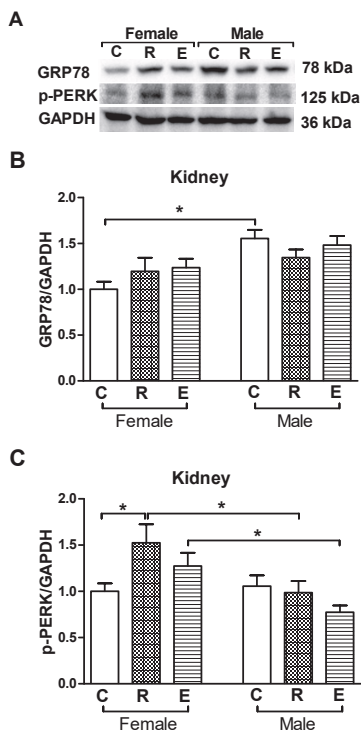


Figure 4. Sex-dependent effects of resveratrol treatment and exercise training on renal GRP78 and p-PERK expression. In females, the renal GRP78 expression was smaller than that of males (A and B). The resveratrol intake increased the renal p-PERK expression in female animals. The expression of p-PERK in the female resveratrol and exercise groups is higher than that of males (A and C). *p < 0.05. Values are expressed as mean ± SEM.

sions of SERCA2 and IκB-α were not good enough to be quantified, these findings were not included in the present study.

All sex-dependent alterations in cardiac and renal protein expressions are summarized in Table-1 and Table2, respectively.

DISCUSSION

In the current study, the effects of resveratrol consumption and exercise-training on various factors related to cellular stress were examined in a tissue- and sex-dependent manner. The differences in TAC levels and p-PERK, TNF-α, and MMP-2 protein expressions in the heart and GRP78, p-IκB-α, MMP-2, p-Akt, and p-ERK expressions in kidney tissue were observed between sexes. Tissue TAC and NO levels and some protein expressions were separately changed by resveratrol intake and regular exercise training in a sex-dependent manner.

The effect of sex in control animals on biomarkers

It has been known that males and females have different responses in both basal and stressful conditions. Revealing sex-specific molecular differences in cellular stress responses in the adult healthy heart and kidney may contribute to a better understanding of the sex-specific aspects of human cardiovascular and kidney disease. In this part, sex- and tissue-dependent differences of the NO and TAC levels and some proteins related to cellular stress response, which play a role in the biology and pathophysiology of cardiovascular and renal diseases, are discussed.

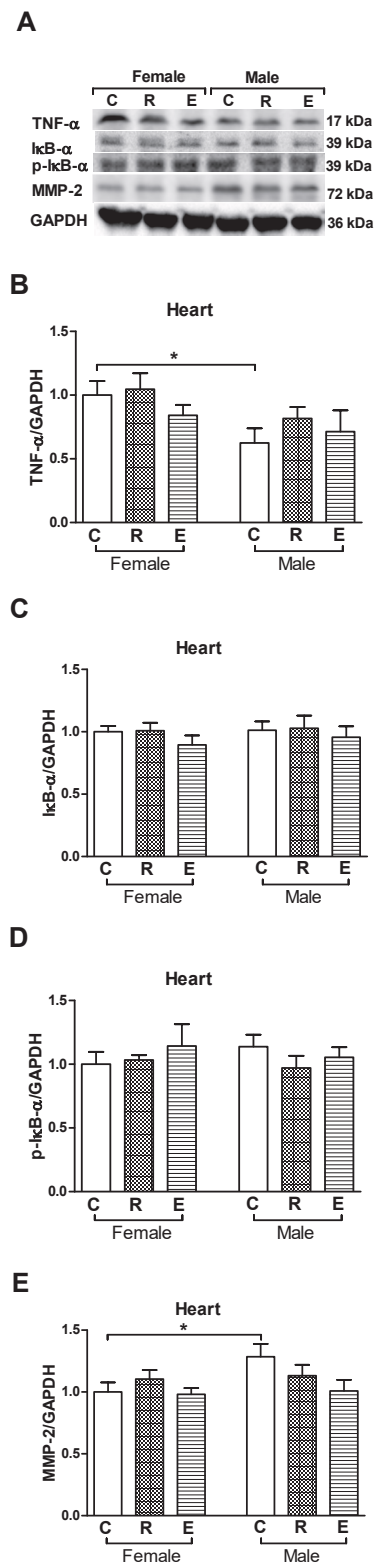


Figure 5. Sex-dependent effects of resveratrol treatment and exercise training on cardiac TNF-α, IκB-α, p-IκB-α, and MMP-2 expression. The cardiac TNF-α (A and B) expression is higher, and the MMP-2 (A and E) expression is lower in the female group compared to those in the male group. IκB-α and p-IκB-α levels were similar in all groups (A, C and D) *p < 0.05. Values are expressed as mean ± SEM.

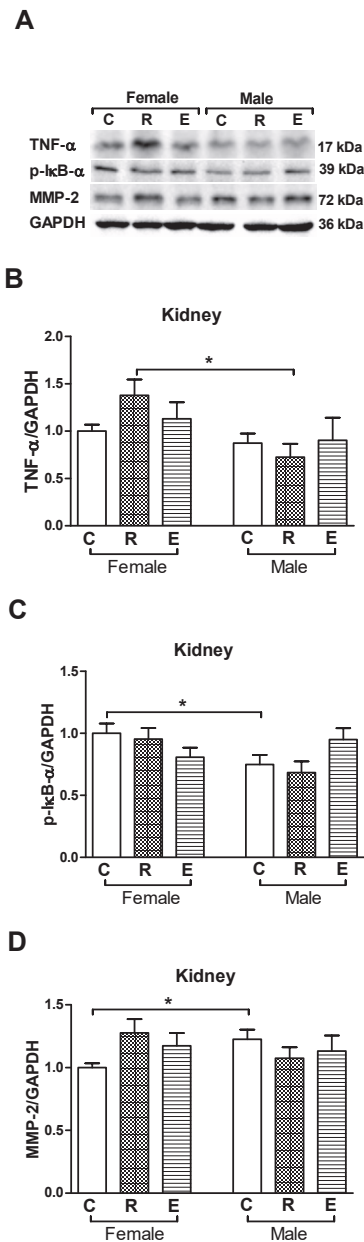


Figure 6. Sex-dependent effects of resveratrol treatment and exercise training on renal TNF- α , p-I κ B- α , and MMP-2 expression. Renal TNF- α levels were greater in resveratrol-treated female rats compared to resveratrol-treated male rats (A and B). The renal p-I κ B- α expression (A and C) was higher, while the MMP-2 expression (A and D) was lower in female rats compared to those of male rats. * $p < 0.05$. Values are expressed as mean \pm SEM.

Increased production of reactive oxygen species or loss of antioxidant defense, defined as oxidative stress, disrupts many physiological processes, leading to detrimental changes. Due to the many different antioxidant components in the body and the relative difficulty of measuring each known antioxidant separately, the total antioxidant capacity was measured as a general indicator of oxidative stress in tissue homogenates. In the current study, cardiac TAC levels were higher in males than in females, but renal TAC levels were similar in both sexes. This finding shows that the sex-related differences in TAC level may

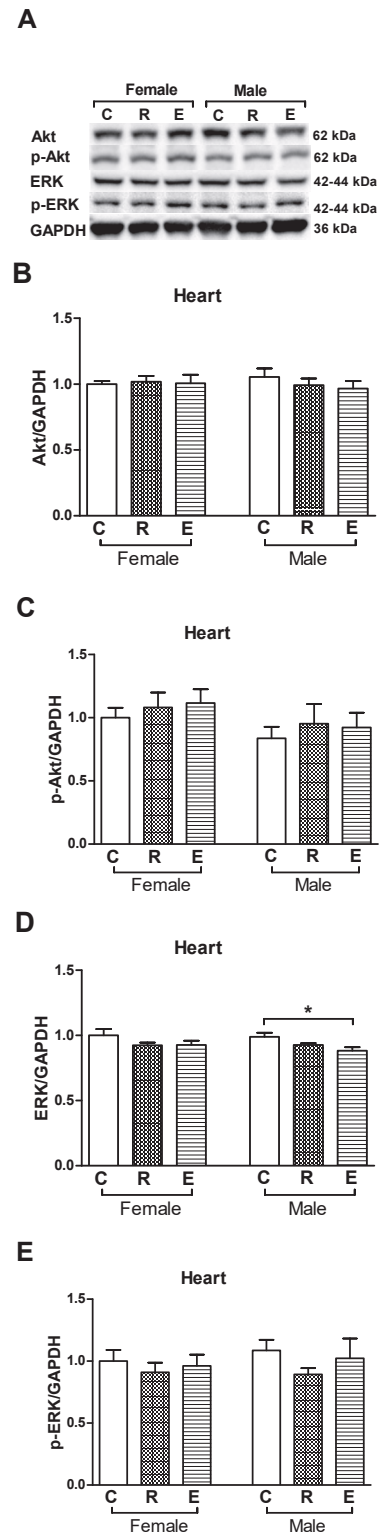


Figure 7. Sex-dependent effects of resveratrol treatment and exercise training on cardiac Akt, p-Akt, ERK, and p-ERK expression. The cardiac Akt, p-Akt, ERK, and p-ERK expressions were not different in females and males (A-E). The exercise only reduced the ERK expression in male rats (A and D). * $p < 0.05$. Values are expressed as mean \pm SEM.

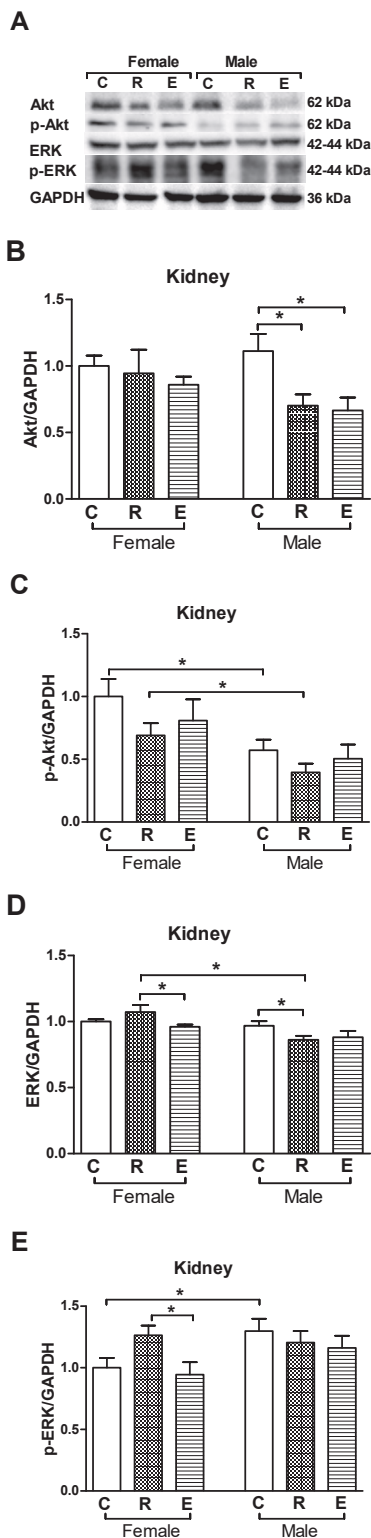


Figure 8. Sex-dependent effects of resveratrol treatment and exercise training on renal Akt, p-Akt, ERK, and p-ERK expression. In females, the renal p-Akt expression (A and C) was higher while the p-ERK expression (A and E) was lower compared to those of males. The resveratrol treatment reduced the Akt and ERK expressions in male animals (A, B and D). * $p < 0.05$. Values are expressed as mean \pm SEM.

vary depending on the tissue. Contrary to our results, cardiac and renal TAC levels have been reported to be lower in 15-16-week-old male Wistar rats than in females (Katalinic, Modun, Music, & Boban, 2005). In another study, the cardiac TAC levels in 8-week-old Wistar rats were found to be similar in both sexes (Saghebjo et al., 2019). These different results in TAC levels may be due to the different measurement methods (FRAP, ABST, etc.) or the use of animals of different ages.

Recent studies have demonstrated the deficiency of NO, which plays a pivotal role as a key secondary messenger that has a role in pathological activities, is both a cause and a consequence of many diseases and organ damages (Baylis, 2012). In the current study, cardiac and renal NO levels were not different in female and male rats. Consistent with our findings, it was reported that cardiac NO levels and renal eNOS expression levels were similar in male and female rats (Erdely, Greenfeld, Wagner & Baylis, 2003; Balci & Pepe, 2012). In light of these data, it can be predicted that NO levels do not change depending on sex or tissue.

The ER regulates protein folding, maturation, quality control, transport, and intracellular Ca^{2+} levels. Disruption of these ER functions leads to the retention of newly synthesized unfolded proteins in the ER, and this condition is referred as ER stress. When ER stress occurs, GRP78 dissociates from the transmembrane sensors (PERK, ATF6 and IRE1), and these signal transduction pathways are activated for the restoration of ER homeostasis (Hong, Kim K, Kim JH & Park, 2017). In the current study, we observed that renal GRP78 expression and cardiac p-PERK expression were higher in males than those of females. These results show that basal expressions of ER stress proteins may vary depending on sex and tissue. Also, it can be argued that the ER stress burden is higher in males than females. SERCA2 is an important pump in the storage of Ca^{2+} back into the ER. The reduction in SERCA2 activity and/or expression leads to the decrease in the ER Ca^{2+} levels and triggers ER stress (Chemaly, Troncone & Lebeche, 2018). In the present study, cardiac SERCA2 expression was similar in both sexes. In light of this result, it can be said that the change in SERCA-mediated calcium regulation in the heart does not contribute to the sex differences in ER stress burden.

It is known that inflammatory response plays a role in the pathogenesis of various acute and chronic diseases and is associated with organ damage. The activation of transcription factor NF- κ B is one of the important inflammatory pathways. Under basal conditions, NF- κ B are bound to I κ B in the cytoplasm and are inactive. During the activation, I κ B is phosphorylated by I κ B kinases and separated from NF- κ B, which leads to the activation of NF- κ B signaling. The activation of this pathway coordinates several gene expression involved in inflammation, fibrosis, and apoptosis (Fiordelisi et al. 2019). However, sex and tissue-dependent alterations of protein expressions related to inflammation are still not studied in detail. In our previous study, it was reported that the mRNA expressions of IL-6, NF- κ B, and TNF- α in thoracic aorta were similar in both sexes. In the current study, the cardiac TNF- α expression was greater in females while the cardiac MMP-2 expression was higher in

Table 1. Sex-dependent effects of resveratrol treatment and exercise training on cardiac protein expression.

HEART						
Protein	Female			Male		
	Control	Resveratrol	Exercise	Control	Resveratrol	Exercise
GRP78	-	F<M	F<M	-	F<M	F<M
p-PERK	F<M	-	-	F<M	-	-
SERCA2	-	-	-	-	-	-
TNF- α	F>M	-	-	F>M	-	-
I κ B- α	-	-	-	-	-	-
p-I κ B- α	-	-	-	-	-	-
MMP-2	F<M	-	-	F<M	-	-
Akt	-	-	-	-	-	-
p-Akt	-	-	-	-	-	-
ERK	-	-	-	C>E	-	C>E
p-ERK	-	-	-	-	-	-

F: Female, M: Male, C: Control, R: Resveratrol, E: Exercise

Table 2. Sex-dependent effects of resveratrol treatment and exercise training on renal protein expression.

KIDNEY						
Protein	Female			Male		
	Control	Resveratrol	Exercise	Control	Resveratrol	Exercise
GRP78	F<M	-	-	F<M	-	-
p-PERK	C<R	F>M, C<R	F>M	-	F>M	F>M
TNF- α	-	F>M	-	-	F>M	-
p-I κ B- α	F>M	-	-	F>M	-	-
MMP-2	F<M	-	-	F<M	-	-
Akt	-	-	-	C>R, C>E	C>R	C>E
p-Akt	F>M	F>M	-	F>M	F>M	-
ERK	-	F>M, R>E	R>E	C>R	F>M, C>R	-
p-ERK	F<M	R>E	R>E	F<M	-	-

F: Female, M: Male, C: Control, R: Resveratrol, E: Exercise

males. On the other hand, in kidney tissue the p-I κ B- α expression was higher; the MMP-2 expression was lower in female rats compared to those of male rats. These findings suggest expressions of some inflammatory and fibrotic proteins may differ depending on tissue and sex. Considering the above results, it can be argued that the inflammatory response in females and the fibrotic response in males are predominant in the basal state.

Akt and ERK-mediated signal transduction have been implicated in many cellular activities ranging from gene expression to proliferation, stress signaling, inflammatory-fibrotic responses, and apoptosis (Zhang et al., 2016; Liu et al., 2017). In the present study, the cardiac expressions of Akt, ERK, and their phosphorylated forms were similar in both sexes, in parallel with the

findings of the previous study on twelve-week-old male and female C57Bl/6 mice (Dworatzek et al., 2014). It was observed that p-Akt expression in females and p-ERK expression in males were greater in kidney tissue. It can be speculated that the Akt signaling pathway in females and the ERK signaling pathway in males is mostly active. In addition, the renal inflammatory state may be mediated by the Akt pathway in females and the renal fibrotic state by the ERK pathway in males, but more evidence is required to confirm this speculation. Also, there was no sex-related difference in these signaling pathways in heart tissue.

The sex specific effect of resveratrol treatment

Resveratrol consumption has beneficial effects in many different pathologies by regulating the oxidant/antioxidant balance, promoting nitric oxide production, and modulating

inflammatory, fibrotic, and mitogenic pathways (Cheng et al., 2020). It has been suggested that resveratrol intake increases antioxidant capacity by modulating antioxidant defense systems. In a recent study, it has been shown that resveratrol administration (10 mg/kg resveratrol per day via gavage, for 12 week) increased plasma TAC levels in 6-week-old male Wistar rats (Nasiri et al., 2021). In the present study, resveratrol intake did not affect the tissue TAC levels in both sexes. On the other hand, cardiac TAC levels were higher in resveratrol-treated males than in the females. This may be because control males also have greater cardiac TAC levels than females.

Promoting nitric oxide production by different mechanisms plays an important role in the cardiovascular and renal protective effects of dietary resveratrol. It has been previously reported that resveratrol can increase NO levels through estrogen receptors (Li, Xia & Förstermann, 2012; Cheng et al., 2020). Also, it has been demonstrated that resveratrol intake increased the blood nitrate/nitrite levels and aortic NO production, eNOS mRNA, and protein levels in rats of both sexes (Soylemez et al., 2008; Soylemez et al., 2009; Pektaş, Sadi & Akar, 2015; Han et al., 2018). In contrast to the previous studies, resveratrol intake did not alter the tissue NO levels in any group in the current study. It can be speculated that this may be due to the differences in the protocol of resveratrol treatment (dose, duration, intensity etc.), age, sex, or tissue of the rats. However, in resveratrol-treated groups, cardiac NO levels were significantly higher in females than in the males. This result is attributable to sex differences in hormonal and/or metabolic status and may be due to the fact that resveratrol increases NO production (mediated by eNOS or iNOS) via cardiac estrogen receptors.

It was reported that resveratrol consumption inhibited ER stress induced by different pathologies (Lin et al., 2016). In contrast to previous studies, it was observed in the current study that resveratrol intake did not affect the expression of ER stress markers GRP78 and p-PERK in both tissues of male and female rats. Also, cardiac SERCA2 expression was similar in resveratrol-treated groups. However, in resveratrol-treated male groups, GRP78 expression was higher in the heart, while p-PERK expression was lower in kidney tissue, compared to females. These findings could be interpreted as the sex- and tissue-dependent variable effects of resveratrol on these stress markers. To the best of our knowledge, the tissue- and sex-dependent or independent effects of resveratrol intake on protein expressions related to ER stress have been demonstrated for the first time in this study.

Although previous studies have reported that resveratrol intake has anti-inflammatory and anti-fibrotic effects (Li et al., 2012; Olesen et al., 2013; Gliemann, Nyberg & Hellsten, 2016), in the present study, resveratrol supplementation did not affect the expression of inflammatory and fibrotic proteins in the heart and kidney tissue of both sexes. In females, only renal expression of TNF- α tended to increase with resveratrol treatment (but was not statistically significant) and probably therefore the renal TNF- α level was higher in resveratrol-treated females than in the males. Based on this finding, it can be said that the dose/duration of resveratrol treatment may not

be enough to affect inflammatory and fibrotic markers in both male and female rats.

In addition, the sex and tissue-related effects of resveratrol on Akt and ERK signaling pathways, which have an important role in the regulation of many cellular processes and stress response, were investigated. Resveratrol administration reduced Akt and ERK expressions only in the kidney tissue of males, but it did not affect the cardiac protein expressions. On the other hand, renal p-Akt expression was lower in resveratrol-treated males than in the females. This may be because control males also have smaller renal p-Akt expression than females. Further studies are needed to elucidate the exact mechanisms and signaling pathways of the resveratrol in a sex- and tissue-dependent manner.

The sex specific effect of regular exercise

Like resveratrol supplementation, regular exercise is a lifestyle change that has beneficial effects on many pathologies by regulating various cellular stress responses (Gliemann et al., 2016). In a recent study, it has been shown that exercise training (5 times *per* week at the speed of 10 m/min for 10 min; the speed gradually increased to 30 m/min for 60 min. for 12 weeks) decreased plasma TAC levels in 6-week-old male Wistar rats (Nasiri et al., 2021). Also, it has been reported that 8-week-exercise training increased plasma TAC levels (TRAP method) but did not alter cardiac TAC levels in male Wistar rats (Farah et al., 2016). In the present study, regular exercise increased the cardiac TAC levels only in female animals. It has been reported that the sex-specific favorable cardiac effects of exercise are modulated by estrogen receptor- β (Dworatzek et al., 2014). Considering these results, it can be suggested that the effects of regular exercise on cardiac TAC levels may be mediated by estrogen receptors.

In our previous study, it was reported that exercise training augmented the mRNA levels of eNOS in the aortas of 3-months old female rats (Han et al., 2018). In this study, regular exercise tended to increase cardiac NO levels in females (but was not statistically significant), while markedly decreasing it in males. Therefore, cardiac NO levels were different between the exercise-treated female and male groups. As with TAC levels, the sex-specific effect of regular exercise on cardiac NO levels may be attributed to hormonal status and cardiac estrogen receptors. In addition, regular exercise did not alter renal NO levels in both sexes. The sex-dependent effects of exercise training on cardiac and renal NO levels have been presented for the first time in the current study. These findings suggest that the effect of regular exercise training on NO levels may be tissue and sex-dependent.

It was reported that regular exercise training inhibited ER stress, inflammation, and fibrosis induced by different pathologies (Dworatzek et al., 2014; Farah et al., 2016; Chengji & Xianjin, 2019; Kosaki et al., 2019). In contrast to previous studies, it was observed in the current study that regular exercise did not alter the protein levels related to ER stress in heart and kidney tissues. Although there is no sex difference between control

groups, in exercise-treated male groups, GRP78 expression was higher in the heart, while p-PERK expression was lower in kidney tissue, compared to females. Also, regular exercise did not affect the expression of inflammatory and fibrotic proteins in both tissue of male and female rats. It can be said that the exercise program that was performed (exercise training intensity, duration, and frequency) may not have been enough to affect the examined inflammatory and fibrotic biomarkers in both sexes. All these findings suggest that the effects of regular exercise on cellular stress response may vary depending on gender and tissue.

Exercise training decreased the cardiac ERK expression and renal Akt expression of male animals. In addition, there was no sex-related difference in Akt, p-Akt, ERK, and p-ERK expressions in both tissues of the exercise-treated groups. Further studies are needed to elucidate the exact mechanisms and signaling pathways of the exercise applications in a sex- and tissue-dependent manner.

Considering above all results, it can be said that regular exercise acts by increasing cardiac TAC and NO levels in females, and decreasing cardiac NO level (unwanted effect), cardiac ERK expression, and renal Akt expression in males.

CONCLUSION

In summary, our results demonstrated that both resveratrol intake and regular exercise altered the markers involved in several pathologies such as tissue TAC/NO levels, GRP78, p-PERK, TNF- α , p-I κ B- α , MMP-2, Akt, p-Akt, ERK, and p-ERK protein expressions, regarding sex and tissue dependencies. These findings suggest that the both physiological and pathophysiological process may not show the same pattern in males and females, therefore the effects of non-pharmacological approaches such as resveratrol and regular exercise also may vary with sex.

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





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An *in-vivo* herb-drug interaction study of *Tinospora cordifolia* extract on the pharmacokinetics of gliclazide in normal Wistar rats

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ABSTRACT

Background and Aims: *Tinospora cordifolia* (Willd.) Miers (TC), from the *Menispermaceae* family, is a well-known traditional herb used to treat diabetes mellitus and a variety of other ailments, according to traditional Indian literature. Herbal treatments are commonly used as alternatives to medicines. Anti-diabetic herbal medicines containing TC are available in the market. TC has been identified as a powerful CYP2C9 inhibitor, boosting the risk of herb-drug interactions (HDI) when used with medications metabolized via the CYP pathway. The present study evaluated the pharmacokinetic HDI of TC extract with Gliclazide (GL) after oral co-administration in Wistar rats using *in vivo* pharmacokinetic studies.

Methods: A simple, sensitive & accurate RP-HPLC/PDA method was developed and validated. Chromatographic separations were performed on a C₁₈ analytical column with a mobile phase of acetonitrile and 40 mM ammonium acetate buffer (55:45 v/v) pH 3.0 and flow rate gradient programming. The drug concentrations in plasma were measured using the RP-HPLC method after oral co-administration of TC extract (100 mg/kg) with GL (8.3 mg/kg) in Wistar rats.

Results: The pharmacokinetic (PK) interaction studies showed that the bioavailability of GL had significantly increased, with a significant influence on C_{max}, AUC₀₋₄₈, and suppression of volume of distribution (V_d), with no change in T_{max}.

Conclusion: The results obtained from this *in vivo* study proposed that there is a potentially significant PK HDI when GL and TC extract are administered together. This knowledge of potential HDI could be beneficial to healthcare providers and diabetic patients on GL medication. Further research is needed to anticipate this pharmacokinetics HDI of GL in humans.

Keywords: Gliclazide, *Tinospora cordifolia*, Herb-drug interaction, RP-HPLC, Pharmacokinetic

INTRODUCTION

Herbal medicines are now widely used by patients worldwide to treat a wide range of conditions (particularly chronic illnesses that necessitate long-term care) in addition to traditional allopathic drugs. This increases the risk of herb-drug interactions (HDIs), which can have additive, synergistic and antagonistic effects, sometimes resulting in catastrophic clinical outcomes (Fugh-Berman & Ernst, 2001; Hu et al., 2005; Posadzki Watson & Ernst, 2013). Cytochrome P450 (CYP450)-mediated inhibition or induction, as well as transport and efflux proteins are among the key routes postulated for HDIs (Shaikh, Thomas & Chitlange, 2020). Due to the complexity of the herbs used and their ability to modulate multiple targets, the study of HDIs remains a major problem when compared to reporting of drug-drug interactions (Tsai, Lin, Simon Pickard, Tsai & Mahady, 2012; Wills, Bone & Morgan, 2000; Brant-

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ley, Argikar, Lin, Nagar & Paine, 2014). However, if these studies are carried out early in the development of herbal medicine, they can reduce the possibility of HDIs and promote the possibility of using any synergism that might be discovered for drug dose reduction and combination therapy promotion (Prabhakara & Doble, 2008).

Tinospora cordifolia (TC), also known as Guduchi, belongs to the *Menispermaceae* family and exhibits a variety of pharmacological actions including anti-diabetic, hypo-lipidemic, cardiotoxic, anti-oxidant, anti-inflammatory, and anti-neoplastic properties (Meshram, Bhagyawant, Gautam & Srivastava, 2013). Tinosporone, tinosporaside, tinocordifolioside, and tinosporic acid, as well as berberine, tembetarine, palmatine, magnoflorine, cordifoliosides A to E, syringen, and sitosterol, are some of the key phytoconstituents of TC. Tinosporine, tinosporide, cordifolide, cordifole, and columbinare the phytoconstituents that help to control cholesterol production and glycolysis (Thikekar, Thomas & Chitlange 2021). Berberine is the major active phytoconstituent and was found to inhibit 50% of CYP2C9 activity in a study conducted by Chatterjee *et al.*, 2003. at a concentration of 500 mM According to Singh *et al.*, 2006 TC has the ability to stimulate carcinogen/drug metabolism and antioxidant system enzymes, inhibiting lipid peroxidation in mice, further proving its CYP interaction potential. Similarly, in a study performed by Bahadur *et al.*, 2016 using particular high-throughput screening assays, the researchers revealed that TC inhibits CYP3A4, CYP2D9, CYP29, and CYP1A2 with IC₅₀ values of 136.45, 144.37, 127.55, and 141.82 g/mL, respectively. According to Sahu *et al.*, 2018 the co-administration of TC extract with glibenclamide resulted in higher C_{max}, T_{max}, increased bioavailability (AUC), and decreased glibenclamide clearance (Cl). TC showed potent inhibition of CYP2C9 activity in CYP inhibition assay. This study postulates that concomitant administration of TC extract/preparations containing TC with glibenclamide (metabolized by the CYP2C9 enzyme) can result in significant HDI. The *in vivo* PK study performed in rats by Asha Thomas *et al.*, 2020 strongly suggested the increase in bioavailability of glimepiride and significant alteration of its PK parameters (increase in C_{max}, AUC₀₋₂₄ and MRT₀₋₂₄ with a decrease in V_d and CL) indicating the potential HDI of glimepiride when administered concomitantly with TC extract via the oral route. Also, an *in silico* molecular docking study using CYP2C9 (PDB ID: 1R90) as the target protein and phytoconstituents (isoquinoline alkaloids) of TC, strongly supported the HDI of glimepiride and TC and demonstrated that berberine and accompanying alkaloidal components in TC extract have a good inhibitory potential against CYP2C9.

Gliclazide 1-(3-azabicyclo[3.3.0]oct-3-yl)-3-(p-tolylsulfonyl) urea is a second-generation sulfonylurea used to treat diabetes mellitus type 2, it works as an insulin secretagogue which stimulates pancreatic cells to secrete insulin. It is completely absorbed and has oral bioavailability of 97 % as gastrointestinal absorption is complete with no interference from meals & absence of pre-systemic hepatic metabolism (Sarkar, Tiwari, Bhasin & Mitra, 2011). GL primarily undergoes oxidative biotransformation by means of CYP2C9, and forms methyl hydroxyl gliclazide and 6β-hydroxy gliclazide metabolites in the liver (Yao *et al.*, 2009). A review of

the literature reveals that various methods for GL bioanalysis have been documented. Chien *et al.* reported GL measurement in rat plasma using HPLC with 70mM disodium tetraborate, pH 7.5, and 26.5 percent acetonitrile as mobile phase (Kuo & Wu, 2005). Lopamudra *et al.*, 2014 performed the HPLC analysis of GL in rat plasma using methanol:acetonitrile:water (60:20:15, v/v) as mobile phase. Xu *et al.*, 2008 reported a LC-MS technique for the estimation of GL in rat plasma employing acetonitrile and 40 mM KH₂PO₄ (pH 4.6, 56%) and acetonitrile (44%).

In the present study an effort was made to employ existing LC methods to evaluate plasma levels of GL in rat plasma following a PK based HDI study involving TC extract. But due to the presence of matrix interferences both from plasma and TC extract, the reported methods were not able to effectively estimate GL. For this reason, there was a need to develop a suitable bioanalytical RP-HPLC/PDA method for the estimation of GL in rat plasma and to validate it as per US-FDA guidelines for HDI study.

MATERIAL AND METHODS

Chemicals and reagents

Gliclazide (GL) and Glimepiride (IS) were obtained as a gift sample from Sava Healthcare Ltd, Pune, India. Merck Chemicals in Mumbai, India, supplied HPLC grade acetonitrile. Millipore water filtration technology was used to prepare the distilled water (Millipore, Sigma). All other reagents were of analytical grade.

Plant extract

TC extract was supplied as a gift sample by Kisalaya Herbal Ltd., Indore, India.

A phytochemical analysis revealed the description (free flowing brown powder with a bitter taste), identification test was positive for bitters, solubility in water: 85.6 %, in 50 v/vethanol: 64.6%, pH of 1% solution (5.11), test for heavy metals: arsenic (0.35 ppm), lead (1.47 ppm), mercury and cadmium (not detected); total ash (5.46%), acid insoluble ash (0.38%), moisture content (3.52 %) and microbiological analysis were within specified limits. The extract contained 3.87 % w/w bitters. For HPTLC fingerprinting of TC extract, the mobile phase used was chloroform: methanol: water (8:2:0.2 v/v/v), and densitometric measurements were performed at 366 nm (Fig 1) (Choudhary, Siddiqui & Khatoun, 2014).

Reverse Phase - High Performance Liquid Chromatography RP-HPLC method and validation

The Shimadzu (LC20AD) HPLC system (Kyoto, Japan) composed of a quaternary pump, manual rheodyne injector with 20μL fixed loop along with the photodiode array (PDA) detector were used for the study. LC solution software was used for processing of data. A Kromasil 100 C₁₈ analytical column (250 mmx4.6 mmx5 μm) was used for chromatographic separations. Mixtures of different solvents with variable polarity and different gradients were employed to find the best chromatographic conditions for generating sharp and well-resolved GL (API) and glimepiride (IS) peaks with minimal tailing. After numerous variations and combinations, in comparison to other mobile phases, it was observed that the mixture of acetonitrile (ACN) and 40mM am-

monium acetate buffer pH 3.0 gave acceptable results. Finally, ACN: 40mM ammonium acetate buffer pH 3.0 adjusted with 1% ortho-phosphoric acid (55:45 v/v) was selected as the mobile phase. The flow rate gradient was set to 0.8 mL/min from 0 to 2 minutes and 1.0 mL/min from 2 to 20 minutes. At 228 nm, the chromatogram was measured. The optimized RP-HPLC/PDA method was validated as per the USFDA bioanalytical method validation guidelines for industry (FDA, 2018).

Animals

The Institutional Animal Ethics Committee (DYPIPSR/IAEC/19-20/P-29), Pune, India, approved the experimental protocol. Wistar rats (Crystal Biological Solutions, Pune, India) with body weights ranging between 250 and 300 g, aged between 6 and 7 weeks were used in this study. The animals were housed in polypropylene cages with solid bottoms and bedding made of autoclaved clean rice husk. The temperature was kept at 22–23°C with a relative humidity of 30–70 % and a 12 hours light/dark cycle with a minimum of 15 air changes *per* hour. All animals were acclimatized to laboratory settings one week previous to the commencement of the experiment. The animals were fasted overnight before the research began but were given *water ad libitum*.

Preparation of calibration standard and quality control standards

Standard stock solutions of GL and IS (100 µg/mL) were prepared separately and subsequently diluted with ACN to obtain working standard solutions of GL (0.5 µg/mL to 32 µg/mL) and IS (50 µg/mL) respectively.

The calibration control standards (CC) of GL-50 ng/mL to 3200 ng/mL and IS-5 µg/mL were prepared by suitably spiking 100 µL of drug free rat plasma with a fixed volume of working standards of GL (100 µL each) and IS respectively. The Quality Control standards (QC); Lower Quality Control (LQC) 150 ng/mL, Middle Quality Control (MQC) 1600 ng/mL and Higher Quality Control (HQC) 2560 ng/mL standards were also prepared.

Extraction procedure

A volume of 0.3 mL of blood from the retro-orbital plexus was collected in Eppendorf tubes (using Gilson micropipettes, P.R. Corporation, Mumbai, India) containing 50 µL of 0.5 M EDTA from individual rats. Blood samples were centrifuged at 3000 rpm for 15 minutes (Bio Era Life Sciences, Pune, India), and the plasma was extracted and stored at -20°C until analysis. The liquid-liquid extraction method (LLE) was then used to extract drugs from plasma. Aliquots of 100 µL of drug-free plasma samples were spiked with 100 µL GL and 100 µL of IS solution (50g/mL) in 2.0 mL eppendorf tubes, to which 1.0 mL of tertiary butyl methyl ether (TBME) was added. The resulting mixture was vortexed for 60 seconds at 2500 rpm (Remi Vortex Mixer, CM-101 Plus) then centrifuged for 10 minutes at 5000 rpm. In a nitrogen evaporator, the organic layer was then separated and evaporated to dryness (Mini-eVap). The leftover mass was reconstituted with 1.0 mL mobile phase, filtered using 0.22 µm syringe filters (Millex-GL), and injected into the RP-HPLC/PDA system for further analysis. The concentration of IS was maintained at 5 µg/mL (Figure 1).

In vivo pharmacokinetic studies in normal Wistar rats

An *in vivo* study was planned to explore the effects of TC extract on the PK of GL in healthy male Wistar rats. Experimental animals weighing 250–300 gm were randomly divided into two groups of 6 animals each.

Group I received a vehicle (1% CMC)

Group II received 100 mg/kg b.w.i.d (bis in die, twice a day) of TC extract for 13 days, by oral route. On the 14th day, group I was treated with GL (8.3 mg/kg, p.o.) and group II was treated with TC (100 mg/kg, p.o.) followed by GL (8.3 mg/kg, p.o.).

A volume of 0.3 mL of blood was collected from individual rats from the retro-orbital plexus in Eppendorf tubes containing 50 µL of 0.5 M EDTA at suitable time intervals (0.25, 0.5, 0.75, 1.0, 2, 4, 6, 8, 24 & 48 hrs.) after drug administration. Blood samples were centrifuged; the plasma was separated and stored at -20°C until analysis. The samples were processed as per the optimized liquid-liquid extraction protocol.

Data analysis

Individual animal plasma concentrations vs time profiles were calculated using WinNonlin software version 5.2. (Pharsight Corporation, Mountain View, CA, USA). The observed individual plasma concentration-time data were used to calculate the peak plasma concentration (C_{max}) and the time it took to achieve C_{max} (T_{max}). The PK parameters evaluated were area under the curve (AUC_{0-48}), clearance (CL), volume of distribution (V_d), elimination rate constant K_{el} , $t_{1/2}$ and mean residence time (MRT). All data were expressed as mean ± SD. Statistical analysis was carried out using t-test on Graph Pad Software, CA, USA. The differences were considered to be significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$.

RESULTS AND DISCUSSION

LC method development and validation for pharmacokinetic studies

As per the United States - Food Drug Administration (US-FDA) bioanalytical method validation guidelines, the developed method was validated for selectivity, linearity, precision and accuracy, extraction recovery, matrix interferences, incurred sample reanalysis and stability study.

Selectivity

Representative chromatograms obtained for blank rat plasma, blank rat plasma spiked with a working solution of GL at a concentration of 50 ng/mL, and plasma samples taken after oral administration of GL in rats showed the method's selectivity as shown in Figure 2.

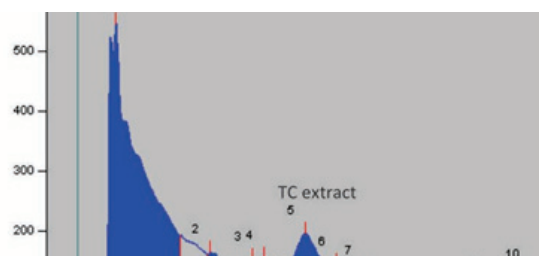


Figure 1. HPTLC densitogram of TC extract.

Linearity

The slopes, intercepts, and correlation coefficients of the GL calibration curve were calculated using the least squares linear regression method in the range of 50-3200 ng/mL. The typical regression equation for GL was $y=0.0024x+ 0.3679$ ($r^2=0.991$) with LLOQ of 50 ng/mL.

Accuracy and precision

At all three QC concentrations, the percent C.V for GL was less than 15% in the intra-day and the inter-day precision of the developed assay method is summarized in Table 1. The accuracy of the method for GL in rat plasma, calculated as relative error (R.E) was within the range of 1.0 to 10%.

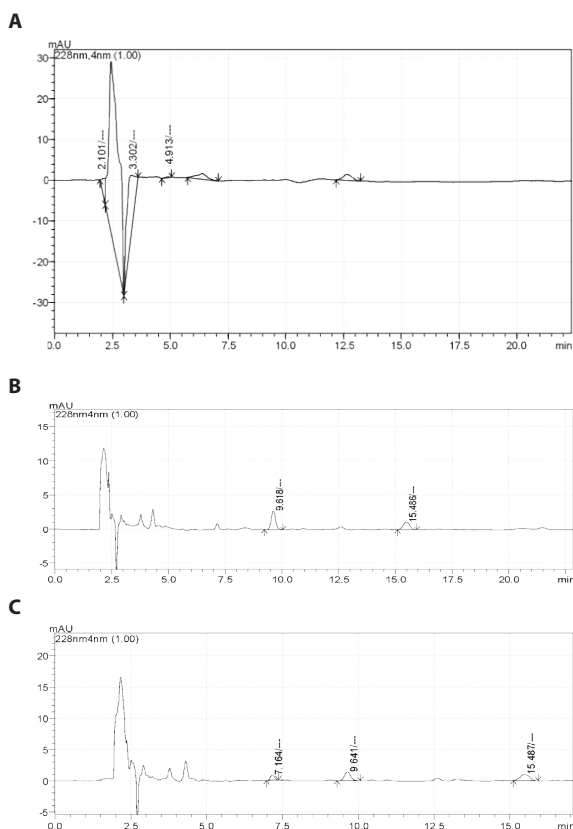


Figure 2. Representative chromatograms of (A) blank rat plasma, (B) blank rat plasma spiked with working solutions at 50 ng/mL of GL and IS and (C) plasma sample collected after oral administration of GL.

Table 2. % Recovery study.

Concentration (ng/mL)	Recovery (%)*		
	Mean	SD	%CV
LQC (150)	89.63	4.34	4.84
MQC (1600)	98.50	0.45	0.46
HQC (2560)	89.74	6.01	6.7

Extraction recovery

As shown in Table 2, the plasma extraction recovery at three concentrations (LQC: 150 ng/mL, MQC: 1600 ng/mL and HQC: 2560 ng/mL) for GL was from 90-95% with % CV below 15%. The mean recovery for Glimepiride (IS) was $90\pm 5\%$.

Stability

The stability of the GL in plasma was studied under various conditions, and the results obtained are reported in Table 3. In rat plasma, GL was found to be stable for 3 hours when held at ambient temperature and for at least 30 days when stored at -20°C with a % CV less than 15% at 2 QC concentrations (LQC and HQC). The % CV of QC samples between the initial concentrations and the concentrations obtained after three freeze-thaw cycles was below 15%. Also, the GL was found to be stable for 24 hrs. at 4°C in the LLE solvent (TBME).

Incurred sample reanalysis, matrix effect

The precision and accuracy of the incurred sample reanalysis of the PK study samples of rats receiving GL (8.3 mg/kg p.o.) were within the acceptance criteria (percent C.V less than 15%, R.E. within the range of 1.0 to 10%) as per USFDA recommendations, as shown in Table 3. In the current method, no significant interference of the matrix at the RT of the GL peak and IS was detected.

In vivo pharmacokinetic studies of Gliclazide after oral administration and co-administration with TC extract

The developed RP-HPLC/PDA method was effectively used to analyze the PK HDI on concomitant administration of GL and TC extract, the latter of which is known to be a powerful CYP2C9 inhibitor. After oral administration of TC, the plasma concentration vs. time profiles of GL are shown in Figure 3. The PK parameters are summarized in Table 4. As per the PK profile obtained; the administration of GL (8.3 mg/kg, p.o.) resulted in C_{\max} of 19129.85 ± 501.96 $\mu\text{g/mL}$ at the T_{\max} 1hr of the study. The value for V_d was found to be $0.000977878\pm$ L with CL of

Table 1. Precision and Accuracy study.

Concentration (ng/mL)	Intraday precision*			Interday Precision*		
	Estimated conc. (ng/mL)	Precision* (%CV)	Accuracy* (%RE)	Estimated conc. (ng/mL)	Precision* (%CV)	Accuracy* (%RE)
LQC (150)	133.85	6.19	10.76	138.71	2.9	7.5
MQC (1600)	1370.94	0.52	14.31	1382.29	0.3	13.6
HQC (2560)	2262.76	7.1	11.61	2219.41	5.6	13.3
Mean of six determinations						

Table 3. Statistical data for stability and other validation parameters.

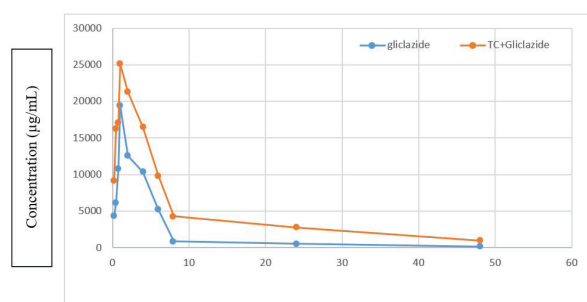
Validation Parameter	Nominal Conc. (ng/mL)*	Determined conc. (ng/mL)*	%CV*
Short term stability	LQC (150)	137.15	1.1
	HQC (2560)	2077.5	2.3
Freeze thaw stability	LQC (150)	135.43	7.5
	HQC (2560)	1901.54	6.4
Long term stability	LQC (150)	121.079	1.1
	HQC (2560)	1659.77	6.2
Incurred sample reanalysis	LQC (150)	128.77	3.41
	HQC (2560)	2260.31	8.68
Matrix effect	-	Nil	-

*Mean of six determinations

Table 4. Pharmacokinetic parameters of Gliclazide after oral administration in rats (n=6).

Group	T _{max} (h)	C _{max} (µg/mL)	AUC ₀₋₄₈ (h*µg/mL)	K _e (hr ⁻¹)	t _{1/2} (hr)	V _d (L)	CL (L/hr)	MRT (hr)
Group-1 (GL)	1	19129.85±501.9	203371.12±11258.4	0.086	8.34	0.00098	0.000081	12.04±1.19
Group-2 (GL+TC)	1	25020.40±295.98****	435102.20±27361.52***	0.067*	10.41±0.85*	0.00052****	0.0000351***	15.03±1.23*

Data are expressed as mean ± SD. **significant with respect to the GL group.

**Figure 3.** Plasma conc. (ng/mL) vs. time in hrs. profile of Gliclazide before and after administration of TC extract.

0.000081 L/hr, K_e 0.083606486 hr⁻¹, $t_{1/2}$ 8.34 ± 0.82 hr and a MRT of 12.04 ± 1.19 hr. Co-administration of TC at a dose of 100 mg/kg, p.o. in rats resulted in a significantly ($p < 0.001$) increased C_{max} 25020.40 ± 295.9 µg/mL, AUC_{0-48} (203371.12 ± 11258.44 to 435102.20 ± 27361.52 h*µg/mL, $t_{1/2}$ 10.41 ± 0.85 hr and MRT 15.02 ± 1.2 hr as shown in Table 4. Consequently a decrease in V_d 0.000525375 L, CL 0.0000351 L/hr, K_e 0.06685169 hr⁻¹ was observed.

CONCLUSION

A simple, sensitive, and accurate HPLC-PDA technique for estimating GL in rat plasma was developed and validated using a simple liquid-liquid extraction method. The developed method was successfully used to study the PK effect of GL in rats *in vivo*. The results of the PK investigation revealed that co-administration of TC extract significantly altered the PK pa-

rameters of GL; co-administration of TC extract resulted in an increase in the C_{max} , AUC_{0-48} , $t_{1/2}$ and MRT, consequently enhancing its bioavailability. As a result, V_d , K_e and CL for GL were reported to be reduced. This shift in PK parameters could be due to the inhibitory ability of berberine and other alkaloids found in high concentrations in TC extract on CYP2C9 activity.

In conclusion, the findings of this evidence-based investigation reveal that GL and TC extract have a considerable HDI. However, thorough clinical PK and pharmacodynamic studies are still required to establish HDI in higher animals. On prolonged administration, increase in bioavailability of GL due to an increase in its plasma concentration in the body may lead to propagation of its toxic manifestation and side effects, which could be fatal. As a result, the concomitant consumption of TC in patients on GL medication must be monitored vigilantly. This information concerning possible HDI of GL and TC may be useful for both physicians and patients on GL therapy.

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Determination of diazepam in human plasma by developed and validated a high-performance liquid chromatographic ultraviolet method

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ABSTRACT

Background and Aims: Diazepam is accepted as a safer drug to medicate in many serious cases, acting as an anticonvulsant, an anxiolytic and a treatment for many types of poisoning. Monitoring it is important in achieving successful treatment and reducing the risk of toxic effects. In this study, it is aimed to develop and validate a sensitive, repeatable, and reliable method based on high-performance liquid chromatographic analysis for the determination in human plasma.

Methods: Separation was carried out using a reverse-phase C18 column (4.0 mm x 150 mm, 3 µm) at 30 °C. The solution was prepared with a 10 mM phosphate buffer and acetonitrile (1:1, v/v) was employed as a mobile phase at the isocratic flow with 0.5 mL/min rate. Quantification was applied at 230 nm. A solid-phase extraction method was established and optimized, which was then used in the preparation of the plasma (0.5 mL) samples to the analysis.

Results: The method was found to be linear ($r^2 = 0.9805$) between 100 and 1200 ng/mL. The analysis run was ≤ 12 min. Intra-day and inter-day accuracy were found between -5.78 and 5.93 and precision was $\leq 1.82\%$. The limit of detection and quantification were calculated as 20.42 and 61.86 ng/mL, respectively. Recovery was found between the range of 95.12% and 106.83%. The method was determined to be robust according to changes in UV, mobile phase organic solvent content, mobile phase pH, column temperature, and operator.

Conclusion: This simple, sensitive and reliable method is suggested for accredited-reference laboratories working on the therapeutic drug monitoring and/or overdose-toxicological quantified analysis of diazepam in human plasma.

Keywords: Diazepam, Solid-phase extraction, Method validation, High-performance liquid chromatography-ultraviolet detection

INTRODUCTION

Diazepam (7-chlorine-1-methyl-5-phenyl-1,3-dihydrobenzo [e] [1,4] diazepam-2-one), a benzodiazepine-derived drug (Figure 1-a), is primarily used in the treatment of mental anxiety (Zhang, Ouyang, Lipina, Wang, & Zhou, 2019), but it also used as a sedative-hypnotic (Cook, Flanagan, & James, 1984) and as an anticonvulsant (Chamberlain et al., 2014). It has skeletal muscle relaxant (Richards, Whittle, & Buchbinder, 2012) and anxiolytic (Faye et al., 2020) properties. It is accepted as a safer drug than others used in the treatment of anxiety (Zhang et al., 2019). It has a widespread use due to its high therapeutic index. Diazepam is used commonly in cholinesterase poisoning (Abou-Donia, Siracuse, Gupta, & Sobel Sokol, 2016), to alleviate some symptoms associated with alcohol (Weintraub, 2017) and barbiturate abstinence syndrome (Perry, Stambaugh, Tsuang, & Smith, 1981), in anti-histamine over-

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dose (Montoro et al., 2013), black widow spider envenomation (Rogers, Stanford, & Dart, 2006) and chloroquine overdose (Hughes, 2020). In addition to that it could be used alone as an anaesthetic (McGrath et al., 2020) or in combination with other drugs for conscious sedation (Zanette et al., 2013), and also as an aid to anaesthesia (Wexler, Peyster, Hakkinen, & Pope, 2005).

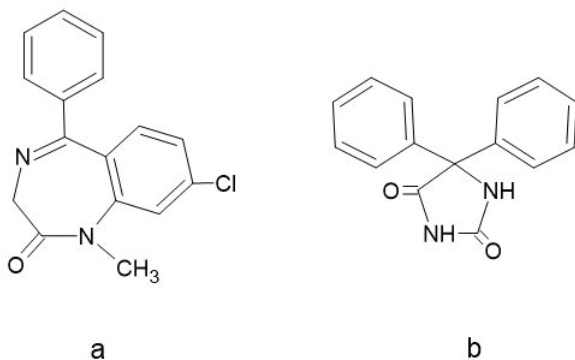


Figure 1. Chemical structures of diazepam (-a) and phenytoin (-b) which used as the internal standard.

Diazepam is commonly used via the oral, rectal, and parenteral routes (Bialer, 2007; Henney, Sperling, & Rabinowicz, 2014). It reaches maximum plasma concentration between 30 and 90 minutes (Gong, Liu, Xu, Fan, & Xue, 2015; Taysse et al., 2003). Diazepam is widely distributed throughout the body (Friedman et al., 1992). Drug interactions associated with protein binding are clinically insignificant, although they are 98% bound to plasma proteins.

Sedation is the most common side effect of the drug. In intravenous administration, the drug side effects emerge more quickly and the toxicity is usually iatrogenic. Chronic treatment with diazepam can lead to addiction. Also, withdrawal symptoms can be observed in cases where cessation and toxic effects occur too (Calcaterra & Barrow, 2014). Therefore, patients under treatment with diazepam need the medical monitoring. Overdose of diazepam in oral or parenteral administration causes a loss of consciousness, hypotension, bradycardia, coma and respiratory failure. Deaths have been reported when diazepam is used with other central nervous system depressants under combination treatment (Calcaterra & Barrow, 2014). Diazepam is reported as a genotoxic agent according to toxicity studies accomplished *in vitro*. Due to diazepam lipid solubility being significantly high, it is known that diazepam passes through the placenta during pregnancy, and it has the ability to cross the blood-brain barrier. Also, it is known that diazepam passes to the infant with maternal exposure by lactation (Ghosh, Reddy, Ramteke, & Rao, 2004; Wexler et al., 2005). For these reasons which could be evaluated as very serious for public health, development of a simple, sensitive and reliable monitoring of diazepam is very important.

Many methods have been developed for determining diazepam from biological samples and pharmaceutical preparations. These methods are based on thin layer chromatography (TLC) (Bakavoli & Kaykhah, 2003), spectrophotometry (Morelli,

1997), gas chromatography mass spectrometry, high-performance liquid chromatography (HPLC) coupled with ultraviolet detector (UV) and tandem mass spectrometry (MS/MS) (Brieudes, Lardy-Fontan, Lalere, Vaslin-Reimann, & Budzinski, 2016; de Araujo, Bauerfeldt, Marques, & Martins, 2019; Gong et al., 2015; Kim et al., 2017; Miller, Wylie, & Oliver, 2008; Tran, Hu, & Ong, 2013), respectively. Also, various extraction methods such as protein precipitation (Pilli et al., 2020), liquid-liquid extraction (Kim et al., 2017), solid-phase extraction (SPE) (Borges, Freire, Martins, & de Siqueira, 2009; Mercolini et al., 2009), and solid-phase microextraction (Yuan & Pawliszyn, 2001) were employed in these investigations.

In this study, it was aimed to develop a high-performance liquid chromatographic method based on solid-phase extraction for monitoring diazepam from human plasma. Subsequently it was then validated in terms of linearity, repeatability, sensitivity, recovery and robustness according to the International Conference on Harmonization guideline Q2(R1) (ICH, 2005). On the other hand, it is aimed to develop a simple, fast, cheaper, repeatable, accurate and reliable high-performance liquid chromatography method for the quantitative determination of diazepam.

MATERIAL AND METHODS

Chemicals and reagents

Diazepam (Figure 1-a) and phenytoin (Figure 1-b) chemical standards (purity $\geq 99.0\%$) were donated from the Forensic Science Institute of Ankara University (Ankara, Turkey) and VEM pharmaceuticals (Istanbul, Turkey), respectively. Methanol and acetonitrile which were HPLC-grade and KCl, NaCl, NaOH and H_3PO_4 which were analytic grade were purchased from Sigma-Aldrich (Missouri, USA). Na_2HPO_4 and KH_2PO_4 were purchased from Merck (Darmstadt, Germany). The Sep-Pac[®] Vac 1 cc (100 mg) solid-phase C18 cartridge was obtained from Waters (Dublin, Ireland). Carboxymethyl cellulose was purchased from Biokim & Wenda Chemicals (Izmir, Turkey). Bovine serum albumin was purchased from Solarbio Life Science (Beijing, PRC). PTFE membrane filter (47 mm DIA, 0.45 μm pore size) was obtained from Millipore (MA, USA). Ultrapure water was supplied from Elga Purelab Water Purification System (Lane End, UK) performing purification as required by the reverse osmosis method. The conductivity and electrical resistance of the ultrapure water obtained were $\leq 0.055 \mu S/cm$ (25 °C) and $\leq 18.2 m\Omega.cm$, respectively. Membrane filters with a pore size of 0.45 μm from Millipore (Massachusetts, USA) were used for the mobile phase filtration.

Instrumentation and chromatographic conditions

The analytical separation and quantification was achieved with an Agilent 1100 series HPLC system (CA, USA) coupled with a UV detector. A high-performance liquid chromatography (HPLC) was employed in this study and consisted of a degasser (G1322A), a gradient pump (G1311A), a manual injector (Rheodyne 7725i) with loop volume 20 μL , a column oven (G1316A, Colcom), and an ultraviolet detector (G1314A VWD). A 100 μL volume glass HPLC needle was utilized for applying samples to the system from the injector port.

A stainless steel end-cap ACE-5 reverse phase (RP) C18 analytical column (Aberdeen, Scotland) with 4.0 mm x 150 mm (i.d. x l) diameters 3 μm (p.s.) column filling material was employed for instrumental analytic separation. A 08.03 version ChemStation® software was used for data collection and system handling.

The column oven temperature was set at 30°C. According to the applied observations, the ultraviolet spectrum ultraviolet detector was set at 230 nm for both diazepam and phenytoin (Figure 2). The mobile phase composed of 10 mM KH_2PO_4 (pH 3.0) and acetonitrile (50:50, v/v) was applied to the analytical system with an isocratic, 0.5 mL/min constant flow. Before application to the system the mobile phase solution was filtered passing through a PTFE membrane disc filter at 20 kPa pressure using a vacuum filter system.

Analytic parameters were determined following the optimization study performed in terms of the mobile phase content and pH, analytical column and column oven temperature, respectively.

Preparation of chemical standards and simulated plasma

Both stock solution (1 mg/mL) and working solutions of diazepam 5, 10, 20, 40 and 60 $\mu\text{g}/\text{mL}$ were prepared by dissolving them in methanol. Diazepam quality control samples (0.1, 0.2, 0.4, 0.8 and 1.2 $\mu\text{g}/\text{mL}$) were prepared by taking 10 μL of the respective working solution and dissolving it in 500 μL volume of simulated plasma sample. The working solution (10 $\mu\text{g}/\text{mL}$) of internal standard was prepared by diluting it with methanol from the main stock solution of phenytoin (1 mg/mL), weekly. Stock solutions were stored at -20°C until analysis and were observed to remain stable for at least 1 month.

During the development of solid-phase extraction and method validation steps, a simulated plasma, the preparation protocol of which is described by Mercolini et al. (2008) was used. According to this, 4 g bovine albumin, 20 mg KCl, 0.8 g NaCl, 20 mg KH_2PO_4 , 144 mg Na_2HPO_4 was dissolved in 100 mL ultrapure water, then its pH was adjusted to 7.4 with 0.1 M KOH or H_3PO_4 . Finally, the formed solution was split up into microtubes in 0.5 mL quantities and stored in -18 °C until use.

Determination of the internal standard

Clozapine, sodium valproate, chlorpromazine, flunarizine, cinnarizine and phenytoin chemicals were tested to determine the internal standard to be used in the analytical method. The obtained results from the selection of internal standard test study showed that the peak shape and structure of chlorpromazine and cinnarizine did not have enough sharpness to be accepted as an internal standard in these chromatographic conditions. Because the chromatographic peaks acquired from flunarizine analysis were so fragmental, and also the obtained clozapine and sodium valproate results were unsuitable in terms of the retention time according to diazepam, these 3 agents could not be utilized as an internal standard. It was decided to use phenytoin as an internal standard because it shows a precise peak structure even at low concentrations, its extraction recovery values are very successful, it gives a nice UV response at 230 nm, and has an acceptable retention time in the chromatogram (Figure 2).

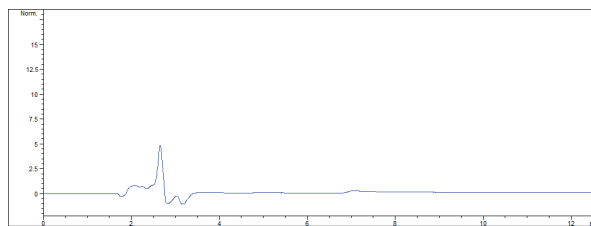


Figure 2. A chromatogram which belong to a blank simulated plasma sample extracted by the SPE method explained.

Optimization of the developed solid-phase extraction method

The solid-phase extraction procedure was optimized in terms of the cartridge conditioning, sample loading, cartridge washing and elution steps according to the results obtained from the comparative chromatographic analyses. As a rule, while performing the optimization study, changes were made in the intended step to monitor, while other variables related to extraction were kept constant.

In the optimization of the conditioning step, the methanol and ultrapure water application volume from 1 mL to 3 mL were tested for the determination of the best efficiency value for the activation of cartridge filling material. In the following step (called sample loading), an attempt was made to apply plasma samples both directly and diluted with water up to 1 mL into the cartridge. In addition, the volume of water to be used in the washing step tested in volumes varying (from 0.5 mL) up to 2 mL. In addition, the dilution values of the acetonitrile: methanol mixture (3: 1, v/v) used in the second stage of the washing step were evaluated at varying rates up to 12 times. The content ratios of the elution solution formed with acetonitrile and methanol were tested to determine the best efficiency value at ratios of 6:1, 3:1, 1:1, 1:3, 1:6 (v/v), respectively. Then, defining the best efficient solution volume value, it was tested from 1 up to 3 mL. The elution volume effect on the extraction efficiency was tested between 1 ml to 3 mL. The optimized extraction values were clearly given under 2.6.2. *Solid-phase extraction* subtitle.

Extraction efficiency was evaluated by comparing the diazepam and phenytoin peak areas recovered from plasma to the results of standard solutions applied directly to HPLC without extraction by dissolving in the mobile phase. Since the internal standard was used in this study, the ratio value obtained by dividing the peak area of diazepam from the same chromatogram by the area of the internal standard was used in both recovery test and also other validation calculations.

Sample preparation

Preparation of spiked samples

10 μL diazepam and 10 μL internal standard (10 $\mu\text{g}/\text{mL}$) working solution were added to 500 μL of blank simulated plasma sample and then its volume was made up to 1 mL with ultrapure water. Then, it was stirred at 1200 rpm for 2 minutes with the vortex mixture before being used as a sample.

Solid-phase extraction

Initially, a C18 solid-phase cartridge was activated and conditioned by applying 3 mL of methanol and 3 mL of ultrapure

water, respectively. Then, 500 μL of the plasma sample were diluted with 1 mL of ultrapure water in a micro-tube containing 10 μL of diazepam and 10 μL of phenytoin internal standard, and this was applied to the solid-phase cartridge with 0.5 mL/min flow. After the samples were loaded into the cartridge, the remains were cleaned twice using 1 mL ultrapure water. Then, the cartridges were cleaned with 1 mL of the acetonitrile: methanol mixture (18:1, v/v) and again and they were fully dried using a constant vacuum. Under 0.5 mL/min constant flow, 1 mL acetonitrile: methanol (3:1, v/v) mixture was applied twice to the cartridges and they were then vacuumed (75 kPa) until completely dry. Finally, the collected extraction liquid (approximately 2 mL) was evaporated to complete dryness using the nitrogen evaporating system heated to 40 $^{\circ}\text{C}$. After the remains were dissolved in 200 μL of the mobile phase by the vortex mixture at 3000 rpm, 1 minute, it was loaded into the HPLC system with a volume of 20 μL .

Method validation

The developed chromatographic method was validated in terms of linearity, sensitivity, accuracy, precision, recovery and robustness in accordance with the International Harmonization Conference (ICH) guidelines (ICH, 2005).

Linearity: Diazepam plasma samples in 5 different concentrations (100, 200, 400, 800 and 1200 ng/mL) that can be anticipated in plasma were prepared on the condition that 3 samples were found for each point. It was plotted according to the versus peak-area ratios to the internal standard. Each plasma sample prepared was delivered to the HPLC device for analysis. The calibration equation and the determination coefficient were calculated by drawing the calibration graph. The linearity test was designed to cover sub-therapeutic, therapeutic, overdose and toxic levels of the diazepam in plasma.

Sensitivity: The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the ICH recommendation based on standard deviation of the response and the slope of the calibration graph. 100 ng/mL was used in the test as the lowest calibration point of diazepam.

$$\text{LOD} = 3.3 \frac{\sigma}{S}; \text{LOQ} = 10 \frac{\sigma}{S}$$

(σ : The standard deviation of the response; S: The slope of the calibration curve).

Accuracy and precision: The accuracy, defined as the relative error (RE%) was calculated as the percentage difference between the added and found diazepam quantity by 5 individual replicates both intraday and inter-day. The precision, defined as relative standard deviation (RSD%), was calculated by five separate replicates of diazepam both intraday and inter-day. Five replicate spiked samples were assayed intraday and inter-day at the three different concentrations (100, 400 and 1200 ng/mL).

Recovery: The efficiency of the extraction procedure from the plasma was determined by comparing pre-extraction spikes with the post-extraction spiked internal standard. Five individual replicates of spiked samples at low, middle and high concentrations (200, 400 and, 800 ng/mL, respectively) of diazepam were prepared with and without the internal standard.

The extraction procedure was carried out as described previously in the sample preparation step.

Robustness: Robustness is a test for evaluating the response of the analytical method to some changes in the analysis parameters. Here, the method's response to changes in analytical parameters is evaluated in terms of reliability by examining the changes in the results obtained. It is the ability of a method to remain unaffected when small changes are applied. The robustness test was performed with 400 ng/mL of diazepam, which is the median concentration of the calibration interval. The response of the method of changes to ultraviolet wavelength (± 1 nm), mobile phase flow rate to column (± 0.1 mL/min), mobile phase organic solvent content ($\pm 5\%$), column temperature (± 5 $^{\circ}\text{C}$), mobile phase final pH (± 0.5) and also the operator changes were evaluated.

RESULTS

Selectivity and specificity

Initially the optimum conditions for mobile phase, column and UV-detector wavelength were determined and plasma samples containing diazepam and phenytoin were injected into HPLC under these conditions. Within the optimum analysis conditions, retention times of diazepam and phenytoin were determined as 4.9 and 10.8 minutes, respectively. Blank and sample chromatograms of 10 $\mu\text{g/mL}$ phenytoin and 400 ng/mL diazepam are given in Figure 2 and 3, respectively.

By adding diazepam and phenytoin analytes into simulated plasma by the standard addition method, concentrations ranging from the lowest calibration point to the highest concentration of diazepam could be successfully detected and quantified from the relevant matrix. This can be clearly seen in the blank simulated plasma chromatogram. All observed analytes peaks were sharply, clearly and obviously detected.

As part of the selectivity study of the method validation, nitrazepam (100 ng/mL), diazepam (400 ng/mL), phenytoin (10 $\mu\text{g/mL}$) and lorazepam (100 ng/mL) were administered to HPLC. Quantitative determination of diazepam and phenytoin was successfully performed under the expressed chromatographic conditions. The chromatogram obtained is given in Figure 4.

Nitrazepam is used in disabling anxiety and insomnia. It has amnestic, sedative, anticonvulsant, and also skeletal muscle relaxant effects. It is defined as a hypnotic drug. It is classified in the benzodiazepine group of drugs (Yasui, et al., 2005). Lorazepam, is also a benzodiazepine medication, used in the treatment of severe agitation, anxiety disorders,

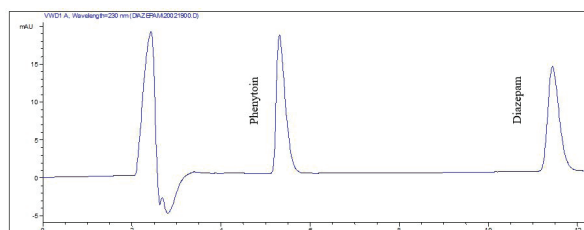


Figure 3. A typical chromatogram which exhibited phenytoin (10 $\mu\text{g/mL}$) and diazepam (400 ng/mL) peaks, respectively.

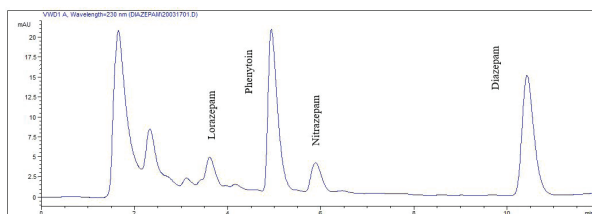


Figure 4. A typical chromatogram obtained in the selectivity study for method validation. Peaks of nitrazepam.

trouble sleeping, active seizures, alcohol withdrawal, and chemotherapy-induced nausea and vomiting (Herman, Van Pharm, & Szakacs, 1989). Nitrazepam and lorazepam were used in the selectivity study because they are structurally very close to diazepam.

Linearity

Calibration curves of diazepam drawn at five points ($n=3$) which are 100, 200, 400, 800 and 1200 ng/mL concentration versus the area of phenytoin as an internal standard by the standard addition method showed excellent correlation with $r^2 = 0.9805$, (Table 1). The linearity study was designed to cover sub-therapeutic, therapeutic and toxic drug levels of the drug. The wide linear range also had a positive effect on the use of the method since the obtained real blood results showed very high standard deviation.

System suitability parameters show that it has good resolution (R_s) and selectivity (α) values. Capacity factor (k') and theoretical plate number (N) show acceptable values for a successful determination of diazepam from plasma as it can be seen in Table 1.

Sensitivity

LOD and LOQ values were 20.42 ng/mL and 61.86 ng/mL, respectively. In addition to the LOD value being approximately 5 times lower than the lowest point on the calibration curve, LOQ value was found to be lower than the last point on the calibration curve. This result shows that the method can be used reliably for the analysis of diazepam in low concentrations in plasma Table 2.

Accuracy and precision

The repeatability study was performed at 100, 400 and 1200 ng/mL diazepam concentrations. The intraday repeatability study was performed with 5 replicate analyses for each concentration on the same day, and the accuracy was found between (RE%) (-5.78) and 3.30, and precision (RSD%) was found between 0.14 and 1.59. The repeatability study between days was carried out for 5 consecutive days and the accuracy was between (-5.78) and 5.93 (RE%), and precision (RSD%) was between 1.22 and 1.82. These data showed that when compared with the literature, this method has high repeatability and can obtain precise and accurate results in diazepam analysis from plasma in the intraday and inter-day reproducibility study (Table 3).

Recovery

The extraction yield values for the 3-replicates recovery study for diazepam concentrations of 200, 400 and 800 ng/mL were in the range of 95.12% to 106.83% (Table 4). These observed excellent values obtained from the extraction method developed and optimized suggest that the analytes, matrix (simulated plasma) and method (solid-phase) are perfectly compatibility. It is thought that it will make a significant contribution to the literature on diazepam analysis since the recovery value observed is 100.39% on average. The raw data used in the calculation of the recovery is given detailed in Table 4.

Robustness

No significant changes in the analytical signals were observed upon changing ultraviolet wavelength value (± 1 nm) (Table 5), mobile phase pH (± 0.5) (Table 6), mobile phase content ($\pm 5\%$) (Table 7). Moreover, change of analysts, sources of chemicals and/or solvents did not lead to significant changes in chromatographic signals and results. The robustness of the experimental results demonstrated that the method is able to create data with acceptable precision and accuracy. Consistent data from selectivity studies and the robustness study demonstrated its suitability in the quantitative determination of diazepam from human blood.

DISCUSSION

Diazepam is widely used as an antiepileptic, muscle relaxant, hypnotic, and anesthetic inductor in pharmacotherapy. However, the

Table 1. Chromatographic properties and system suitability parameters of the suggested method. Because of phenytoin was used as an internal standard; calibration range, calibration equation, determination coefficient (r^2), selectivity factor (α), resolution (R_s) values belong to this agent did not be calculated.

Analyte	Retention time (min, t_R)	Capacity Factor (k')	Theoretical plate number (N)	Selectivity factor (α)	Resolution (R_s)	Calibration range (ng/mL)	Calibration equation	Determination coefficient (r^2)
Diazepam	10.8	5.35	3808.65	2.85	8.44	100 – 1200	$y = 0.0019x + 0.1641$	0.9805
Phenytoin	4.9	1.88	153.64	null	null	null	null	null

Capacity factor (k') = $\frac{t_R - t_0}{t_0}$; Theoretical plate number (N) = $16 \left(\frac{t_R}{W_t} \right)^2$; Specificity factor (α) = $\frac{k_2}{k_1}$; Resolution (R_s) = $\frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k + 1)}$; Abbreviations: t_R : retention time of the analyte peak; t_0 : retention time of first peak; W_t : peak width

Table 2. Sensitivity test data applied at 100 ng/mL concentration of diazepam.

No	Concentration (ng/mL)	Area (mAu)			STD/ISTD			LOD (ng/mL)	LOQ (ng/mL)
		Diazepam	Phenytoin	Rationed Values	Average	SD	RSD%		
1	100	96.5	261.0	0.370					
2	100	98.6	266.8	0.370					
3	100	97.9	259.6	0.377					
4	100	94.6	259.9	0.364					
5	100	92.9	264.3	0.352					
6	100	105.2	288.2	0.365	0.368	0.012	3.193	20.42	61.86
7	100	109.7	303.2	0.362					
8	100	97.3	271.6	0.358					
9	100	105.5	274.8	0.384					
10	100	110.9	291.3	0.380					

Table 3. Confidence parameters of the method that including intraday and inter-day precision, accuracy and recovery values. These results were obtained from individual samples (n=3) prepared as quality control samples in real plasma.

Expected Concentration (ng/mL)	Intraday				Inter-day				Average Recovery (%)
	No. Obs.	Observed concentration \pm SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	No. Obs.	Observed concentration \pm SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	
100	5	99.82 \pm 3.30	1.59	-0.18	5	102.26 \pm 3.84	1.82	2.25	99.22
400	5	381.42 \pm 4.03	0.78	-4.65	5	384.97 \pm 6.43	1.22	-3.76	95.12
1200	5	1173.50 \pm 1.97	0.14	-2.21	5	1210.93 \pm 24.19	1.22	0.91	106.83

drug is connected with abuse due to its high potential for side effects such as addiction. Therefore, toxicological analysis of diazepam for therapeutic dose impression studies is important for forensic and clinical toxicologists. Some studies involving the monitoring of diazepam from various samples are summarized below.

In the study by Uddin et al. (2008), diazepam and 5 other benzodiazepines and two metabolites: in human plasma, urine and saliva by RP-HPLC-diode array detection method were determined. Analytes were quantitated at 240 nm. Methods were based on a solid phase extraction method. The separation was carried out on a C₈ (250 mm x 65 mm, 5 μ m) analytical column with a mobile phase containing methanol, acetonitrile, and 50 mM ammonium acetate. Linearity was maintained in the range of 300-20000 ng/mL. r^2 was found \geq 0.997. Intraday and inter-day precision test implementation were applied at concentrations of 2000, 4000 and 8000 ng/mL. Method precision values were found between 1.3% and 7.9% in plasma and between 2.1 and 7.8% in the urine. Linearity study of saliva was applied to 500, 1000 and 2000 ng/mL concentrations. The results were found between 2.2 and 8.1%, respectively. LOD and LOQ values were found as 20-470 and 70-1570 ng/mL, respectively (Uddin, Samanidou, & Papadoyannis, 2008).

Muchohi et al. (2001) developed an ultraviolet based reverse phase HPLC method to determine the amount of diazepam in plasma samples from children with severe malaria. After precipitation of plasma proteins, liquid-liquid extraction was performed with the mixture of acetonitrile, ethyl acetate and n-hexane. Diazepam was eluted from a reverse phase C₁₈ column with an acidic (pH: 3.5) aqueous mobile phase (10 mM KH₂PO₄-acetonitrile, 69:31, v/v) at ambient temperature. The calibration curve between 10 and 200 ng/mL in the plasma after centrifugation was linear and the determination coefficient (r^2) was \geq 0.99. Relative recovery values at 25 and 180 ng/mL were greater than 87%. The relative standard deviation during the day and between days was less than 15%.

Dragica Zendelovska et al. (2018) developed a high-performance liquid chromatographic (HPLC) method with UV-detection for direct determination of diazepam in whole blood and serum. The isolation of diazepam and internal standard bromazepam from serum and whole blood samples was performed using reverse phase cartridges with the solid-phase extraction method. The analytes were separated using a reverse phase C8 column with a mobile phase of 0.1% (v/v) triethylamine (pH 3.5) and acetonitrile (63:37, v/v) in water. The UV detector wavelength was

Table 4. Recovery data of the developed analysis method and obtained total recovery values.

Diazepam concentration (ng/mL)	Extraction	Area values (mAU)			STD/ISTD			Average Recovery (%)
		STD	ISTD	Rationed Values	Mean (\bar{X})	SD	RSD%	
200	Non-extracted samples	150.7	327.7	0.461	0.455	0.007	1.440	99.22
		158.5	345.9	0.458				
		154.9	347.9	0.445				
200	Extracted samples	145.1	304.6	0.476	0.451	0.019	4.085	
		131.5	303.4	0.434				
		141.2	318.8	0.443				
400	Non-extracted samples	256.6	259.6	0.988	0.963	0.039	4.053	95.12
		259.0	261.0	0.992				
		246.8	271.9	0.908				
400	Extracted samples	264.4	288.9	0.915	0.915	0.001	0.078	
		275.4	300.7	0.916				
		278.9	305.1	0.914				
800	Non-extracted samples	561.9	327.2	1.717	1.720	0.002	0.008	106.83
		565.0	328.5	1.721				
		584.1	339.0	1.723				
800	Extracted samples	456.3	250.9	1.819	1.837	0.018	0.997	
		473.7	258.8	1.830				
		507.4	272.5	1.862				

Note: Diazepam as the analytic agent and the phenytoin used as the internal standard employed in the study were abbreviated as STD and ISTD, respectively. Standard deviation was abbreviated as SD and relative standard deviation was abbreviated as RSD% calculated with the formula

Table 5. Robustness test results by changing the detector wavelength ± 1 nm % within the standard optimization conditions.

UV (nm)	Area			STD/ISTD		
	Diazepam	Phenytoin	Rationed Values	Mean (\bar{X})	SD	RSD%
229	278.0	330.8	0.840	0.842	0.002	0.170
	291.3	346.2	0.842			
	289.7	345.7	0.838			
230	304.7	309.5	0.985	0.937	0.040	4.289
	278.9	297.1	0.939			
	292.0	329.5	0.886			
231	262.8	232.2	1.132	1.132	0.003	0.248
	272.4	240.0	1.135			
	287.0	254.4	1.128			
Average (\bar{X})					0.969	
Standard deviation (SD)					0.149	
RSD%					15.330	

240 nm. Linearity for serum and whole blood was achieved in the range of 10 -1000 ng/mL. In this method, after oral administration of 10 mg diazepam, plasma proteins were precipitated and applied to real biological samples.

Borges et al. (2008) developed a simultaneous HPLC method for determination of diazepam and individual 6 benzodiazepines from both human plasma, using the liquid-liquid and solid-phase extraction. 5 mM KH_2PO_4 buffer solution (pH 6.0):methanol:diethyl

Table 6. Robustness test results performed by changing mobile phase pH \pm 0.5 % within the standard optimization conditions.

pH value	Area			STD/ISTD		
	Diazepam	Phenytoin	Rationed Values	Mean (\bar{X})	SD	RSD%
pH 2.5	211.7	232	0.913	0.921	0.012	1.338
	212.3	232.5	0.913			
	224.6	239.2	0.939			
pH 3	304.7	309.5	0.985	0.937	0.040	4.289
	278.9	297.1	0.939			
	292	329.5	0.886			
pH 3.5	251.9	293.1	0.860	0.854	0.014	1.682
	256.6	307.7	0.834			
	247.7	285.5	0.868			
Average (\bar{X})					0.969	
Standard deviation (SD)					0.149	
RSD%					15.330	

Table 7. Robustness test results performed by changing the mobile phase content \pm 5% within the standard optimization conditions.

Mobile phase ingredients (Acetonitrile:KH ₂ PO ₄ , v/v)	Area			STD/ISTD		
	Diazepam	Phenytoin	Rationed Values	Mean (\bar{X})	SD	RSD%
45:55	276.7	332.8	0.832	0.817	0.012	1.426
	278.2	346.5	0.803			
	272.2	333.0	0.818			
50:50	304.7	309.5	0.985	0.937	0.040	4.289
	278.9	297.1	0.939			
	292.0	329.5	0.886			
55:45	278.0	318.1	0.874	0.874	0.001	0.132
	276.4	316.9	0.872			
	287.0	328.0	0.875			
Average (\bar{X})					0.876	
Standard deviation (SD)					0.060	
RSD%					6.812	

ether (55:40:5, v/v/v) mixture was used as a mobile phase at 0.8 mL/min flow rate. LC-18 DB column (250 mm x 4.6 mm, 5 μ m) was used in isocratic conditions. The UV detector wavelength detector was set at 245 nm. When using liquid-liquid extraction, the best conditions were obtained by double extraction of 0.5 mL of plasma for pH 9.5 using ethyl acetate and Na₂HPO₄. Using SPE, the best conditions were achieved with 0.5 mL of plasma and 3 mL of 0.1 M borate buffer pH 9.5. In both methods, the solvent was evaporated under a stream of nitrogen at 40 °C. For LLE plasma linearity range 50–1200 ng/mL and r^2 was found as 0.9927. Plasma linearity study was implemented in the range of 30–1200 ng/mL and r^2 was found as 0.9900 (Borges et al., 2009).

In this study, an optimization study was performed on wavelength, mobile phase, column and column furnace param-

eters in order to determine diazepam from plasma by HPLC coupled with UV. Optimized method: in accordance with the ICH Q2(R1) guidelines, it is validated in the parameters of linearity, precision, repeatability, recovery and robustness (ICH, 2005). All samples were prepared successfully with an optimized solid phase extraction method. Simulated plasma was used in all quantification steps, and the results obtained clearly showed that the artificial plasma provided appropriate results in the development and validation of the method.

Sample preparation in the study: this was made using simulated plasma and solid-phase extraction method. The determination coefficient for 5 points in the 100-1200 ng/mL diazepam concentration range was found to be greater than 0.98, which was found to be a good value for linearity. Sensitivity values

were 20.42 and 61.86 ng/mL for LOD and LOQ, respectively. The fact that the LOQ value was lower than the last point in the calibration curve suggests that the method can safely use for the analysis of diazepam at low concentrations. The intraday repeatability study was performed with 5 replicate analyses for each concentration on the same day, and the accuracy was between (RE%) (-5.78) and 3.30, and precision (RSD%) between 0.14% and 1.59%. The repeatability study between days was performed for 5 consecutive days, and the accuracy was between (RE%) (-5.78) and 5.93, and precision (RSD%) was between 1.22% and 1.82%.

The developed method has outstanding features when compared to the methods in the literature. This method was carried out between 100 and 1200 ng/mL concentrations including sub-therapeutic, therapeutic, and toxic doses of diazepam in human blood. The fact that the related method has been developed using diazepam concentrations that can be encountered in human blood further increases the importance of the relevant validation tests. The values of sensitivity data determined in both LOD and LOQ (20.42 and 61.86 ng/mL, respectively) enable the developed method to work safely at sub-therapeutic doses. It was determined that the RE% (accuracy) values obtained both within and between days were maximum (-5.78) and 5.93. These values, obtained by the analysis of independent samples on 5 different days and on the same day, are a clear statement that the method can be used safely both during and between days. In addition, the value of 1.82, obtained as the maximum RSD% value within and between days, is one of the assertive values obtained in the literature. In addition, the low mobile phase flow (0.5 mL/min) applied to the HPLC system during the analysis, the high efficiency and ease of application obtained in the developed solid-phase extraction application, the low organic solvent volume used during the extraction (≤ 6 mL), are other important features that make the study stand out from other studies in the literature. It was seen that the yield values obtained from the recovery tests between 95.12% and 10.83% were directly related to the successful values obtained in the basic validation parameters such as sensitivity, selectivity, robustness and reproducibility.

CONCLUSION

In order to perform diazepam analyses on plasma, an HPLC-UV analysis method was developed, which was simple, sensitive and reliable. The analysis method, which was developed and validated and applied to simulated plasma, has a simple sample preparation method with the use of 500 μ L of plasma and 1 mL of solvent, and the total analysis time is less than 11 minutes. The method was found to be linear between 400 ng/mL and 1200 ng/mL. Recovery from plasma was performed at concentrations of 200, 400 and 800 ng/mL and a high recovery value was achieved with an average of 100.39%.

It was concluded that the developed and validated HPLC-UV method is a simple, fast, sensitive, and reliable analysis method that can be used in reference laboratories that make therapeutic and toxicological impressions of diazepam.

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Synthesis, antifungal activity and *in silico* ADMET studies of benzyl alcohol derivatives

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ABSTRACT

Background and Aims: Fungal infections continue to pose a serious threat to human well-being due to the increasing cases of resistance against the existing antifungal drugs. Consequently, efforts are increasingly focused towards investigating new moieties with potential activity against different fungal species. The object of this work was to synthesize and evaluate the antifungal activity of five benzyl alcohol derivatives against *Candida albicans* and *Trichophyton rubrum* and also to study the biochemical as well as the ADMET properties of the compounds *in silico*.

Methods: The target compounds were obtained by the reduction of the appropriate aldehyde using sodium borohydride. Subsequently the compounds were tested against the two fungal species using ketoconazole as the positive control. The biochemical activities as well as the ADMET properties were calculated at DFT/B3LYP/6-311G* basis set with the help of Gaussian09 (G09) software.

Results: The findings revealed that some of the compounds exhibited interesting inhibitory action against *C. albicans* while others against *T. rubrum*. However, the MIC and MFC results demonstrated that none of the compounds were fungicidal at the concentrations tested. *In silico* ADMET studies showed that all the compounds have a good cell permeability index, their human intestinal absorption values were within the recommended scale and with good plasma protein binding.

Conclusion: The benzyl alcohol derivatives studied in this work have exhibited some encouraging antifungal activity and favorable biochemical as well as pharmacokinetic properties.

Keywords: ADMET properties, Antifungal activity, Benzyl alcohol, *Candida albicans*, *Trichophytonrubrum*

INTRODUCTION

Microbial infections, specifically those of fungal origin reportedly account for over 1.5 million deaths *per annum* globally despite the tremendous improvements in the diagnosis and treatment of these infections (Koushlesh et al., 2020). The growing resistance for the existing antimicrobial drugs has been attributed to this unfortunate situation, and this has led to the quest for structurally diverse compounds with a view of discovering alternative drugs (Durante-Mangoni, Grammatikos, Utili, & Falagas, 2009; Giamarellos-Bourboulis, 2008; Gonzalo-Garijo, Rodriguez-Nevalo & de Argila, 2006; Bozdogan, & Appelbaum, 2004).

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Benzyl alcohol (BA) is an aromatic organic compound containing the phenyl methanol moiety. It is used as a local anesthetic, flavor enhancing agent, fragrance in cosmetics, and as a preservative (Almoughrabie et al., 2020; Beata, Anna, & Anna, 2014). BA is naturally found in many plants where it exists as a free molecule and as esters in a variety of essential oils such as hyacinth, ylang-ylang and jasmine (Wei & Shibamoto, 2007). Previously some biological potentials of benzyl alcohol derivatives have been reported, for instance, Wang et al., (2020) reported the use of nitro-benzyl alcohol as a photo-reactive group with amine selectivity which enabled its applications for photo-affinity labeling and cross-linking of biomolecules. Lim et al., (2008) also reported the anti-angiogenic, anti-inflammatory, and anti-nociceptive activities of vanillin. These reports prompted us to further investigate the antifungal activity of some benzyl alcohol derivatives.

MATERIAL AND METHODS

All reagents and solvents were purchased from Sigma-Aldrich (Darmstadt, Germany) and used as received. ¹H and ¹³C NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. FTIR spectra were recorded on a Perkin-Elmer BX spectrophotometer (Bayero University, Kano, Nigeria). Melting points were determined on an Electro-thermal melting point apparatus and are uncorrected.

Methods

General procedure for the preparation of benzyl alcohol derivatives (A-E)

In a round bottom flask, aldehyde (26.6mmol) was dissolved in ethanol (8 mL), and the mixture placed on an ice-bath to cool. Using a small vial, NaBH₄ (26.4 mmol) was dissolved in 1M NaOH (7.6mL), and the resulting solution was then added slowly to the solution of the aldehyde over a period of 10 min. The mixture was stirred at room temperature for 10 min, and then cooled down. While stirring, HCl solution (6 M) was added dropwise, this led to the precipitation of the respective product. The addition continued until the evolution of H₂ gas stopped. Then, the pH was checked to ensure that the solution was acidic. The product was collected by filtration, washed twice with ice-cold water, transferred to a dry piece of filter paper and air dried. However in the case of compound **E**, after the addition of HCl solution (6 M), the resulting mixture was treated with chloroform (30 mL), and then separated. The chloroform layer was evaporated to afford the corresponding solid product.

Characterization

4-Bromobenzyl alcohol, Compound A

White powder; 95% yield; mp 212-215 °C; ¹HNMR (400MHz, DMSO₄-d₆) δ ppm 4.46 (2H, s, CH₂), 5.38 (1H, s, OH), 7.27 (2H, d, J = 8.0 Hz, Ar-H), 7.48 (2H, d, J = 8.0 Hz, Ar-H); ¹³C-NMR (100MHz, DMSO₄-d₆) δ ppm 62.40 (CH₂), 119.81 (Ar-C), 128.79 (2×ArCH), 131.12 (2×ArCH), 142.06 (Ar-CBr); FTIR cm⁻¹ 3272 (OH), 1002 (C-O).

4-Nitrobenzyl alcohol, Compound B

Yellow powder, 93% yield, mp 131-134 °C. ¹HNMR (400MHz, DMSO₄-d₆) δ ppm 3.56 (2H, s, CH₂), 5.61 (1H, s, OH), 7.57 (2H, d, J = 8.0

Hz, Ar-H), 8.17 (2H, d, J = 4.0 Hz, Ar-H); ¹³CNMR (100MHz, DMSO₄-d₆) δ ppm 62.21 (CH₂), 123.49 (2×ArCH), 127.25 (2×ArCH), 148.50 (Ar-C), 150.89 (Ar-CNO₂); FTIR cm⁻¹ 3503 (OH), 1054 (C-O).

4-Hydroxybenzyl alcohol, Compound C

White powder, 87% yield, mp 260-265 °C; ¹HNMR (400MHz, DMSO₄-d₆) δ ppm 4.37 (2H, s, CH₂), 5.07 (1H, s, OH), 6.71 (2H, d, J = 8.0 Hz, Ar-H), 7.11 (2H, d, J = 8.0 Hz, Ar-H), 9.36 (1H, s, Ar-OH); ¹³CNMR (100MHz, DMSO₄-d₆) δ ppm 63.09 (CH₂), 115.10 (2×ArCH), 128.43 (2×ArCH), 132.94 (Ar-C), 156.41 (Ar-COH); FTIR cm⁻¹ 3377 (OH), 1207 (C-O).

4-Hydroxy-3-methoxybenzyl alcohol Compound D

White powder, 93% yield, mp 112-114 °C. ¹HNMR (400MHz, DMSO₄-d₆) δ ppm 3.74 (3H, s, OCH₃), 4.37 (2H, s, CH₂), 5.14 (1H, s, OH), 6.70 (2H, s, 2×Ar-H), 6.88 (1H, s, Ar-H), 8.88 (1H, s, ArOH); ¹³CNMR (100MHz, DMSO₄-d₆) δ ppm 55.80 (OCH₃), 63.36 (CH₂), 111.32 (Ar-C), 115.35 (Ar-CH), 119.51 (Ar-CH), 133.72 (Ar-CH), 145.54 (Ar-C-OCH₃), 147.67 (Ar-COH); FTIR cm⁻¹ 3503 (OH), 1121 (C-O).

4-Methoxybenzyl alcohol, Compound E

White solid; 94% yield; mp 212-215 °C; ¹HNMR (400MHz, DMSO₄-d₆) δ ppm 3.71 (3H, s, OCH₃), 4.40 (2H, s, CH₂), 5.15 (1H, s, OH), 6.87 (2H, d, J = 8.0 Hz, Ar-H), 7.22 (2H, d, J = 8.0 Hz, Ar-H); ¹³CNMR (100MHz, DMSO₄-d₆) δ ppm 55.22 (OCH₃), 62.79 (CH₂), 113.67 (Ar-C), 128.21 (2×ArCH), 128.82 (2×ArCH), 158.36 (Ar-COCH₃); FTIR cm⁻¹ 3326 (OH), 1244 (C-O).

Antifungal activity

Test microorganisms

The test microorganisms used in this study were clinical isolates of *Candida albicans* and *trichophyton rubrum* obtained from the Department of Microbiology, Umaru Musa Yar'adua University, Katsina, Nigeria.

In vitro assay of the activity of the synthesized compounds on *Candida albicans* and *Trichophyton rubrum*

An 80 mg/mL solution of each of the compounds including the positive control, ketoconazole was prepared by dissolving 0.2 g each in 2.5 mL of 50% DMSO in separate test tubes. Sterilized discs were made from filter paper and 100 discs were transferred into each tube so that each disc absorbed 100 µL (equivalent to 800 µg of the compound). After the disc preparation, each of the fungal isolates was separately inoculated on the freshly prepared SDA plates using a streak plate method. Four (4) discs impregnated with a compound, standard drug or 50% DMSO were then introduced into each of the inoculated plates using an aseptic technique. The plates were inoculated at room temperature for 7 days after which the zones of inhibition were recorded for each plate (Singh, Dar, & Sharma, 2012). This process was repeated three (3) times.

Preparation of different concentrations of the compounds for MIC and MFC

About 0.1 g of each compound was dissolved in 100 mL DMSO to obtain a stock solution of 1000µg/mL. Dilution was carried out using sterile syringes to obtain concentrations of 1000, 800, 600, 400, 200, 100 and 50 µg/mL.

Determination of minimum inhibitory concentration (MIC)

The MIC of the compounds was determined using the tube dilution method. Serial dilutions of the solutions of the compounds were carried out in well labeled test tubes using Muller-Hinton broth as diluents. The tubes were then inoculated with 0.1 mL of standard inoculums and incubated for 72h until turbidity was observed. The least concentration showing no visible sign of growth, which gave no turbidity of the medium, was taken as the MIC. Broth with no inoculum, broth containing the test organism and ketoconazole and broth with test organisms only, were included as controls (EUCAST, 2003).

Determination of the minimum fungicidal concentration (MFC)

The contents of the tubes used for the MIC determination study were streaked onto SDA plates using a wire-loop and the plates were incubated aerobically at 37°C for 3 days. The MFC values were read as the least concentrations that killed the test organisms, which was indicated by the absence of growth (Imanirampa & Alele, 2016).

Statistical analysis

Data were statistically analyzed using SPSS 16.0 software by comparison of means (one-way ANOVA) using the Tukey post hoc test, at the significance level of $p < 0.05$.

Biochemical activity and *in silico* ADMET analysis

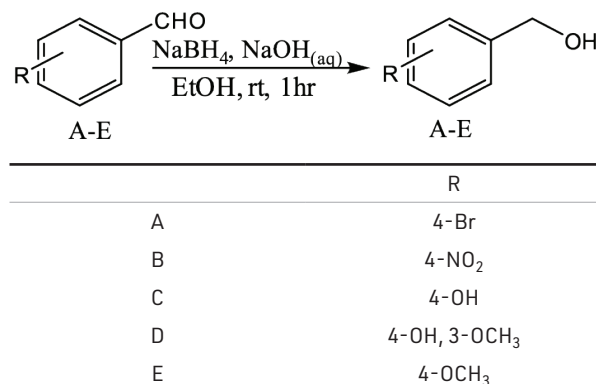
All compounds (A-E and ketoconazole as positive control) were optimized and the vibrational frequencies of the benzene's derivatives were calculated at DFT/B3LYP/6-311G* basis set with the help of Gaussian 09 (G09) software (Gaussian et al., 2009) to investigate the chemical and biochemical activities of the benzene's derivatives. Afterwards, the eigenvalues of HOMO and LUMO, the global hardness (η), the chemical potential (μ), electrophilicity index (ω) and dipole moments (DM) on each compound were calculated at same level of theory. Further, Lipinski (Lipinski et al., 2001) and Veber rules (Veber et al., 2002) were performed using Discovery Studio (DS) 3.5 for the compounds. Then *in silico*, an ADMET (Absorption, Distribution, Metabolism, Extraction and Toxicity) analysis for the compounds was predicted by using the sub-protocol of DS 3.5 software and pkCSM server (Cheng et al., 2012) in order to elucidate the pharmacoinformatic information of the compounds examined. Human intestinal absorption (HIA), Caco2 permeability for the prediction of oral drug absorption, skin permeability, blood brain barrier penetration (BBB), Human

ether-a-go-go related gene (hERG) inhibition, Cytochrome P450 (CYP1A2, CYP1C19, CYP2C9, CYP2D6, CYP3A4) inhibition, AMES toxicity, Tetrahymena Pyriformis Toxicity and plasma protein binding (PPB) descriptors were calculated.

RESULTS

Chemistry

The benzyl alcohol derivatives, **A-E** were obtained by reducing the appropriate aldehyde with NaBH_4 as shown in Scheme 1, and their structures were confirmed by the ^1H and ^{13}C NMR, and FTIR spectroscopy techniques.



Scheme 1. Synthesis of benzyl alcohol derivatives.

Antifungal activity

The synthesized compounds were screened for *in vitro* antifungal activity against *C. albicans* and *T. rubrum* using a disc diffusion method. Generally, both fungi were sensitive to all the compounds except C which had no activity on *C. albicans* (Table 1).

The MIC and MFC of the compounds against both organisms are given in Tables 2 and 3 respectively.

Biochemical activity and *in silico* ADMET analysis

In the present study, we firstly investigated the five compounds and selected a positive control (ketoconazole) based on quantum chemical parameters. The obtained direct and indirect parameters for each compound are summarized in Table 4 and Figure 1.

The five compounds were examined according to both the Lipinski and Veber rules, and the result is presented in Table 5.

Table 1. Comparative activities of the compounds against *C. albicans* and *T. rubrum* at 800 µg/disc.

		Ketoconazole	Compound A	Compound B	Compound C	Compound D	Compound E	Negative control
<i>C. albicans</i>	Mean ZI (mm)	22.98±4.13	13.74±3.4 ^a	18.56±1.3 ^b	06.93±5.2 ^c	14.62±3.7 ^{ab}	10.66±1.8 ^{ac}	07.15±0.3 ^c
<i>T. rubrum</i>	Mean ZI (mm)	25.84±6.18 ^b	13.52±3.3 ^a	21.48±5.1 ^b	12.23±3.5 ^a	10.55±1.0 ^{ac}	07.20 ±2.58 ^c	00.00

Values are expressed as mean ± standard deviation of three experiments conducted in quadruplicates. Those with the same superscript are not significantly different statistically ($p > 0.05$)

Table 2. MIC of the compounds against <i>C. albicans</i> and <i>T. rubrum</i> .							
<i>C. albicans</i>							
Compound	1000 µg/ml	800 µg/ml	600 µg/ml	400 µg/ml	200 µg/ml	100 µg/ml	50 µg/ml
A	-	+	+	+	+	+	+
B	+	-	+	+	+	+	+
C	+	+	+	+	+	+	+
D	-	+	+	+	+	+	+
E	+	+	+	+	+	+	+
<i>T. rubrum</i>							
Compound	1000 µg/ml	800 µg/ml	600 µg/ml	400 µg/ml	200 µg/ml	100 µg/ml	50 µg/ml
A	-	+	+	+	+	+	+
B	-	+	+	+	+	+	+
C	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+

+ = Presence of growth; - = Absence of growth; Ketoconazole = 50 µg/ml for both organisms

Table 3. MFC of the compounds against <i>C. albicans</i> and <i>T. rubrum</i> .							
<i>C. albicans</i>							
Compound	1000 µg/ml	800 µg/ml	600 µg/ml	400 µg/ml	200 µg/ml	100 µg/ml	50 µg/ml
A	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+
<i>T. rubrum</i>							
Compound	1000 µg/ml	800 µg/ml	600 µg/ml	400 µg/ml	200 µg/ml	100 µg/ml	50 µg/ml
A	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+

+ = Presence of growth; - = Absence of growth Ketoconazole; = <50 µg/ml for both organisms

Table 4. Biochemical activity analysis of the compounds and the positive control (ketoconazole).						
Name	ϵ HOMO (eV)	ϵ LUMO (eV)	Dipole M (Debye)	Hardness (η)	Chem. Pot. (μ)	Electrophilicity index (ω)
A	-6.536	-0.583	3.692	5.953	-3.559	1.064
B	-7.437	-2.455	6.529	4.982	-4.946	2.455
C	-5.953	-0.194	1.587	5.759	-3.074	0.820
D	-5.841	-0.048	2.774	5.793	-2.944	0.748
E	-6.040	-0.205	1.702	5.835	-3.122	0.835
Ketoconazole	-5.167	-1.277	5.039	3.890	-3.222	1.334

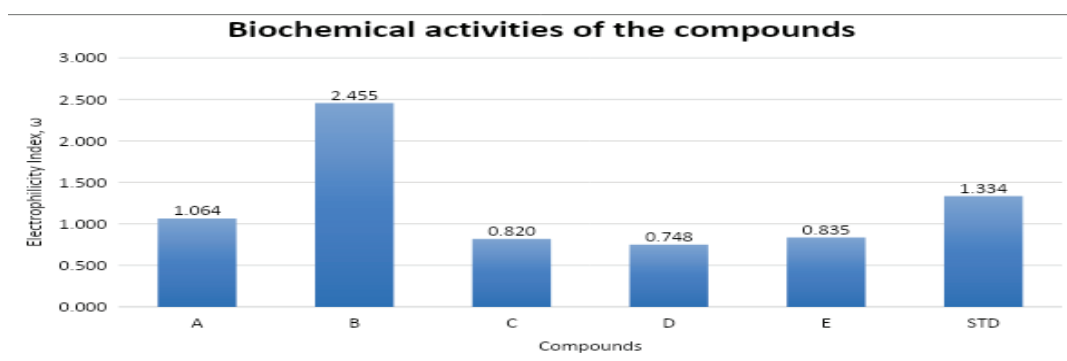


Figure 1. Biochemical activity plot.

The ADMET analysis was also exerted for the same structures to estimate their pharmacokinetic and toxicological properties in the biological system. Thus, the ADMET plots was built for all the compounds and ketoconazole (Tables 6 and 7).

in the aromatic region and was assigned to the two aromatic protons closer to the methylene group, while the signal at 8.18 ppm was assigned to the aromatic protons closer to the nitro group, (-NO₂).

Table 5. The Lipinski and Veber rules analysis of the compounds and positive control (ketoconazole).

Name	MW	AlogP	MPSA	NRB	HA_Lipinski	HD_Lipinski	HA_Veber	HD_Veber
	(≤500 g/mol)	(≤5)	(≤140 Å ²)	(≤10)	(≤10)	(≤5)	(≤12)	(≤12)
A	153.135	1.119	66.05	2	4	1	3	1
B	187.034	1.973	20.23	1	1	1	1	1
C	124.137	0.983	40.46	1	2	2	2	2
D	138.164	1.209	29.46	2	2	1	2	1
E	154.163	0.967	49.69	2	3	2	3	2
Ketoconazole	531.431	3.61	69.06	7	8	0	6	0

Abbreviations: ALogP, octanol/water partition coefficient, a measure for lipophilicity; MW, molecular weight; MPSA, molecular polar surface area; Num_H_Acceptors_Lipinski, Number of hydrogen bond acceptors; Num_H_Donors_Lipinski, Number of hydrogen bond donors; NRB, Number of Rotatable bonds; Num_H_Acceptors, Number of hydrogen bond acceptors based on Veber; Num_H_Donors, Number of hydrogen bond donors based on Veber.

Table 6. ADMET analysis of the compounds and positive control (ketoconazole).

Comp.	PSA_2D(< 140 Å ²)	AlogP98(< 5)	HIA	Solubility	BBB	PPB
A	20.815	1.973	0(good)	4(optimal solubility)	1(good)	-2.6545
B	63.638	1.119	0(good)	4(optimal solubility)	3(low)	-4.1726
C	41.631	0.983	0(good)	4(optimal solubility)	2(medium)	-6.351
D	50.561	0.967	0(good)	4(optimal solubility)	3(low)	-4.4769
E	29.745	1.209	0(good)	4(optimal solubility)	2(medium)	-1.9019
Ketoconazole	67.405	3.61	0(good)	2(low)	2(medium)	46.479

Abbreviations: PSA, polar surface area; AlogP98, the logarithm of the partition coefficient between n-octanol and water; BBB blood brain barrier; CYP2D6 cytochrome P450 2D6 binding; PPB plasma protein binding, more than 90% for PPB: Chemicals strongly bound. Less than 90% for PPB: Chemicals weakly bound.

Table 7. ADMET analysis of the compounds and positive control (ketoconazole).

Name	hERG-inhibition	CYP1A2 inhibitor	CYP1C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
A	No	No	No	No	No	No
B	No	Yes	No	No	No	No
C	No	No	No	No	No	No
D	No	No	No	No	No	No
E	No	No	No	No	No	No
Ketoconazole	Yes	Yes	Yes	Yes	Yes	Yes

DISCUSSION

Typically the ¹H NMR spectrum of compound **B** gave a signal at 4.62 ppm which was assigned to the two protons of the methylene group (-CH₂). The hydroxyl proton was found at 5.62 ppm. Further down, the signal at 7.57 ppm appeared

The ¹³C NMR spectrum gave the signal for the methylene carbon at 62.21 ppm, and the signal at 123.49 ppm was assigned to the aromatic carbon to which the methylene group is attached. The signals at 123.49 ppm and 127.25 ppm were assigned to the four carbon atoms further away. Lastly, the signal at 150.89 ppm was assigned to the carbon atom attached to the nitro group (NO₂).

The FTIR spectrum gave the hydroxyl (-OH) band at 3503 cm^{-1} , while the C-O stretch was found at 1054 cm^{-1} .

The antifungal activity (Table 1) revealed that for *Candida albicans*, compound **B** showed very good activity with an inhibition zone of 18.56 ± 1.32 mm compared to the standard antifungal ketoconazole (ZI = 22.98 ± 4.13 mm), followed by compound D (ZI = 14.62 ± 3.76), A (ZI = 13.74 ± 3.44), E (ZI = 10.66 ± 1.81) and the least being compound C that showed no significant activity. In the case of *T. rubrum*, compound **B** gave an excellent inhibition zone of 21.48 ± 5.18 mm quite comparable to the 25.84 ± 6.18 mm exerted by the control drug. Compounds **A, C, D** and **E** gave ZI of 13.52 ± 3.31 , 12.23 ± 3.53 , 10.55 ± 1.06 and 07.20 ± 2.58 zones of inhibition respectively. In the case of MIC (Table 2) against *Candida albicans*, Compounds **A** and **D** were both found to show inhibition at concentrations of $1000 \mu\text{g/mL}$ while compound **B** showed an MIC of $800 \mu\text{g/mL}$. On the other hand, compounds **C** and **E** exhibited no inhibitory actions at all the concentrations tested. For *Trichophyton rubrum*, its MIC showed that only compounds **A** and **B** at $1000 \mu\text{g}$ had inhibitory actions on the fungus. This implied that their activities were concentration-dependent. Compounds **C, D** and **E** showed no inhibitory effect at all on the concentrations tested. The MFC results (Table 3) indicated that none of the compounds were fungicidal against any of the pathogens at all on the concentrations tested. This showed that the compounds **A, B** and **D** only succeeded in inhibiting the growth of the organisms but could not kill them. They were therefore, fungistatic and not fungicidal.

The biochemical activity analysis (Table 4 and Figure 1) revealed that compound B exhibited the best electrophilicity index value compared to the rest of the compounds including the positive control (ketoconazole). According to the Lipinski and Veber rules, it is suggested that potential lead compounds that conform to their rules of 5 tend to have lower withdrawal rates during phases of clinical trials and have an increased chance of reaching the markets (Leeson & Springthorpe, 2007). As shown in Table 5, all the tested compounds were found to be located within the borders of tests. The ADMET plot (Tables 6 and 7), the two parameters; polar surface area (PSA) and lipophilicity (AlogP98) served as indicators of cell permeability. Any compound with $\text{PSA} < 140 \text{ \AA}^2$ and $\text{AlogP98} < 5$ has optimum cell permeability. In this work, all the studied compounds followed these rules. In addition to these parameters, the human Intestinal absorption (HIA) and Caco-2 permeability presented in Table 6 serve as good indicators for drug absorbance in the intestine and for predicting the gut blood barrier penetration. While for all the studied compounds including the control, the HIA values were found to be good in terms of Caco2 permeability and have permeability coefficient values at the recommended scale ($> 0.90 \log \text{ cm/s}$), the BBB partition coefficient was estimated to predict the blood brain barrier permeability for each compound. It indicates accessibility of bioactive for the central nervous system. As shown in Tables 6-7, while ketoconazole has a low BBB coefficient, others have a medium BBB value. The PPB coefficient is a prediction of plasma-protein binding. The binding of the compound to the plasma proteins such as lipoprotein, glycoprotein, human serum albumin, a, b,

and c globulins can greatly affect the quantity of the drug in blood circulation. In general, the less degree of PPB is desirable for designing drugs with more cell availability and cell membrane traverse/diffusion. The computed values show that all the compounds are in the acceptable range (< 90 -chemicals weakly bound) in terms of PPB.

CONCLUSION

Five benzyl alcohol derivatives were successfully obtained via NaBH_4 reduction of the appropriate aldehyde. For the first time, the antifungal activities of the compounds were evaluated against *Candida albicans* and *Trichophyton rubrum* using disc diffusion method. And it was found that compounds A, B and D exhibited fungistatic effect against *C. albicans*, while compounds A and B against *T. rubrum*. However, none of the compounds were fungicidal at the concentrations tested. In terms of a broad spectrum activity, the nitro-substituted compound (compound B) was found to exhibit a promising efficacy almost competing with that of the standard drug (ketoconazole). In order to understand the mode of interaction of the compounds, an *in silico* ADMET analysis was exerted for the same structures to estimate their pharmacokinetic and toxicological properties in the biological system. Fortunately, all the compounds were found to be in the acceptable range of the pharmacokinetics parameters.

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Aromatherapeutic essential oils and their pharmaceutical combinations: Tools for inhibition of quorum sensing activity and biofilm formation of human pathogens*

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ABSTRACT

Background and Aims: Aromatherapy, as one of the complementary therapies, uses essential oils as the main therapeutic agents to treat several diseases. In the present study, it was aimed to investigate inhibition of quorum sensing (QS) and biofilm formation of aromatherapeutic essential oils (AEOs) and their pharmaceutical combinations (PC-I and PC-II).

Methods: The anti-QS potential of AEOs were determined using the biosensor strains *Chromobacterium violaceum* ATCC 12472 and *Pseudomonas aeruginosa* PAO1. Anti-QS activity was detected by agar-well diffusion and violacein pigment inhibition assays. Blocking of PAO1 swim and swarm motilities and biofilm formation was also performed.

Results: Most of the AEOs demonstrated highly active (>95%) violacein pigment inhibition. Additionally, they inhibited swarming (40.34%-72.80%) and swimming (20.06%-50.08%) motilities of PAO1. Moreover, the majority of AEOs also decreased the biofilm formation, particularly on *P. aeruginosa* and *S. aureus*.

Conclusion: Consequently, aromatherapeutic formulations might be a complementary or prophylactic cure for infectious disease by their anti-QS and antibiofilm activities rather than just antimicrobial effects.

Keywords: Aromatherapeutic essential oils, Antibiofilm, quorum sensing, *Pseudomonas aeruginosa* PAO1, Synergistic effect

INTRODUCTION

Increasingly becoming fundamental in the global health system due to better patient tolerance, renewability and better biodegradability, aromatherapy is one of the complementary therapies, which uses essential oils to cure or support the cure of numerous physical or mental problems including bacterial or viral born infectious diseases, respiratory, digestive and urinary problems, headache, depression, insomnia, muscular pain, as well as skin ailments such as acne and dermatitis (Ali et al., 2015; Yan, Wang, Cruz Flores, & Su, 2019). The therapeutic effects of essential oils have been attributed to their chemical components mainly terpenoids as well as nonterpenoid compounds such as phenols, esters and oxides. Essential oils can be mostly obtained from dried

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flowers, leaves, fruits, roots, barks or whole aerial parts of plants by using several techniques including water or steam distillation, solvent extraction, supercritical fluid and subcritical water extractions. In addition to the advantages of their use, with their well-known antibacterial and antiviral properties, they are claimed to be very good complementary products and alternatives to anti-pathogenic drugs and antibiotics (Boire, Riedel, & Parrish, 2013; Yap, Yap, Ping, & Lim, 2014).

Over the past decade, the uncontrollable spread of bacteria that are simultaneously resistant to various drugs have increased in the community because of the inappropriate use of antibiotics. With antibiotic resistance, these bacteria could cause wider infection control problems such as treatment failure, increased fatality rate, and dispersion of resistant bacteria from hospital to community. Biofilm formation, in which bacteria stick to each other and to a surface, also creates a serious problem in medical facilities, inducing resistant hospital infection. Moreover, biofilm formation represents the main indicator of bacterial infection, especially those caused by devices such as catheters, prosthetic valves, orthopedic devices, etc. Therefore, the eradication of the biofilm matrix from these kinds of surfaces becomes extremely difficult (Li et al., 2018; Vipin, Mujeeburahiman, Ashwini, Arun, & Rekha, 2019; Yap et al., 2014; Zhang et al., 2019; Zhang et al., 2018).

Biofilm formation and bacterial virulence are correlated with quorum sensing (QS), a process of bacterial cell to cell communication in which cells regulate the transcription of the specific genes responsible for the production of antibiotics, biofilm differentiation, cell division, bioluminescence and the other virulence features (Ahmad, Viljoen, & Chenia, 2015; Brun, Bernabè, Filippini, & Piovan, 2019; Bali, Erkan Türkmen, Erdönmez, & Sağlam, 2019). The QS system permits bacteria to assess their population density via the production and sensing of QS signaling molecules called N-acyl homoserine lactones (AHLs) and oligopeptides in Gram-negative and Gram-positive bacteria, respectively (Y. Zhang et al., 2018). Blocking QS signaling molecules or the bacterial QS system is considered as a significant alternative strategy for controlling persistent infections due to bacterial resistance and a promotive target to discover the anti-infective properties of natural products (Vasavi, Arun, & Rekha, 2014; Ahmad et al., 2015; Doğan, Gökalsın, Şenkardeş, Doğan, & Sesal, 2019). Therefore, the objective of our research was to evaluate the anti-QS activity of aromatherapeutic essential oils (AEOs) and their combinations via *Chromobacterium violaceum* ATCC 12472 and *Pseudomonas aeruginosa* PAO1 as biosensor strains and their antibiofilm effects against Gram-negative and Gram-positive human pathogens.

MATERIAL AND METHODS

Preparation of aromatherapeutic essential oils (AEOs) and their pharmaceutical combinations

Eleven aromatherapeutic essential oils (AEOs) of *Cedrus atlantica* (Endl.) G.Manetti ex Carrière (cedrus), *Citrus aurantium* L. var. *bergamia* (bergamot), *Citrus limon* L. (lemon), *Citrus sinensis* L. (orange), *Eugenia caryophyllus* (Spreng.) Bullock & S.G.Harrison (clove), *Eucalyptus globulus* Labill. (eucalyptus), *Lavandula angustifolia* Mill. (lavender), *Melaleuca alternifolia* (Maiden &

Betche) Cheel. (tea tree), *Mentha piperita* L. (mint), *Rosmarinus officinalis* L. (rosemary), *Thymus vulgaris* L. (thyme) and their pharmaceutical combinations were applied on QS biosensor strains and human pathogens to detect their anti-QS and antibiofilm activities. AEOs were purchased from Florame (St. Remy de Provence, France). The combinations of these AEOs were coded as pharmaceutical composition-I (PC-I) and II (PC-II). PC-I is the combination of thyme:bergamot:lemon:tea tree:lavender:mint essential oils (1:2:4:5:5:5) whereas, PC-II is the combination of thyme:tea tree essential oils (1:1). The stock solution of AEOs was prepared in dimethyl sulfoxide (DMSO) and diluted as 0.05% DMSO when used in the experiments.

Bacteria and culture conditions

The bacterial strains were obtained from the American Type Culture Collection (ATCC, USA). Gram-positive human pathogen bacteria: *Bacillus cereus* American Type Culture Collection (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* Wild-type, *Enterococcus faecalis* (ATCC 29212), *Micrococcus luteus* (ATCC 7468), and Gram-negative human pathogen bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603), *Klebsiella oxytoca* (ATCC 43165), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14074), *Serratia marcescens* (ATCC 27117), *Acinetobacter baumannii* (ATCC 19606) and *Proteus mirabilis* (ATCC 7002) were used to detect the antibiofilm activity of AEOs. The wild-type strain *Chromobacterium violaceum* (ATCC 12472) used as a biosensor strain for anti-QS activity was a kind gift by Prof. Dr. Robert Mclean from the Department of Biology, Texas State University-San Marcos, USA. *P. aeruginosa* PAO1 was also kindly gifted by Daniel Lopez, PhD from the National Centre for Biotechnology (CNB), Autonomous University of Madrid, Spain. The bacterial cultures were grown in Brain Heart Infusion (BHI) broth medium (Merck, Germany) at 37°C for 24h. *C. violaceum* 12472 and PAO1 were cultivated in Luria-Bertani broth (LB) medium (Sigma-Aldrich, USA, pH=7.0) at 30°C and 37°C for 24 h, respectively. All strains were subcultured until the optical density (OD) of 0.4 at 600 nm was reached.

Minimum inhibitory concentrations (MICs) of AEOs

Minimum inhibitory concentrations (MICs) of the AEOs against human pathogens and biosensor strains were performed using the broth microdilution method (Zgoda & Porter, 2001). Briefly, 100µL of essential oils were diluted in sterile 96-well microplates in 95 µL of Brain Heart Infusion (BHI) broth and 5 µL of the tested bacteria (10⁸ CFU/mL) were added to each well. The final volume in the wells was 200 µL and the microplates were incubated at 30°C or 37°C for 24 hours. The 96-well microtiter plates were then measured in a microplate reader at 600 nm according to the control to determine the growth inhibition of the essential oil on the microorganisms. Gentamicin (10µg/ml) (Sigma, Saint Louis, USA) was used as the positive antibiotic control.

Anti-quorum sensing (Anti-QS) assays

qualitative detection: Agar well diffusion method

Anti-QS activity of AEOs, at the sub-MIC of 0.4% v/v was performed with the biosensor strain *C. violaceum* ATCC 12472 (Zahin et al., 2010). Briefly, Luria-Bertani (LB) agar plates were

inoculated with 0.1 ml of overnight bacterial cultures and wells of 6 mm diameter were opened at the bottom of soft agar. Each oil was added into the wells and the petri dishes were left for incubation at 30 °C for 48 hours. QS inhibitions of each oil were detected as an opaque zone with loss of purple pigmentation around each well. The measurements were made from the outer edge of the disks to the edge of the opaque zones suggesting anti-QS inhibition.

Quantitative detection: Violacein pigment inhibition assay

Inhibitory effects of violacein pigment production by AEOs at the sub-MIC of 0.4% v/v were also measured spectrophotometrically (Blosser & Gray, 2000). Briefly, each oil was added to 200 µL of bacterial culture and incubated at 30°C until complete pigmentation was achieved in the blank, i.e., the untreated culture. First, 200 µL of treated (test) and untreated cultures (control) were placed in a microcentrifuge tube and lysed by addition of 200 µL of 10 % SDS, vortexed for 5 sec. and incubated at room temperature for 5 min. Subsequently, 900 µL of water-saturated butanol (50 mL n-butanol mixed with 10 mL distilled water) was added to the cell lysate, followed by vortexing for 5 sec. and centrifugation at 13,000×g for 5 min. The upper (butanol) phase containing the violacein was collected and the absorbance was read at 585 nm in UV-Vis spectrophotometer. The percentage of violacein inhibition was calculated using the following formula:

$$\text{Violacein inhibition (\%)} = \frac{(A_{585\text{nm}}(\text{Control}) - A_{585\text{nm}}(\text{Test}))}{(A_{585\text{nm}}(\text{Control}))} \times 100$$

Motility assays of *Pseudomonas aeruginosa* PAO1 strain

The swimming and swarming motility assays were performed using a previously described, slightly modified method (Packiavathy, Agilandeswari, Musthafa, Pandian, & Ravi, 2012). In the swimming assay, 5 µL of overnight culture of *P. aeruginosa* PAO1 (A600 nm=0.4) was point inoculated at the center of an agar medium consisting of 1% tryptone, 0.5% NaCl and 0.3% agar with 0.0125% v/v sub-MIC concentration of the materials. For swarming assays, the agar medium comprised 1% peptone, 0.5% NaCl, 0.5% agar and 0.5% filter-sterilized D-glucose with the same sub-MIC concentration. The plates were then incubated at 37°C in an upright position for 16 h. The reduction in swimming and swarming migration was recorded by measuring the swimming and swarming zones of the bacterial cells after 16 h compared to the negative controls.

Antibiofilm activity assay

Antibiofilm effects of AEOs at the sub-MIC of 0.0125% v/v were performed in 96-well U-bottom polystyrene microtiter plates according to the slightly modified method (O'Toole & Kolter, 1998). An overnight culture of *C. violaceum* ATCC 12472 was diluted 1:100 with LB broth and grown for another hour. After the addition of each oil and the combinations, 100 µL of the culture was pipetted into the wells of the microtiter plates and the plates were incubated for 24 h at 30°C. Then, the medium was removed and washed with 1×PBS buffer in triplicate. The plates were dried at 65°C in a universal oven and then 100 µL of a 1% m/V crystal violet aqueous solution was added. The stain was allowed to fix at room temperature for 20 min, after which the dye was removed from the wells by washing thoroughly

with sterile water. For the quantification of the attached biomass, the bound dye was dissolved with 30% acetic acid solution, and the absorbance was determined at 595 nm. Inhibitor-mediated reduction of biofilm formation was assessed by comparing it to the control without the oils, and the standard antibiotic amoxicillin (2µg/ml) was also used as a positive control. Amoxicillin and gentamicin (10µg/ml) were used as positive controls since they are widely used in infectious diseases and their effectiveness is known. The percentage inhibition of biofilm was calculated as:

$$\text{Biofilm inhibition (\%)} = \frac{(\text{Control OD}_{595\text{nm}} - \text{Test OD}_{595\text{nm}})}{\text{Control OD}_{595\text{nm}}} \times 100$$

Statistical analysis

The results of all the experiments were performed in triplicate and repeated at least twice. All values are expressed as means ± standard deviations (SD). Statistical analyses were performed using the statistical program SPSS version 20.0 (Statistical Package for the Social Sciences). Differences among means were performed by analysis of variance (ANOVA) and averages were compared using Bonferroni test. Differences at *p<0.05, **p<0.01 and ***p<0.001 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Minimal inhibitory concentrations of AEOs and their pharmaceutical combinations

Minimal inhibitory concentration (MIC) values of AEOs and their pharmaceutical combinations (PC-I and PC-II) were detected to select the sub-MIC concentrations to study their effects on QS and biofilm inhibition. The MIC values of AEOs against biosensor strains and human pathogens were found to be in the ranges of 0.025%- 1.6% (v/v) and 0.2%- 1.6% (v/v), respectively (Table 1). The majority of AEOs was detected to inhibit bacterial growth (Table 1). Eucalyptus (its main components: 1,8-cineole and α-pinene), clove (eugenol, eugenyl acetate, and β-caryophyllene) AEOs and the combinations (PC-I and PC-II) showed higher antimicrobial effect than gentamycin as a positive control on *S. typhimurium*, indicating the lower MIC values (<1.2% v/v). Eucalyptus, lavender (its main components: linalyl acetate, linalool and cis-β-ocimene), mint (menthol and menthone), and clove AEOs also displayed higher growth inhibition than gentamycin on *S. marcescens*, indicating lower MIC values (<0.3% v/v) (Table 1). Orange (its main components: limonene and myrcene) and lemon (limonene, β-pinene and γ-terpinene) AEOs exhibited the best growth inhibition with the MIC value of 0.2% (v/v) on *S. aureus*. Furthermore, rosemary AEO, including the main components of 1,8-cineole, β-pinene and α-pinene, displayed the best antimicrobial effect on *S. epidermidis* and *A. baumannii* (Table 1). Except lavender and rosemary AEOs, *P. aeruginosa* ATCC 27853 demonstrated as the most resistant strain against all AEOs; however, *P. aeruginosa* PAO1 as the QS biosensor strain was found to be the most susceptible to all AEOs. The MIC results revealed that AEOs exhibited more effective inhibition against Gram positive bacterial growth than Gram negatives (Table 1). The antibacterial properties of essential oils have mostly been reported as being more effective against Gram positive

than Gram negative bacteria (Bharti et al., 2020; Pellegrini et al., 2014). Due to the existence of hydrophobic lipopolysaccharide in the outer membrane structure of Gram-negative bacteria, their external membrane could be impermeable to AEOs (Zgurskaya, López, & Gnanakaran, 2015). Therefore, our results are in agreement with these reports (Pellegrini et al., 2014; Zgurskaya et al., 2015; Bharti et al., 2020).

where thyme (40.33±1.15 mm) and tea tree (32.33±2.52 mm) AEOs presented the highest activity with larger zones (Figure 1). Our qualitative QS results were more encouraging than the study by Mokhetho et al., in which they observed an anti-QS activity with the highest diameter zones of 5.50±1.10 mm (Mokhetho, Sandasi, Ahmad, Kamatou, & Viljoen, 2018).

Table 1. MIC values of aromatherapeutic essential oils on pathogen bacteria and biosensor strains.

Aromatherapeutic Essential Oils % (v/v)/ Biosensor Strains/ Human Pathogen Bacteria	Biosensor Strains			Gram negative human pathogens						Gram positive human pathogens					
	<i>C. violaceum</i> ATCC 12472	<i>P. aeruginosa</i> PA01	<i>A. baumannii</i> ATCC 19606	<i>E. coli</i> ATCC 25922	<i>K. oxytoca</i> ATCC 43165	<i>K. pneumoniae</i> ATCC 700603	<i>P. aeruginosa</i> ATCC 27853	<i>P. mirabilis</i> ATCC 7002	<i>S. thyphimurium</i> ATCC 14074	<i>S. marcescens</i> ATCC 27117	<i>B. cereus</i> ATCC 6633	<i>S. epidermidis</i> wt.	<i>S. aureus</i> ATCC 29213	<i>E. faecalis</i> ATCC 29212	<i>M. luteus</i> ATCC 7468
PC-I	1,6	0.025	0,8	0,8	0,8	1,6	-	1,6	02	0,8	04	0,8	04	0,8	04
PC-II	1,6	0.025	04	04	0,8	0,8	-	0,8	0,8	0,8	0,8	04	04	04	04
Eucalyptus	1,6	01	1,6	0,8	1,6	0,8	-	1,6	08	02	0,8	0,8	0,8	1,6	0,8
Bergamot	0,8	0.025	-	0,8	-	0,8	-	-	-	-	0,8	0,8	0,8	1,6	0,8
Cedrus	-	0.025	-	1,6	-	-	-	-	-	-	1,6	-	-	-	-
Lavender	1,6	01	-	0,8	-	04	1,6	-	-	02	04	0,8	04	04	04
Orange	1,6	0.025	-	0,8	-	0,8	-	-	-	-	04	0,8	02	-	0,8
Mint	1,6	005	-	1,6	1,6	0,8	-	1,6	-	02	0,8	0,8	04	-	0,8
Tea Tree	0,8	005	0,8	04	1,6	0,8	-	08	1,6	04	0,8	0,8	04	08	04
Thyme	-	005	04	04	0,8	0,8	-	1,6	1,6	04	04	0,8	04	08	04
Lemon	-	0.025	-	04	04	1,6	-	-	-	-	1,6	04	02	0,8	0,8
Clove	0,8	005	1,6	0,8	1,6	0,8	-	1,6	0,8	02	0,8	1,6	0,8	1,6	0,8
Rosemary	0,8	01	02	04	1,6	04	16	-	-	-	08	02	0,8	0,8	0,8
Gentamycin	N.D	N.D	01	004	016	016	12	008	12	03	03	01	0.025	008	0.025

N.D: Not detected.

Inhibition of quorum sensing (QS) formation in *C. violaceum* 12472

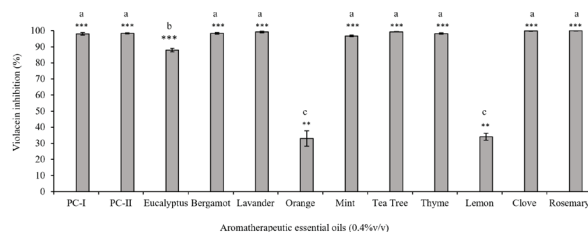
In this study, QS inhibition of *C. violaceum* 12472, a biosensor strain, by AEOs was assessed qualitatively using the agar-well diffusion method shown in Figure 1. and Table 2. The synthesis of purple pigment violacein by the strain was comprised by QS. The indicators of QS inhibition were loss of its purple pigmentation and the formation of opaque halos around the wells including the AEOs. All AEOs, except cedrus AEO, exhibited a colorless, opaque zone of different diameters, which inferred that they displayed detectable anti-QS effects at the sub-MIC concentration of 0.4%v/v (Figure 1). The QS inhibitory diameters of AEOs on violacein production varied from 10.00±1.00 mm to 40.33±1.15mm (Table 2). In this study, the qualitative QS results of AEOs were found on all tested AEOs, except cedrus AEO, which displayed significant QS inhibition zones on *C. violaceum* 12472 in the agar well diffusion assay

Quantitative QS inhibition of *C. violaceum* 12472 was also measured spectrophotometrically using AEOs at the sub-MIC concentration of 0.4% v/v. Excluding cedrus AEO, all the AEOs showed significant (p<0.01 and p<0.001) inhibitory effect on violacein production without inhibition of bacterial growth (Figure 2). Anti-QS effects of the AEOs ranged from 33.01±4.70% to 99.96±0.02%. The inhibition of violacein by orange (33.01%±4.70) and lemon (34.10%±2.20) AEOs was significant (p<0.01) but less than the others. Eucalyptus AEO exhibited a remarkable anti-QS effect with the value of 87.96%±1.13 (p<0.01), which was much better than the anti-QS potentials of orange and lemon AEOs (Figure 2). The AEOs with high QS inhibitory effects were PC-I (98.10%±0.77), PC-II (98.34%±0.42), bergamot (98.37%±0.60), thyme (98.28%±0.47), lavender (99.20%±0.47), mint (96.77%±0.46), tea tree (99.38%±0.17), clove (99.86%±0.03) and rosemary (99.96%±0.02) AEOs (Figure 2).

Table 2. Qualitative QS inhibition of the aromatherapeutic essential oils (AEOs) by agar well diffusion method.

Aromatherapeutic essential oils (0.4 v/v)	Violacein Inhibition Zones (mm)
PC-I	16.67±1.15 ^a
PC-II	22.00±2.00 ^b
Eucalyptus	15.33±1.15 ^a
Bergamot	20.50±1.50 ^{a,b}
Cedrus	-
Lavender	22.50±1.00 ^{a,b}
Orange	10.00±1.00 ^c
Mint	21.33±1.53 ^{a,b}
Tea Tree	32.33±2.52 ^d
Thyme	40.33±1.15 ^e
Lemon	10.67±1.15 ^c
Clove	23.00±2.65 ^b
Rosemary	20.00±2.00 ^{a,b}

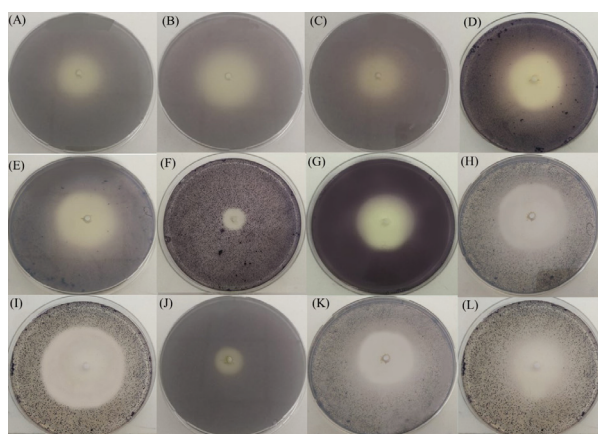
The values of violacein inhibition zone (mm) represent averages ± standard deviations (SD) for triplicate experiments. Values in the same column with different superscripts are significantly different (p<0.05).

**Figure 2.** Quantitative analysis of violacein pigment inhibition of *Chromobacterium violaceum* CV12472 treated with the aromatherapeutic essential oils (AEOs) and combinations (PC-I and PC-II). Bars represent the mean of three independent experiments±SD (ANOVA). **Statistically different from the control (**p<0.01, ***p<0.001). Values with different superscripts are significantly different from each AEO (p<0.05).

Torres, & Ortiz, 2020; Husain et al., 2015; Mokhetho et al., 2018; Raut & Karuppaiyl, 2014). Moreover, anti-QS effect of eucalyptus AEO (87.96%±1.13) was in line with an earlier report (Luís, Duarte, Gominho, Domingues, & Duarte, 2016) while the violacein inhibitions of orange (33.01%±4.70) and lemon (34.10%±2.20) AEOs were more effective than previous reports (Kerekes et al., 2013; Mukherji & Prabhune, 2014). Although there are some reports about AEOs with anti-QS activity (Husain et al., 2015; Mokhetho et al., 2018; Alibi et al., 2020; Cáceres et al., 2020), our results also reveal that AEOs with high anti-QS effects (96.77%-99.96%) could be used as promising anti-QS compounds.

Inhibition effect of AEOs on swarming and swimming motility and biofilm formation of *P. aeruginosa* PAO1

P. aeruginosa PAO1 is a swarming opportunistic Gram-negative pathogen that mostly causes nosocomial infections by forming permanent biofilms. To create an efficient infection, it synthesizes many virulence factors like biofilm formation, swarming and swimming motility via the quorum sensing (QS) process (Ilic-Tomić et al., 2017; Önem, Tüzün, & Akkoç, 2021). In the present study, the inhibition effect of AEOs and their pharmaceutical combinations (PC-I and PC-II) on motility ability and biofilm formation of *P. aeruginosa* PAO1 was tested at the sub-MIC concentration (0.0125% v/v). The motility inhibition results showed that all AEOs significantly (p<0.05) blocked the swarming and swimming motility of PAO1 in the inhibition rate value of 40.34%-72.80% and 20.06%-50.08%, respectively. The swimming and swarming inhibition effects of each essential oil were mostly higher than the combinations, PC-I and PC-II (Table 3). Lavender and thyme AEOs presented the best inhibition on the motility ability of PAO1 with the lowest swarming (15.84±1.20 mm) and swimming zones (37.23±1.24 mm), respectively. In the swarming inhibition activity, after lavender AEO, eucalyptus and orange AEOs also exhibited high effects with the inhibition values of 68.68% and 70.05%, respectively (Table 3). Previous studies showed that *E. globulus* essential oil (EO) at 100 µg/ml, *R. officinalis* EO at 0.02% (v/v) and *M. piperita* EO at 3% (v/v) reduced the swarming motility of PAO1 in the inhibition values of 25%, 61.53% and 81.3%, respectively (Bai A & Vittal, 2014; Husain et al., 2015; Merghni et al., 2018). Compared to these studies, in our results, each EO and their combinations at low concentration (0.0125% v/v) presented remarkable anti-swarming activity (Table 3). Furthermore, a review study showed that *Thymus vulgaris*, *Lavandula angustifolia*, and

**Figure 1.** Violacein inhibition of *Chromobacterium violaceum* ATCC 12472 by AEOs. The opaque zones with loss of purple pigmentation around the wells show violacein inhibition (A)PC-I, (B) PC-II, (C) Eucalyptus EO, (D) Bergamot EO, (E) Lavender EO, (F) Orange EO, (G) Mint EO, (H) Tea tree EO, (I) Thyme EO, (J) Lemon EO, (K) Clove EO, (L) Rosemary EO.

In quantitative results, the combinations and individual EOs bergamot, lavender, mint, tea tree, thyme, clove and rosemary were found to have highly active QS inhibition (>95%), but showed no statistical difference between each other and the AEO combinations (p>0.05). These findings demonstrated that since QS inhibition of AEO combinations was not stronger than individual AEOs, the QS inhibition potentials of AEOs are mostly associated with their major constituents such as menthol, thymol, carvacrol, eugenol, geraniol and geranial. Therefore, our results are in agreement with previous reports (Cáceres, Hidalgo, Stashenko,

Table 3. Inhibition effect of AEOs (0.0125% v/v) on swimming and swarming motilities of *Pseudomonas aeruginosa* PAO1.

Aromatherapeutic Essential Oils	Swarming Zone Diameter (mm)	Swarming inhibition (%)	Cell Viability (Log CFU/ml)	Swimming Zone Diameter (mm)	Swimming inhibition (%)	Cell Viability (Log CFU/ml)
PC-I	33.15±0.23	43.09	6.28	55.18±1.17	24.67	7.02
PC-II	28.48±1.30	51.10	6.32	53.24±1.26	27.32	6.98
Eucalyptus	18.17±1.65	68.80	6.42	60.02±1.30	20.06	6.97
Bergamot	26.33±0.45	54.79	6.27	43.18±1.06	25.21	6.86
Cedrus	27.87±1.35	52.15	6.23	50.07±1.20	28.81	7.12
Lavender	15.84±1.20	72.80	6.28	45.19±1.00	38.31	6.93
Orange	17.56±0.50	70.05	6.24	51.27±0.69	30.01	6.91
Mint	30.06±1.25	50.09	6.51	50.02±0.55	32.88	6.88
Tea Tree	34.75±0.50	40.34	6.53	54.71±0.75	25.32	6.97
Thyme	27.38±1.23	53.00	6.17	37.23±1.24	50.08	6.89
Lemon	20.34±1.38	66.79	6.78	51.46±0.25	30.05	7.10
Clove	23.18±1.38	60.20	6.18	46.81±1.02	36.10	7.16
Rosemary	32.21±1.84	55.29	6.37	46.28±0.84	36.82	7.24
PAO1	60.12±1.24	0	6.72	59.12±1.10	0	7.10

Values in the same column with different superscripts are significantly different ($p < 0.05$).

clove EOs inhibited the swarming and swimming motility of PAO1 or different strains (D. Zhang et al., 2020) (34). Our results are in agreement with these results, indicating significant reduction of the motilities (Bai A & Vittal, 2014; Husain et al., 2015; Mergagni et al., 2018; Zhang et al., 2020).

In the results of PAO1 antibiofilm activity, all AEOs (0.0125% v/v) showed a statistically significant ($p < 0.05$) inhibition in the range of 38.11% to 77.36%. Eucalyptus, orange and lemon AEOs also showed the best activity on the biofilm formation of PAO1 (Table 4). PC-I and PC-II, lavender, thyme and clove AEOs also exhibited noteworthy antibiofilm effects on PAO1. Husain et al. (2015) showed that *M. piperita* EO at 0.375% (v/v) inhibited biofilm formation by 42.8% (23). Our data is considered to be more promising than their findings (Table 4). Additionally, spice and clove EOs were also reported to have a significant antibiofilm effect on PAO1 strain (Eris & Ulusoy, 2013; D. Zhang et al., 2020). These findings are in line with our results.

Antibiofilm effects of AEOs and their pharmaceutical combinations on human pathogens

In the antibiofilm results of Gram-negative bacteria, PC-I exhibited the most powerful antibiofilm effect on *E. coli*, *P. aeruginosa*, *A. baumannii*, *K. oxytoca* and *P. mirabilis*, which were found to be as effective as amoxicillin (Amx). No statistically significant difference was observed among the means of antibiotic and bacteria ($p > 0.05$). The antibiofilm effect of thyme AEO was also as powerful as Amp for *E. coli* ($p > 0.05$) (Table 4). Antibiofilm activity of lemon, clove and rosemary AEOs also exhibited a remarkable inhibition on *P. aeruginosa* ($p < 0.01$) (Table 4). As for the Gram-negative bacteria, PC-I presented the best biofilm inhibition on *S. aureus* and *M. luteus*. Compared to Amp, PC-I showed significantly higher ($p < 0.001$) antibiofilm effect on *S.*

aureus, and as powerful as Amp on *M. luteus* ($p > 0.05$). Moreover, PC-I significantly ($p < 0.05$) decreased the biofilm formation of *S. epidermidis* wt and *E. faecalis* ATCC 29212. While PC-I exhibited high biofilm inhibition (max. 80.09%) on the majority of the Gram-positive bacteria, PC-II inhibited biofilm formation up to 50.70% (Table 5). One of the major and most commonly used AEO, thyme, also exhibited a significant ($p < 0.05$) antibiofilm effect on *S. epidermidis* wt. Actually, its biofilm inhibitions on *S. epidermidis* wt. and *S. aureus* were as powerful as Amp ($p > 0.05$) (Table 5). Furthermore, the antibiofilm effect of eucalyptus, bergamot, cedrus, lavender and orange AEOs were also found to be as powerful as Amp ($p > 0.05$) on *S. aureus* (Table 5).

In the antibiofilm results of all human pathogens, PC-I containing six aromatherapeutic EOs, thyme+bergamot+lemon+teatree+lavender+mint (1:2:4:5:5:5), exhibited excellent antibiofilm effect on *S. aureus*, *M. luteus*, *P. aeruginosa*, *A. baumannii*, *S. typhimurium*, *K. oxytoca*, *P. mirabilis* and *S. marcescens* than the individual effect of each oil in the combination, signifying its synergistic activity. PC-II also displayed synergistic antibiofilm effect on *S. marcescens* (Table 4 and Table 5). These results confirm previous reports indicating the significant increase of biological activities by the combinations of essential oils (Yap et al., 2014; Vieira et al., 2017). Additionally, the antibiofilm effects of PC-I on *M. luteus*, *P. aeruginosa*, *E. coli*, *K. oxytoca* and *P. mirabilis* were found to be as powerful as amoxicillin (Amx) and, its activity on *S. aureus* was more powerful than Amp. These findings are compatible with an earlier study (Kavanaugh & Ribbeck, 2012) in which cassia, Peru balsam, and red thyme EOs eradicated *Pseudomonas* sp. and *S. aureus* biofilms with higher efficiency than selected antibiotics.

On the other hand, PC-I exhibited no significantly different antibiofilm effect ($p > 0.05$) from thyme and/or lemon EOs on *E. coli*.

Table 4. Antibiofilm activity of aromatherapeutic essential oils against some pathogenic Gram negative bacteria tested with micro-dilution assay .

AEOs / Biofilm inhibition (%)	<i>P. aeruginosa</i> ATCC 27853	<i>P. aeruginosa</i> PAO1	<i>A. baumannii</i> ATCC 19606	<i>K. pneumoniae</i> ATCC 700603	<i>S. thyphimurium</i> ATCC 14074	<i>K.oxytoca</i> ATCC 43165	<i>P. mirabilis</i> ATCC 7002	<i>S. marcescens</i> ATCC 27117	<i>E. coli</i> ATCC 25922
PC-I	75.08±4.03 ^{a,g}	61.08±0.03	61.38±5.35 ^a	50.36±9.30 ^{a,c}	47.25±1.30 ^a	63.07±1.15 ^a	66.64±4.16 ^{a,h}	57.37±4.00 ^a	58.73±7.12 ^{a,c}
PC-II	50.51±2.24 ^b	60.52±0.10	25.08±0.61 ^b	15.68±4.10 ^b	18.20±6.03 ^b	23.08±2.80 ^{b,e}	55.66±9.11 ^{b,e}	57.78±2.43 ^a	-
Eucalyptus	41.00±1.02 ^c	74.20±0.07	30.32±5.21 ^{b,c}	15.28±1.60 ^b	16.58±3.72 ^{b,c}	35.13±7.40 ^{c,g}	51.00±2.00 ^{a,f}	22.08±4.02 ^b	-
Bergamot	22.05±4.05 ^d	38.11±0.01	22.26±0.30 ^b	16.22±0.56 ^b	11.25±0.28 ^c	4.36±0.20 ^d	21.54±2.11 ^{c,d}	8.00±0.80 ^c	-
Cedrus	20.78±5.45 ^d	55.76±0.10	22.25±0.30 ^b	16.20±0.50 ^b	11.28±0.30 ^c	15.73±5.45 ^e	15.11±0.80 ^{c,d}	11.60±0.33 ^{c,d}	-
Lavander	21.80±2.44 ^d	65.50±0.04	33.00±7.04 ^{b,c}	40.00±6.18 ^a	4.70±1.04 ^d	30.73±7.40 ^{b,c}	63.24±6.40 ^{a,e}	23.55±1.60 ^{b,e}	-
Orange	22.00±1.60 ^d	77.36±0.02	23.30±3.20 ^b	23.57±8.70 ^b	40.02±0.02 ^e	6.64±0.40 ^d	20.50±1.30 ^{c,d}	17.17±5.14 ^{b,d}	-
Mint	23.28±4.22 ^d	46.70±0.01	23.00±0.65 ^b	16.44±0.56 ^b	17.83±4.02 ^{b,c}	28.40±2.42 ^{b,c}	51.61±1.22 ^b	30.34±2.43 ^e	-
Tea Tree	20.80±3.00 ^d	58.10±0.03	28.02±7.06 ^b	20.03±6.15 ^b	11.32±0.66 ^b	33.00±1.50 ^{c,f}	55.66±2.60 ^{b,e}	38.41±1.11 ^f	-
Thyme	67.67±2.20 ^e	63.40±0.06	40.00±1.00 ^{c,e}	47.04±1.18 ^{a,c}	3.82±0.04 ^d	41.08±1.71 ^g	41.17±0.33 ^f	40.25±1.30 ^f	55.20±5.11 ^{a,c}
Lemon	63.60±2.10 ^e	73.30±0.10	10.50±0.26 ^d	54.45±0.60 ^c	1.60±0.03 ^d	25.54±1.45 ^{b,c}	27.17±0.04 ^c	18.53±0.91 ^{b,d}	48.53±5.71 ^{a,b}
Clove	60.17±3.75 ^e	60.36±0.01	18.35±0.06 ^d	43.17±1.00 ^{a,c}	46.12±0.50 ^{a,e}	36.80±1.82 ^g	35.75±0.78 ^g	40.76±0.70 ^f	44.68±0.52 ^b
Rosemary	56.68±0.57 ^b	45.31±0.02	45.68±1.45 ^e	26.63±1.17 ^b	-	33.25±1.44 ^{b,c}	15.86±0.52 ^d	20.80±0.50 ^b	50.02±6.54 ^{a,b}
Ampicillin (Amp)	80.25±0.32 ^g	N.D.	75.85±0.10 ^f	71.42±0.20 ^d	75.26±0.08 ^f	63.85±0.80 ^a	74.64±0.30 ^h	68.07±0.31 ^g	60.87±0.67 ^c

Biofilm inhibition of the aromatherapeutic essential oils (AEOs) and combinations (PC-I and PC-II) at the sub-MIC concentration of 0.1% v/v and 0.0125% v/v against Gram-negative human pathogens and PAO1 as a biosensor strain, respectively. Amoxicillin at 2 µg/ml was used as a positive control. The values of biofilm inhibition (%) represent averages ± standard deviations (SD) for triplicate experiments. Values in the same column with different superscripts are significantly different (p < 0.05). ND: Not detected.

Table 5. Antibiofilm activity of aromatherapeutic essential oils against some pathogenic Gram positive bacteria tested with micro-dilution assay.

AEOs / Biofilm inhibition (%)	<i>B. cereus</i> ATCC 6633	<i>S. epidermitis</i> wt	<i>S. aureus</i> ATCC 29213	<i>E. faecalis</i> ATCC 29212	<i>M. luteus</i> ATCC 7468
PC-I	-	46.84±6.04 ^a	80.09±3.00 ^a	26.64±0.31 ^a	78.00±4.07 ^a
PC-II	-	21.40±5.06 ^b	50.70±0.50 ^b	24.34±4.12 ^a	31.23±5.42 ^b
Eucalyptus	-	20.28±4.28 ^b	55.11±7.74 ^{c,e}	24.60±4.20 ^a	31.70±1.33 ^b
Bergamot	-	20.03±4.25 ^b	55.38±6.41 ^{c,e}	20.01±1.77 ^a	53.37±0.70 ^c
Cedrus	-	17.83±5.62 ^b	55.22±0.55 ^{c,e}	21.11±0.70 ^a	10.44±2.80 ^d
Lavander	-	27.72±5.07 ^b	61.30±5.84 ^{c,e}	35.58±3.30 ^b	64.72±2.04 ^e
Orange	-	23.40±7.22 ^b	57.53±7.48 ^{c,e}	12.73±2.05 ^c	51.48±1.45 ^c
Mint	-	18.00±2.80 ^b	52.70±1.00 ^c	20.47±2.15 ^a	57.55±0.52 ^c
Tea Tree	-	26.62±5.57 ^b	40.32±3.18 ^d	36.08±1.01 ^b	30.23±1.00 ^b
Thyme	33.11±1.70 ^a	70.74±0.07 ^{c,e}	58.55±1.30 ^{c,e}	50.37±2.00 ^d	65.36±0.53 ^e
Lemon	15.04±1.60 ^b	62.36±1.21 ^{c,d}	35.56±0.65 ^d	48.34±1.26 ^d	57.52±0.50 ^c
Clove	12.08±3.44 ^b	61.24±0.72 ^{c,d}	33.50±2.57 ^d	11.50±1.60 ^c	57.37±0.42 ^c
Rosemary	11.50±2.06 ^b	56.83±1.54 ^{a,d}	51.03±0.80 ^{b,c}	2.67±0.04 ^e	53.22±0.56 ^c
Ampicillin	60.76±0.72 ^c	80.04±0.32 ^e	67.00±1.09 ^e	66.02±0.75 ^f	77.33±0.31 ^a

Biofilm inhibition of the aromatherapeutic essential oils (AEOs) and combinations (PC-I and PC-II) at the sub-MIC concentration of 0.1% (v/v) against Gram-positive human pathogens. Amoxicillin at 2 µg/ml was used as a positive control. The values of biofilm inhibition (%) represent averages ± standard deviations (SD) for triplicate experiments. Values in the same column with different superscripts are significantly different (p<0.05).

Also, its inhibitory effect on *K. pneumoniae* was not higher than each EO, signifying an indifferent effect defined as the absence of interaction between Eos (Table 5). Unlike PC-I, PC-II including thyme and tea tree Eos (1:1) displayed an antagonistic antibiofilm effect on *E. faecalis* and *K. oxytoca*; a lower antibiofilm effect of PC-II than each oil was observed. It also exhibited an indifferent antibiofilm effect on *S. typhimurium* and *P. mirabilis* (Table 4 and Table 5). These findings showed that thyme EO displayed a better effect on biofilm formation of many pathogens than PC-II and, the antibiofilm effect of tea tree EO on most of the pathogens was no different from PC-II. These results confirmed the study (Oh et al., 2017), where single essential oil also had a better result on antibiofilm formation than blended essential oil.

Our findings also revealed that PC-I exhibited remarkable synergistic effect on most of Gram-negative and some Gram-positive human pathogens; however, PC-II displayed indifferent and antagonistic effects on most of the pathogens. The antibiofilm actions of EOs combinations could be related to the content of EOs, interaction between EOs in the mixture, type of pathogen, or evaluation methods of biofilm inhibition. Thus, these findings are also in line with previous reports where various interactions of EOs were explained (Yap et al., 2014; Luís et al., 2016; Tariq et al., 2019).

Aromatherapeutic EOs with the advantages of possessing low mammalian toxicity, relative accessibility, and quick degradation in water and soil are used in the medicinal industry. EOs obtained from plants belonging to the families, especially, Lamiaceae, Myrtaceae and Rutaceae are known to have important potentials in terms of medicinal practices (Kavanaugh & Ribbeck, 2012; Raut & Karuppaiyil, 2014). In our study,

cedrus EO, from the family Pinaceae, at sub-MIC of 0.4% (v/v) displayed a low antibiofilm effect on most of the pathogens; nevertheless, thyme EO, except on *S. typhimurium*, Lamiaceae, and also clove EO, Myrtaceae, at 0.4% (v/v) exhibited higher antibiofilm inhibition on most of the pathogens (Table 1-2). These results are mostly in harmony with a previous study (Alibi et al., 2020) in which thyme and clove EO, at sub-inhibitory concentrations, showed remarkable antibiofilm effect on all the tested multidrug-resistant clinical strains (1). In another research, it was found that EOs, also derived from thyme, orange and rosemary, significantly (p<0.05) inhibited the biofilm formation of *S. epidermidis* ATCC 12228, *E. coli* O33 and O157:H7 strains (Cáceres et al., 2020). Our results also exhibited that thyme and rosemary EOs hampered the biofilm formations of *S. epidermidis* wt and *E. coli* ATCC 25922 significantly (p<0.05); however, orange EO did not inhibit the biofilm formation of *E. coli* strain. This discrepancy might have occurred due to the methods used to obtain the EOs and different strains, changeable experimental conditions and the variable volatile content of EOs.

The current studies also indicated that mint, tea tree, lavender, lemon, eucalyptus, and rosemary EOs prevented the biofilm formation of different clinical and/or standard strains of methicillin-resistant *S. aureus* (MRSA), *B. cereus*, *P. aeruginosa*, *P. putida*, *S. aureus*, *E. coli* and mixed-culture biofilms, which were compatible with our antibiofilm results revealing higher antibiofilm potentials of many EOs on especially *S. aureus* 29212 and *P. aeruginosa* 27853 (Kerekes et al., 2013; Vieira et al., 2017; Merghni et al., 2018). Also, in our results, the biofilm formation of *E. coli* and *B. cereus* strains was only hampered by thyme, lemon, clove and rosemary EOs. It was reported that biofilm inhibitory differences between the strains indicate species-

specific activity of the oils and the specific mechanisms of resistance to the oils might be at work (Kavanaugh & Ribbeck, 2012). For instance, certain EOs can work on the bacterial cell wall or cell membrane. Therefore, the composition of these cellular components could be key to specifying susceptibility to EOs.

Essential oils are an excellent alternative to use as antibiotics against resistant strains of bacteria. Most antibiotics on the market, which are based on inhibiting growth and killing bacteria, are out of use due to the development of microbial resistance and there is a need for alternatives that can be used instead (Chatterjee & Vittal, 2021; Hong, Wang, Chen, & Zhu, 2021). In this context, in order to include essential oils in therapeutic treatments, they should be included in various cell culture studies and the results should be evaluated. In the study by Alibi et al. to determine the cytotoxicity of EOs in the Vero cell line, essential oils were found to have a higher affinity for the bacterial species evaluated (Alibi et al., 2020). Most of the resistance and virulence traits in bacteria occur through quorum sensing mechanisms involving bacterial cell-cell communication. Therefore, breaking the quorum sensing system would be a good strategy. Overall, the high antibiofilm and anti-QS activities detected for essential oils position them as promising natural products for the development of new and better therapeutic strategies for emerging clinical problems (Saeki, Kobayashi, & Nakazato, 2020; Boudiba et al., 2021). Consequently, essential oils can be used as an alternative to synthetic antioxidants, as natural products may be more compatible with living systems and safer than synthetic ones. Clinical trials are needed to confirm the place of EOs in clinical medicine.

CONCLUSIONS

This is the first detailed study that confirms the anti-quorum sensing and antibiofilm potentials of essential oils and their pharmaceutical combinations applied in aromatherapy. The research results clearly demonstrate that all aromatherapeutic essential oils (AEOs), especially PC-I and thyme AEOs, hamper the biofilm formation of most of the pathogens, particularly on *P. aeruginosa* and *S. aureus* in relation with respiratory infections. All AEOs, especially thyme, lavender, eucalyptus and orange AEOs, also significantly ($p < 0.05$) inhibited the swarming and swimming motility of PAO1 strain, which could be considered as antipseudomonad agents. PC-I, PC-II, bergamot, lavender, mint, tea tree, thyme, clove and rosemary AEOs displayed highly active QS inhibition (>95%). This study also proved that each AEO, specifically PC-I and thyme EO, could have a potential to use an alternative therapy for bacterial infections in particular for those caused by biofilm formation, and all AEOs could be a candidate of anti-QS agents. Moreover, the remarkable synergistic action of PC-I, demonstrating more powerful antibiofilm effect than amoxicillin on *S. aureus*, suggests that the combined use of AEOs could enhance their therapeutic actions by eradicating bacterial biofilm. Consequently, new aromatherapeutic formulations should be produced for the cure of especially respiratory infections associated with the bacterial QS and biofilm formation, and the mechanism of actions of QS and bacterial biofilm formation for AEOs are needed to be investigated to discover new complementary and alternative therapies against infectious diseases, moreover, to reduce the tragic effects of antibiotic resistance.

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Chemical constituent of *Isochrysis galbana* microalgae extract and its cytotoxic activities on leukemic cell lines

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ABSTRACT

Background and Aims: This study was aimed to investigate the anti-cancer effect of *Isochrysis galbana* microalgae extract.

Methods: In the study, the chemical composition of *Isochrysis galbana* microalgae extract was analyzed, and its cytotoxic effect against K562, HL60, U937, MOLT-4, and Raji cancer cells was investigated. ECV304 endothelial cells were used as a healthy cell line for the understanding of its selective cytotoxicity. To determine the effects of *Isochrysis galbana* extract, an MTT cytotoxicity assay was performed.

Results: According to the results of the experiments, the highest cytotoxic effect of *Isochrysis galbana* microalgae extract was shown at about 24.07±6.48% cytotoxicity against Raji cells. There were a large number of bioactive molecules in the extract, and these molecules showed a specific response to Raji cells when considering the synergistic and antagonistic effects of these molecules on each other.

Conclusion: According to the results of GC-MS analysis of *Isochrysis galbana* microalgae extract, the most intense molecules in the content were Dodecanoic acid, 3-hydroxy- (CAS) Beta-Hydroxy Dodecanoic Acid, and Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-isonide. The investigation of the effect of these molecules specifically against Raji cells is important to determine the possible anti-leukemic molecules and their combinations that show cytotoxicity against this cell line.

Keywords: Microalgae, Cancer, Cytotoxicity, GC-MS

INTRODUCTION

Algae, which can be unicellular or multicellular, are classified as micro or macroalgae according to their size. There are more than 50,000 species of microalgae, one of the oldest life forms in the world, that can live in fresh and salt water, moist soils, and trees (Kuşoğlu Gültekin, 2020; Richmond, 2003). Different species of microalgae are known to be rich in carbohydrates, proteins, lipids, and molecules with therapeutic properties (Becker, 2003). Due to their special cellular components, microalgae have been used by humans for various purposes for many years.

Microalgae are becoming the focus of attention in drug discovery day by day due to their high biodiversity, ease of production, and the diversity of bioactive molecules they have. It is known that many bioactive molecules obtained from macro and micro-

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algae have cytotoxic effects on cancer cells. However, the fact that a substance has shown cytotoxicity to cancer cells in anti-cancer drug research is not an adequate result. For a substance to have drug potential, it is important that it exhibits selective cytotoxicity.

Isochrysis galbana, a marine flagellated microalgae, belongs to the phylum Haptophyta, class Coccolithophyceae, subclass Prymnesiophycidae, order Isochrysidales, family Isochrysidaceae. Due to its small size (4–7 µm), it can be easily taken into cells by marine animals, and these animals can be added to the food chain by being consumed by other living things (Sadovskaya et al., 2014). *I. galbana* cells have high levels of soluble and insoluble polysaccharides and proteins as well as high amounts of polyunsaturated fatty acids (Sánchez, Martínez, & Espinola, 2000). It has been determined that the carbohydrate components, which make up approximately 13% of the dry weight of *I. galbana*, are a complex mixture of biopolymers containing various proportions of glucose, galactose, mannose, xylose, arabinose, fucose, and rhamnose (Batista, Gouveia, Bandarra, Franco, & Raymundo, 2013). According to the results of the *I. galbana* GC-MS analysis conducted by Hafsa et al. in 2017, *I. galbana* aqueous extract has been reported to contain 56.88% glucose, 38.8% mannitol, and 20.32% inositol (Hafsa et al., 2017).

An abnormality resulting from uncontrollable proliferation in one or more hematopoietic cell lines is called leukemia. There is a balance between differentiation and proliferation in the cell. Disruption of the coordination between differentiation and proliferation, chromosome translocations, mutations of proliferation suppressor genes and accumulation of oncogenes cause the development of leukemia. These mutated genes may be genes that synthesize proteins or growth factors that are active in the cell cycle or that are functional in the intracellular signal transduction pathway. Excessive increase or suppression of the expression of these genes causes the cell to be unbalanced, blocking differentiation and increasing proliferation (Barata et al., 2004).

Acute leukemias occur as a result of cancerization and clonal spread of hematopoietic progenitor cells during their differentiation stages. Some features of leukemia cells are the same as normal cells, but they cannot respond to cytokines that transmit differentiation information. This suppresses differentiation in leukemia cells and causes an increase in proliferation (Karawajew et al., 2000).

The aim of this study was to investigate the anti-cancer effect of *I. galbana* microalgae extract. For this reason, the chemical composition of *I. galbana* microalgae extract was analyzed with a gas chromatography-mass spectrometry, and its cytotoxic effect against chronic myelogenous leukemia cell line (K562), Caucasian promyelocytic leukemia (HL60), Human Caucasian histiocytic lymphoma (U937), Human acute T lymphoblastic leukaemia (MOLT-4), and Human Burkitt's lymphoma (Raji) cancer cells was investigated with an MTT cytotoxicity assay. ECV304 endothelial cells were used as healthy cell line to understand the selective cytotoxicity of the extract.

MATERIAL AND METHODS

Isochrysis galbana culture conditions

Isochrysis galbana was obtained from the University of Texas (USA) Algae Culture Collection. The culture medium recommended by the collection was used for the microalgae strain *I. galbana* (UTEX Collection Culture No #LB987, Prymnesiophyceae, Erdschreiber's Medium). The culture was done in temperature and humidity controlled Erlenmeyer flasks.

Preparation of *Isochrysis galbana* microalgae extract and gas chromatography-mass spectrometry (GC-MS) samples

The *Isochrysis galbana* microalgae culture was incubated in Erdschreiber's Medium for 10 days and their development was followed by daily spectrophotometric measurements. At the end of the 10-day incubation period, the liquid microalgae culture was centrifuged at 8000 rpm for 10 minutes. The resulting microalgae pellet was dried at 60°C overnight at room temperature. 100 mg of dried microalgae samples were weighed, homogenized in 10 mL of methanol with the help of sonication, and then incubated for 1 day at 4°C in the dark with stirring. At the end of the incubation, the sample was centrifuged at 1200 rpm at 4°C for 10 minutes, the supernatant was collected and kept in the dark at -80°C until use. The concentration to be used in the study was determined according to the volume/volume (µg/mL) calculation since the amounts of bioactive components were not known. *I. galbana* GC-MS samples prepared in this way were measured by injecting 2 µL into the sample chamber in the GC-MS device.

Characterization of *Isochrysis galbana* microalgae extract using gas chromatography-mass spectrometry (GC-MS) method

The content of the *I. galbana* microalgae extract prepared using methanol was determined by a Shimadzu brand gas chromatography device (QP5050, NY, USA) carrying an Rtx®-5MS column (30 mx 0.25 mm ID, 0.10 µm film thickness).

Nitrogen gas was used as a carrier gas in the analysis; the average gas flow rate was set as 1 mL/min. The oven temperature program was set from 110 °C (2 min) to 200 °C, increasing by 10 °C per minute, then increasing to 300 °C by 5 °C per minute, and finally, incubation at 300 °C for 9 minutes after analysis. Injector and detector (FID) temperatures were determined as 250 °C and 280 °C, respectively. The MS electron impact mode was set to 70 eV, and the ion source temperature was set to 200 °C. Mass spectrum results were obtained in the scan mode, in the range of m/z 45-450 (Paul, De Nys, & Steinberg, 2006).

Analyzing GC-MS results

The National Institute Standard and Technology (NIST) database (<http://www.nist.gov/srd/nist1a.cfm>), which contains more than 62,000 patterns, was used for mass spectrum analysis. For *I. galbana* microalgae, 3 GC-MS samples were prepared, and the potential peaks (considering the area under the peak) observed during the GC-MS analysis of all 3 samples were automatically compared and characterized with the patterns in the NIST database. The molecular weights, structural properties, and names of the components were verified with informa-

tion from the NIST database. The percentages of the identified molecules in the cell relative to each other were calculated.

Cell lines and culture conditions

In this study, leukemic cell lines K562, HL60, U937, MOLT-4, Raji obtained from the American Type Culture Collection (ATCC), and ECV304 endothelial cell line as healthy cells were used. Leukemia cells were grown in an RPMI medium containing penicillin/streptomycin, L-glutamine, and 10% Fetal Bovine Serum (FBS) while ECV304 endothelial cells were grown in a DMEM medium containing penicillin/streptomycin, L-glutamine, and 10% Fetal Bovine Serum (FBS). Cells were stored frozen in liquid nitrogen at -196°C in an ultra-cold environment.

Cytotoxicity test

An MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) cytotoxicity assay was performed using K562, HL60, U937, MOLT-4, Raji leukemia cells, and ECV304 cells to determine the drug potentials of *I. galbana* microalgae.

K562, HL60, U937, MOLT-4, Raji leukemia cells, and ECV304 cells were counted and prepared for the cytotoxicity experiment at 100,000 cells per mL. Five different concentrations, selected according to preparatory studies, of *I. galbana* microalgae species (500, 50, 10, 5, 1 µg/mL) were placed on microplates, and 10 µL of the medium was added as a control. 90 µL of cells prepared as 100,000 cells per mL were added to these wells. In addition, the culture plates were incubated for 48 h in a humid environment in a 37 °C incubator containing 5% CO₂, and then 10 µL of MTT (5 mg/mL) was added to each well and incubated for 4 hours in the incubator. After incubation, 100 µL of

a mixture of sodium dodecyl sulfate (SDS, pH:5.5) dissolved in 50% isopropyl alcohol was added to the microplate. The prepared SDS mixture broke up the formazan crystals formed by MTT. The resulting color was measured at 570 nm, according to the reference wavelength of 630 nm, in the ELISA spectrometer. Cells that were incubated with only the medium were used as the negative control. For negative control, cells were incubated with 10 mL medium. This experiment was repeated 6 times independently. Cytotoxicity values of microalgae were calculated by comparison with controls (Arslan, Isik, Gur, Ozen, & Catal, 2017; Kaya, Atasever-Arslan, Kalkan, Gür, & Ülküseven, 2016). Six independent repeats were carried out, and the results were evaluated using the GraphPad Prism® 8 program.

RESULTS

The cytotoxic effects of *I. galbana* microalgae extract against HL60, K562, U937, MOLT-4, and Raji cells were investigated using the MTT cytotoxicity test. Non-cancer ECV304 cells were used as controls. According to the experimental results, *I. galbana* microalgae extract showed cytotoxicity below 50% in all cell lines (Figure 1). Therefore, IC₅₀ values of *I. galbana* microalgae were more than 500 µg/mL for these cells. It showed that 500 µg/mL concentration had the highest cytotoxic effect against Raji cells at a rate of 24.07±6.48%. There are many bioactive molecules in the extract and considering the synergistic and antagonistic effects of these molecules with each other, it shows 25% cytotoxicity against Raji cells while the absence of this effect against other cells shows that it creates a specific response to Raji cells.

Table 1. Molecules found over 1% in *Isochrysis galbana* extract as a result of GC-MS analysis.

Molecules	rt*	<i>I. galbana</i> (%)
Dodecanoic acid, 3-hydroxy- (CAS) Beta-Hydroxy Dodecanoic Acid	2.58	24.61
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-isonide	6.14	23.19
Isopropyl dodecanoate	6.57	4.02
Decyl sulfide	6.94	8.43
2,6-Diisopropyl-naphthalene	7.56	1.42
Iron, tricarbonyl [N-(phenyl-2-pyridinylmethylene) benzenamine-N,N']-	7.75	2.12
Decanoic acid, decyl ester	7.83	3.74
Benzoic acid, 2-ethylhexyl ester	7.99	2.81
Tetradecanoic acid, methyl ester (CAS) Methyl myristate	8.15	2.54
Octadecane	9.12	1.02
Tricosane (CAS) n-Tricosane	9.24	1.10
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	10.05	1.68
Hexadecanoic acid, methyl ester (CAS) Methyl palmitate	10.55	4.96
Pentadecanal-	11.68	1.21
Octadecanoic acid, methyl ester	12.77	5.13
1H-Purin-6-amine, [(2-fluorophenyl) methyl]-	15.53	3.09
Eicosanoic Acid, 2,3-Bis[(Trimethylsilyl)Oxy]Propyl Ester (Cas) Trimethylsilylether Derivative Of 1-Monoarachidin	16.37	1.39

*rt, retention time (min)

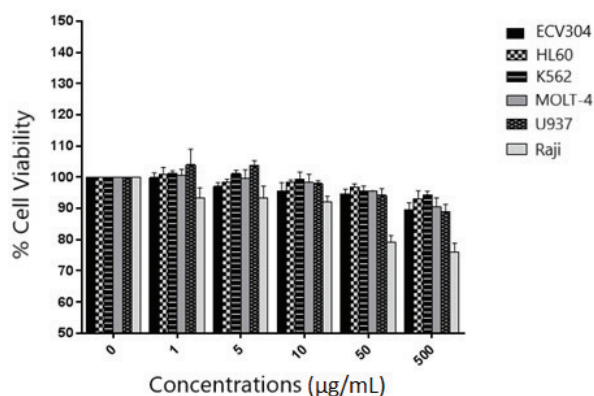


Figure 1. The figure shows the cytotoxic effects of *Isochrysis galbana* microalgae extract against ECV304, HL60, K562, MOLT-4, U937, and Raji cells. The control was accepted as 100% and considered as baseline, then the amount of decrease in cell viability was shown at 1, 5, 10, 50 and 500 µg/mL, respectively.

Therefore, investigation of the molecules that make up the chemical composition of *I. galbana* microalgae extract and their combinations in these cells may lead to the identification of agents with specific anti-leukemic effects against Raji cells.

The extract showed $10.37 \pm 4.79\%$, $7 \pm 5.99\%$, $11.1 \pm 5.48\%$, and $9.45 \pm 6.39\%$ cytotoxicity against ECV304 (control cells), HL60, U937, and MOLT-4 cells, respectively, and $5.44 \pm 2.44\%$ cytotoxicity against K562 cells. It is an important result that shows low cytotoxicity against control cells.

According to the GC-MS analysis results of *I. galbana* microalgae extract, the most concentrated molecules found as Dodecanoic acid, 3-hydroxy- (CAS) Beta-Hydroxy Dodecanoic Acid and Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-isonide (Table 1). The investigation of the effects of these molecules against Raji cells is important in terms of determining the molecules and their combinations showing cytotoxicity against this cell line.

DISCUSSION

In recent years, the search for natural products that can be an alternative to chemicals used in the maintenance of health or the treatment of various diseases has accelerated. Algae are seen as potential natural therapeutic molecule producers for researchers due to their relatively cheap production cost, rapid proliferation under favorable conditions, and being rich in various bioactive molecules. Algae have a very rich content in terms of various bioactive compounds, minerals, polysaccharides, polyunsaturated fatty acids, and vitamins. Due to these properties, it is important to investigate its anti-cancer activity potential and to identify a new anti-cancer agent in cancer research.

Folmer et al. showed that some algal species have an inhibitory effect on the NF- κ B transcription factor (Folmer et al., 2009). Kwon and Nam showed that *Capsosiphon fulvescens* algae inhibit the anti-apoptotic molecule Bcl-2 in gastric cancer cells (Kwon & Nam, 2007). However, the effect of this species on healthy cells has not been demonstrated. In recent years, studies on algae species have shown that various algae species

have hepatic and renal protective activities, antioxidant, anti-tumor, anti-inflammatory, anti-coagulant, and anti-viral activities. However, the effects on healthy cells are still not fully clear (Samarakoon & Jeon, 2012; Gamal-Eldeen, Ahmed, & Abo-Zeid, 2009; Mayer & Gustafson, 2004; Samarakoon & Jeon, 2012). Harada and Kamei found that the algae species they used in their study showed cytotoxic effects against leukemia cell lines in the concentration range of 15-375 µg/mL (Harada & Kamei, 1997). Machana, Weerapreeyakul, Barusru, Thumanu, & Tanthanuch, found that *Polyalthiaevecta* algae species showed anti-cancer effects on HepG2 hepatoma cells (Machana, Weerapreeyakul, Barusru, Thumanu, & Tanthanuch, 2012). However, in this study, a very high concentration of 500 µg/mL was observed, and *Polyalthiaevecta* also showed cytotoxicity against Vero cells used as healthy cells (Ferdous, Norhana, & Yusof). It has been shown that fucoxanthin, one of the tetraterpenoids isolated from *Isochrysis galbana*, has antioxidant activity, and violaxanthin has antioxidant and anti-inflammatory activities (Ferdous, Norhana, & Yusof, 2021). In the study conducted by Matos et al. various biological properties of *I. galbana* such as anti-oxidant, cytotoxicity, and hypocholesterolemic were examined; it has been shown that ethanolic extraction of *I. galbana* had significant cytotoxicity against HeLa human cervical cancer cells, with IC_{50} values of 0.32 and 0.28 mg/mL⁻¹ (Matos et al., 2019). With the increase of *I. galbana* content, cell proliferation decreases. Cell proliferation reached 42.7% and 13.8% at *I. galbana* concentrations of 31.25 and 500 µg/mL, respectively. Cytotoxic activity was evaluated on human HeLa cervical cancer cells. HeLa cell proliferation has been shown to be completely inhibited after treatment with *I. galbana* (1 mg/mL) (Hafsa et al., 2017). In their study, Sadovskaya et al. showed that polysaccharide extracts from *I. galbana* inhibited the proliferation of U937 human leukemic monocyte lymphoma cells (30% at 100 µg/mL) and consequently had potential anti-tumor activity (Sadovskaya et al., 2014). Ho et al. observed a 41% cytotoxic effect in HeLa cells at a concentration of 200 µg/mL. In this study, low cytotoxicity values were found compared to the high concentration (Ho et al., 2007). Bechelli et al. found that the algae species they studied showed cytotoxic effects against both AML cell lines and normal hematopoietic cells at low concentration (Bechelli, Coppage, Rosell, & Liesveld, 2011). In various studies, they detected algae species showing cytotoxicity on leukemia cell lines (Zandi et al., 2010; Ishikawa et al., 2008; Kotake-Nara, Terasaki, & Nagao, 2005).

CONCLUSION

After the data obtained, it was found that there are a wide variety of bioactive molecules in the microalgae extract. According to GC-MS results, Dodecanoic acid, 3-hydroxy- (CAS) Beta-Hydroxy Dodecanoic Acid and Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-isonide are the most abundant molecules in the microalgae extract. Among the cell lines studied, it was determined that *Isochrysis galbana* extract had a high cytotoxic effect especially against Raji cells. For this reason, this study is important in terms of investigating the effects of the mentioned molecules against leukemic Raji cells and determining possible molecules and combinations that have cytotoxicity against this cell line.

Peer-review: Externally peer-reviewed.

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Antioxidant activity and three phenolic compounds from the roots of *Taraxacum gracilens* Dahlst.

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ABSTRACT

Background and Aims: The genus *Taraxacum* is a member of the family Asteraceae. *Taraxacum* species are widely distributed in the warmer temperate zones of the Northern Hemisphere. The aim of this study was to evaluate the phenolic contents and antioxidant activity of the ethanol extract and its chloroform and ethyl acetate fractions from the roots of *Taraxacum gracilens* Dahlst. and isolate some of its phenolic compounds.

Methods: The roots of *T. gracilens* Dahlst. were first macerated with EtOH and fractionated to obtain chloroform and ethyl acetate fractions, and then phenolic compounds were isolated by column chromatography. The polyphenolic contents and antioxidant activities of the fractions were evaluated by measuring their abilities to inhibit lipid peroxidation induced by Fe³⁺-ascorbate, their reducing powers, and their hydrogen-donor activities.

Results: Three phenolic compounds [vanillic acid (**1**), caffeic acid (**2**) and luteolin (**3**)] were isolated from the roots of *T. gracilens* Dahlst. The ethyl acetate fraction from the ethanol extract of *T. gracilens* Dahlst. roots showed the highest antioxidant activity due to its richest phenolic contents, whereas the ethanol extract containing the least phenolics was the weakest in activity.

Conclusion: The fractions have potential to act as antioxidant agents. *T. gracilens* Dahlst. roots were investigated for the first time in literature data in terms of phenolic contents and antioxidant activity.

Keywords: *Taraxacum gracilens* dahlst., Root, Phenolic compounds, Antioxidant activity

INTRODUCTION

Plants of the genus *Taraxacum* F. H. Wigg. are members of the family Asteraceae, Cichorioideae subfamily, Lactuceae tribe. *Taraxacum* species are widely distributed in the warmer temperate zones of the Northern Hemisphere and are cosmopolitan, perennial weeds of the Asteraceae family commonly found in meadows, gardens, roadsides, and uncultivated areas (Schütz, Carle & Schieber, 2006; Martinez et al., 2015). *Taraxacum* species, the name is derived from the Greek words "taraxis" for inflammation and "akeomai" for curative, are known as dandelion (Schütz et al., 2006).

Taraxacum genus has long been used in folk medicine in the form of decoctions and infusions of its roots, leaves, or flowers due to its pharmacological effects as a mild laxative, aperitive, stimulant, tonic, and diuretic (Schütz et al., 2006; Martinez et al., 2015; Stefano, Bruna, Virginia & Giuliano Bonanomi, 2019). Many phytochemicals have been found in the plants of *Taraxacum*

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genus, such as sesquiterpenoids, phytosterols, triterpenoids, flavonoids, phenolic acids, organic acids, inulin, indole alkaloids, coumarins, and lignans (Schütz et al., 2006; Martinez et al., 2015; Hu, 2018).

The aim of this study was to evaluate the phenolic contents and antioxidant activity of the ethanol extract and its chloroform and ethyl acetate fractions from the roots of *T. gracilens* Dahlst. and isolate some of its phenolic compounds.

MATERIAL AND METHODS

Plant material

The plant material was collected from Istanbul in April 2013. A voucher specimen (ISTE 81948) identified by Prof. Dr. Aynur Sari is deposited at the Herbarium of the Faculty of Pharmacy, Istanbul University, Turkey.

Extraction and isolation

The air-dried roots of *Taraxacum gracilens* Dahlst. (540 g) were exhaustively macerated with ethanol (EtOH) at room temperature. After solvent evaporation, the yield of EtOH extract was dissolved in methanol/water (MeOH/H₂O) (1:2), and then successively extracted with chloroform (CHCl₃) and ethyl acetate (AcOEt). The ethyl acetate and chloroform fractions were dried in vacuo yielding 8.0329 g and 8.2133 g, respectively.

The ethyl acetate fraction was separated by silica gel column chromatography using a stepwise gradient of CHCl₃ and MeOH to give 8 fractions. Fraction 1 (0.1511 g) was subjected to preparative thin layer chromatography (prep. TLC) (silica gel; CHCl₃/ MeOH 95:5) and then to column chromatography (Sephadex LH-20; MeOH) to provide pure **1** (4.3 mg). Fraction 7 (0.3847 g) was subjected to column chromatography (Sephadex LH-20; MeOH and Acetone) and then to prep. TLC (silica gel; Toluol/AcOEt/HCOOH 5:4:1) to provide pure **2** (10.9 mg) and **3** (6.9 mg), respectively.

Antioxidant activity

Antioxidant activity of the ethanol extract, ethyl acetate, and chloroform fractions from the ethanol extract of *T. gracilens* roots was assayed by 4 different methods: total phenols assay by Folin-Ciocalteu (FCR) (Slinkard & Singleton, 1977), ferric ion reducing antioxidant power (FRAP) (Benzie & Strain, 1996), DPPH free radical activity (Brand-Williams, Cuvelier, & Berset, 1995), and thiobarbituric acid test using the lipid peroxidation of liposomes (LPO) (Duh, Tu, & Yen, 1999). The antioxidant activities exhibited by the fractions evaluated by these assays reflect the capacity of fractions to act as electrons or hydrogen atom donors, a necessary requirement for antioxidant function in biological systems.

Statistical analysis

Results were expressed as the mean ± the standard deviation of triplicate analysis. Statistical comparisons were performed using the Student's t-test. Differences were considered significant at p<0.05.

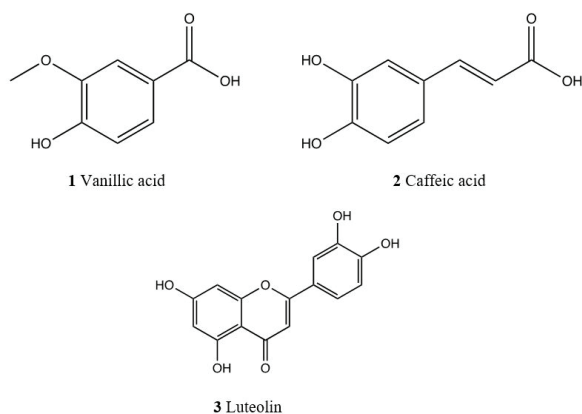
RESULTS AND DISCUSSION

Dandelion extract may be prepared as the whole plant or as different parts, such as roots, leaves, stem, and flowers. The ex-

traction procedure and solvents were properly designed and selected in order for the desired bioactive compounds to be efficiently extracted and the chemical composition of the produced extracts to be identified. The phytochemical research of the *Taraxacum* root extracts reported the presence of phenolic compounds, sesquiterpenes, triterpenes, and phytosterols (Schütz et al., 2006). Among the phenolic compounds, hydroxycinnamic acid derivatives like chlorogenic acid, caffeic acid, ferulic acid, and coumaric acid are generally reported (Williams, Goldstone, & Greenham, 1996; Ivanov, 2014; Kenny, Smyth, Hewage & Brunton, 2014).

Compared to the roots, dandelion leaves and flowers are more enriched in flavonoids (luteolin and its glycoside derivatives, chrysoeriol) and coumarins (cichoriin and aesculin), but hydroxycinnamic acid derivatives (caffeic, chlorogenic, chicoric, and monocaffeoyltartaric acids) were also reported to be present (Williams et al., 1996; Budzianowski 1997).

In this study, phytochemical investigations resulted in the isolation of vanillic acid (**1**), caffeic acid (**2**), and luteolin (**3**) which are new for the species *Taraxacum gracilens* Dahlst. roots. Their structures were established conclusively by ultraviolet visible spectroscopy (UV) and nuclear magnetic resonance (1H-NMR) spectra analyses in comparison with literature data.



All these compounds had already been identified in *T. officinale* and *T. formosanum* root extracts (Leu, Wang, Huang & Shi, 2005; Kenny et al., 2014; Jedrejek, Lis, Rolnik, Stochmal & Olas, 2019). This results are in agreement with a study reported by Kenny, Smyth, Hewage & Brunton (2015) where chlorogenic acid was the most abundant chemical compound in a dandelion root extract, followed by caffeic acid. Also, ferulic, syringic vanillic, coumaric, and gallic acids were identified. Research about the antioxidant properties of these compounds have revealed strong antioxidant activities (Kenny et al., 2014; Kenny et al., 2015). Therefore, the antioxidant activities of the ethyl acetate extract could be explained by the presence of these compounds.

The highest total phenolic level was detected in the ethyl acetate fraction of the *T. gracilens* root ethanol extract. The lowest total phenolic level was detected in the ethanol extract of the root (Table 1). The order of total phenolic content was established as follow: Ethyl Acetate fraction > Chloroform fraction > Ethanol extract (p < 0.05).

Table 1. Total phenolic compounds (PC) (as gallic acid equivalents), total flavonoids (as catechin equivalents), and EC₅₀ values of *T. gracilens* root ethanol extract and the fractions obtained from EtOH extract.

Extract	Anti-LPO EC ₅₀ * (mg/mL)	DPPH EC ₅₀ * (mg/mL)	FRAP** (mM)	Phenol (mg/g extract)	Flavonoid (mg/g extract)
Chloroform fraction	6.36±0.03 ^d	5.04±0.24 ^d	0.76±0.04 ^b	26.14±1.69 ^b	14.33±1.03 ^b
Ethyl acetate fraction	1.28±0.11 ^c	0.83 ±0.14 ^c	3.94±0.13 ^d	122.54±9.28 ^c	110.85±5.59 ^c
Ethanol extract	20.39±0.55 ^e	9.01 ±0.26 ^e	0.49±0.02 ^e	16.85±0.65 ^d	9.33±1.02 ^d
Quercetin	0.059±0.001 ^f	0.034±0.002 ^f	3.24±0.13 ^a	-	-

*EC₅₀ value: The effective concentration at which the anti-LPO activity was 50% and DPPH radicals were scavenged by 50%; EC₅₀ value was obtained by interpolation from linear regression analysis; **Expressed as mM ferrous ions equivalents
Values were the means of three replicates ± standard deviation. Values with different letters in the same column were significantly (p<0.05) different.

From the effective concentrations (EC₅₀) (Table 1), it was seen that among the three fractions selected for the study, the ethyl acetate fraction showed the highest antioxidant activity due to its richest phenolic content. This activity may be contributed to significant ferric reducing power (3.94 mM Fe²⁺/g extract), DPPH radical scavenging activity (EC₅₀=0.83 mg/ml), and inhibition of lipid peroxidation on liposomes (EC₅₀=1.28 mg/ml).

These results are in accordance with the literature data. Kenny et al. (2014) also demonstrated high antioxidant activity in the reducing power and free radical activity assays, which are correlated with a high content of total phenolics of the extracts of *Taraxacum officinale* roots (Kenny et al., 2014).

The vanillic acid and caffeic acid compounds, although mono-nuclear aromatic ones, have *ortho* and *para* OH groups leading to higher activity. The presence of at least one free OH group, attached to either ring A or B, is necessary for the antioxidant activity (Pratt, 1976).

CONCLUSION

The aim of this study was to isolate phenolic compounds from the roots of *T. gracilens* Dahlst. and investigate antioxidant activity of the ethanol extract and the ethyl acetate and chloroform fractions obtained from the ethanol extract of the roots. As a result, luteolin, vanillic acid, and caffeic acid were isolated from *T. gracilens* roots.

It is concluded that the ethyl acetate and chloroform fractions as well as the ethanol extract acted as antioxidant agents i.e. reducing the thiobarbituric acid reactive substances (TBARS) produced from lipid peroxidation and showing the proton and electron-donating ability. So this herbal drug may serve as a dietary source of phenolic substances, which may act as antioxidants for disease prevention and general health promotion through improved nutrition.

T. gracilens Dahlst. roots were investigated for the first time in the literature data in terms of phenolic contents and antioxidant activity.

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In vitro inhibitory potential of *Amaranthus lividus* L. against therapeutic target enzymes

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ABSTRACT

Background and Aims: The search for new enzyme inhibitors in plants is attractive because they can be used as drugs in the treatment of various diseases. *Amaranthus* spp. (Amaranthaceae) includes about 70 different species, some of which are edible and some of which are used in traditional medicine to treat various ailments. *Amaranthus lividus* L. is a vegetable whose stems and leaves are used for human consumption in Turkey.

Methods: In this study, the *in vitro* enzyme inhibition potential of *A. lividus* on α -amylase, α -glucosidase, acetylcholinesterase (AChE), elastase, lipase, neuraminidase and tyrosinase was investigated for the first time. For this purpose, water extract of *A. lividus* was prepared. The tests of enzyme inhibitory activity were carried out by spectrophotometric and fluorometric methods.

Results: The water extract showed significant α -glucosidase and α -amylase inhibitory activities. Also, it displayed good elastase, lipase and tyrosinase inhibitory activities. However, it exhibited low inhibitory activity on AChE and neuraminidase.

Conclusion: The plant and its active constituents may be used as an antidiabetic enzyme inhibitor with future phytochemical constituent analysis.

Keywords: α -Amylase, α -Glucosidase, Acetylcholinesterase, Elastase, Lipase, Tyrosinase

INTRODUCTION

Enzymes continue to be important targets for drug development because altering enzyme activity has led to immediate therapeutic effects (Ramsay & Tipton, 2017). The search for natural enzyme inhibitors in plants is one of many investigations in the discovery and development of new drugs. Many compounds isolated from medicinal plants have the potential to inhibit enzymes and these compounds are used as therapeutic agents (Rauf & Jehan, 2017).

Diabetes mellitus is a chronic disease characterized by high blood glucose levels. Type 2 diabetes mellitus is the most common form of diabetes (more than 90% of all cases). One of the most widely used classes of oral antidiabetic drugs for the treatment of type 2 diabetes mellitus are the α -glucosidase inhibitors (Şöhretoğlu & Sari, 2020). α -Glucosidase (EC 3.2.1.20) is responsible for catalyzing the hydrolysis of disaccharides (maltose and sucrose). One of the other enzymes responsible for catalyzing starch hydrolysis is α -amylase (EC 3.2.1.1). The function of α -amylase is to catalyze the hydrolysis of α -(1 \rightarrow 4)-glycosidic bonds of starch (Papoutsis et al., 2021). α -Glucosidase inhibitors reduce hyperglycemia by delaying glucose absorption and lowering postprandial blood glucose and insulin levels (Şöhretoğlu & Sari, 2020). Miglitol, acarbose, and voglibose are some of the clinically used

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α -glucosidase and α -amylase inhibitors (Papoutsis et al., 2021). There is a need to find new antidiabetic agents as the efficacy of therapy mainly depends on the patients (Şöhretoğlu & Sari, 2020).

Alzheimer's disease is one of the major causes of dementia and is characterized by a progressive neurodegenerative disorder. Cholinergic dysfunction has been closely associated with early cognitive decline in Alzheimer's disease patients. Most of the drugs currently used to treat dementia are acetylcholinesterase (AChE; EC 3.1.1.7) inhibitors, namely donepezil, rivastigmine, and galantamine. AChE inhibitors increase synaptic acetylcholine levels and improve cholinergic function in the brain (Santos, Gomes, Pinto, Camara, & Paes, 2018).

Elastase (EC 3.4.4.7) is responsible for the degradation of elastin and other proteins such as collagen and fibronectin (which are fundamental for the elasticity of the extracellular matrix). Excessive hydrolysis of elastin leads to loss of skin elasticity and subsequently skin aging. Elastase inhibitors are used as skin lighteners, anti-aging and anti-wrinkle agents. They are also used in the treatment of dermatological diseases and to promote the maintenance of skin elasticity (Chiocchio et al., 2018).

Inhibitors of lipase (EC 3.1.1.3) play a critical role in human lipid metabolism. A major cause of obesity is often excessive fat intake/absorption and accumulation. Obesity and hyperlipidemia are associated with a number of risk factors such as insulin resistance, impaired glucose tolerance, and hypertension, which may increase mortality rates. Lipase inhibitors have lipid-lowering effects by reducing the digestion and absorption of lipids from food and controlling the fat that enters the blood. Lipase inhibitors such as orlistat have become important in the treatment of obesity (Liu, Liu, Chen, & Shi, 2020).

Influenza is an infectious disease that leads to thousands of deaths and is caused by the influenza virus (Mahal et al., 2021). Neuraminidase (EC 3.2.1.18) is a membrane-bound glycoprotein, playing an important role in the viral life cycle (Wang et al., 2017). Neuraminidase inhibitors have potency against influenza A and B viruses responsible for the annual influenza epidemics. These inhibitors prevent the cleavage of sialic acid by viral neuraminidase, thereby inhibiting cell interactions between virus-host (Mahal et al., 2021). These inhibitors, including oseltamivir, zanamivir, and peramivir, are the main class of antivirals available for clinical use. However, the development of resistance to neuraminidase inhibitors is a public health concern (Lee & Hurt, 2018).

Melanin is produced by melanogenesis which is a major protective factor against ultraviolet radiation damage and it is the main pigment responsible for the pigmentation of skin, hair and eyes in humans (Zolghadri et al., 2019). However, uncontrolled and excessive melanogenesis may cause skin disorders, including freckles, age spots and hyperpigmentation (Chatatikun, Yamauchi, Yamasaki, Aiba, & Chiabchalard, 2019). Tyrosinase (EC 1.14.18.1) is a multifunctional metalloenzyme that acts as a rate-limiting enzyme in the synthesis of melanin. Therefore, inhibition of tyrosinase activity is essential for

the treatment of pigmentation disorders in mammals (Zolghadri et al., 2019). Tyrosinase inhibitors are used in medical and cosmetic products such as skin lighteners, anti-aging and anti-wrinkle agents, and in the treatment of dermatological diseases (Chiocchio et al., 2018).

Many natural compounds obtained from plants are used in the treatment of various diseases due to their inhibitory effects on various enzymes. Therefore, numerous plants are still being studied to identify effective enzyme inhibitors and to use them in medical and cosmetic products (Zolghadri et al., 2019).

The genus *Amaranthus* (Amaranthaceae) includes 70 different species of annual herbs, some of which are edible (Peter & Gandhi, 2017). Some species of *Amaranthus*, such as *A. cruentus* and *A. hypochondriacus*, are consumed as grains and are highly nutritious (Alegbejo, 2013). Natural crude extracts of *Amaranthus* spp. have been used in traditional medicine to treat various ailments including urinary tract infections, gynecological disorders, diarrhea, pain, respiratory disorders, diabetes and also as a diuretic. Interest in *Amaranthus* (which means "immortal" in Greek) as a nutraceutical and natural protective agent against chronic diseases continues unabated. Many phytochemical studies undertaken to analyse various *Amaranthus* species have shown that they contain active constituents such as alkaloids, flavonoids, glycosides, phenolic acids, steroids, saponins, vitamins, terpenoids and carotenoids (Peter & Gandhi, 2017).

Amaranthus lividus L. (locally known as "dari mançari" in Turkey) is a vegetable whose stems and leaves are used for human consumption (Ozsoy, Yilmaz, Kurt, Can, & Yanardag, 2009). Limited studies conducted using different methods have thus far shown that extracts of *A. lividus* possess antioxidant activity (Al-Mamun et al., 2016; Amornrit & Santiyonont, 2016; Gunathilake & Ranaweera, 2016; Yilmaz-Ozden et al., 2016; Sarker, Oba, & Daramy, 2020). According to our literature search, no study has thus far been conducted on the enzyme inhibitory activity of *A. lividus*. The aim of this study is to search for new/natural α -amylase, α -glucosidase, AChE, elastase, lipase, neuraminidase and tyrosinase inhibitors by measuring *in vitro* enzyme inhibitory potential of *A. lividus*.

MATERIAL AND METHODS

Chemicals

α -Amylase, quercetin, kojic acid were purchased from Fluka Chemical Co. (Buchs, Switzerland). α -Glucosidase, AChE, acetylthiocholine iodide (ATChI), galantamine hydrobromide, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), neuraminidase, N-succinyl-Ala-Ala-Ala-p-anilide (STANA), tyrosinase, ursolic acid, p-nitrophenyl α -D-glucopyranoside (PNPG), 4-methylumbelliferyl- α -DN-acetylneuraminic acid sodium salt hydrate, rutin, lipase, 4-nitrophenyl caprate, orlistat were purchased from Sigma-Aldrich (St. Louis, MO, USA). Elastase was purchased from Calbiochem. Starch were purchased from Merck & Co. (Darmstadt, Germany). All other reagents were of analytical grade.

Plant material

Amaranthus lividus L. were collected from Bartın, Turkey. Voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Istanbul University, Istanbul, Turkey (ISTE 83401).

Preparation of the extract

Plant materials were washed and dried at room temperature. The dried stems, leaves and flowers of *A. lividus* were manually ground to a fine powder. A water extract was prepared by heating the powdered plant material (10 g) with 100 mL distilled water for 30 minutes, stirring constantly. The extract was filtered and evaporated to dryness under reduced pressure at 40°C. The extract was stored at -20°C and dissolved in distilled water before use.

α-Amylase inhibitory activity

The inhibitory activity of *A. lividus* on α-amylase (from hog pancreas) was determined according to the modified method of Al-Dabbas, Kitahara, Suganuma, Hashimoto, & Tadera, (2006). The extract (30 µL) was mixed with 400 µg/mL soluble starch (100 µL) in phosphate buffer (0.25 M, pH 7.0). After 5 min of incubation at 37°C, 50 µg/mL α-amylase solution (10 µL) was added to the mixture, followed by the addition of 10 µL phosphate buffer (0.25 M, pH 7.0). After further incubation of the mixture for 7.5 min, 0.01 N iodine solution (100 µL) and then distilled water (500 µL) were added, followed by measurement of absorbance at 660 nm. Rutin was used as the standard compound. The percentage inhibition of enzyme activity was calculated according to the following formula:

$$\alpha - \text{Amylase inhibitory activity (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100$$

A₀ is the enzyme activity without an inhibitor.

A₁ is the enzyme activity in presence of an inhibitor.

α-Glucosidase inhibitory activity

The inhibitory activity of *A. lividus* on α-glucosidase (from *Saccharomyces cerevisiae*) was determined according to the modified method of Matsui, Yoshimoto, Osajima, Oki, & Osajima, (1996), in which the enzyme solution was adjusted to 32 mU/assay volume of α-glucosidase in a phosphate buffer (50 mM, pH 7.0) containing 100 mM NaCl. For each assay, the extract (10 µL) and the enzyme solution (40 µL) were pre-incubated for 5 min at 37°C. The reaction was started by adding 0.7 mM PNPG (950 µL) in buffer, and the solution was then incubated for 5 min at 37°C. After the reaction was stopped by adding 0.5 M Tris (1.0 mL), the absorbance of p-nitrophenyl released from PNPG was measured spectrophotometrically at 400 nm. Acarbose was used as the standard compound. The percent of enzyme activity inhibition was calculated according to the formula given below:

$$\alpha - \text{Glucosidase inhibitory activity (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100$$

A₀ is the enzyme activity without an inhibitor.

A₁ is the enzyme activity in presence of an inhibitor.

Acetylcholinesterase (AChE) inhibitory activity

The inhibitory activity of *A. lividus* on AChE was determined by the modified method of Ellman, Courtney, Andres Jr, & Featherstone, (1961). The extract (20 µL) and Ellman solution (220 µL; 318 mM DTNB, and 955 mM ATChI in phosphate buffer; pH 7.5) were mixed. Then, 0.5 U/mL AChE solution (10 µL) was added and the absorbance change was monitored at 412 nm for 10 min. A sample without inhibitor was treated the same and used as a negative control. Galantamine was used as a standard substance. The percentage inhibition of enzyme activity was calculated according to the following formula:

AChE inhibitory activity (%) =

$$\left(\frac{\text{Rate of control reaction} - \text{Rate of sample reaction}}{\text{Rate of control reaction}} \right) \times 100$$

Elastase inhibitory activity

Elastase inhibitory activity was determined using STANA as substrate and by measuring the release of p-nitroanilide at 410 nm (James, Timothy, & Gordon, 1996). The extract (50 µL) was preincubated with 0.16 U/mL elastase solution (50 µL) and 900 µL of Tris-HCl buffer (0.2 M, pH 7.8) at 37°C for 15 min before addition of the substrate solution. Then 5 mM STANA solution (50 µL) was added and the mixture was incubated at 37°C for 30 min. The release of p-nitroaniline was measured at 410 nm. Ursolic acid was used as a standard. The percentage inhibition of elastase was calculated using the following formula:

$$\text{Elastase inhibitory activity (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100$$

A₀ is the enzyme activity without an inhibitor.

A₁ is the enzyme activity in presence of an inhibitor.

Lipase inhibitory activity

Lipase inhibitory activity was determined according to the method of Conforti et al. (2012). Pig pancreatic-type II lipase was prepared by dissolving 10 mg/mL of the enzyme in 0.1 M Tris-HCl buffer (pH 8.5), followed by centrifugation at 7000 g, for 10 min, and the collected supernatants were stored at -20°C until use (Lehner & Verger, 1997). Lipase activity was determined by measuring the hydrolysis of 4-nitrophenyl caprate to 4-nitrophenol. The reaction solution containing the extract (5 µL), 10 mg/mL lipase (10 µL), and 200 µL of Tris-HCl buffer (0.1 M, pH 8.5) was incubated at 37°C for 25 min, after which 5 mM 4-nitrophenyl caprate (5 µL) was added. The absorbance was read at 405 nm after incubation at 37°C for 15 min using a microplate reader. Orlistat was used as the standard inhibitor of lipase in this study. Percent lipase inhibition was calculated as follows:

$$\text{Lipase inhibitory activity (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100$$

A₀ is the enzyme activity without an inhibitor.

A₁ is the enzyme activity in presence of an inhibitor.

Neuraminidase inhibitory activity

The inhibitory activity of *A. lividus* on neuraminidase (from *Clostridium perfringens*) was determined according to the modified method of Myers et al. (1980). The reaction was carried out with 2.5×10^{-3} U/mL the enzyme solution (10 μ L), 360 μ L of acetate buffer (pH 5.0), the extract (10 μ L) and 4-methylumbelliferyl- α -DN-acetylneuraminic acid sodium salt hydrate (20 μ L). The mixture were incubated for 10 min at 37°C. After the reaction was stopped by adding 3.5 mL of glycine-NaOH buffer (pH 10.4), the fluorescence of the reactions was measured spectrofluorometrically. The emission wavelength was 440 nm, while the excitation wavelength was 360 nm. Quercetin was used as the standard compound. The percentage of inhibition was determined by the following equation:

Neuraminidase inhibitory activity (%) =

$$\left(\frac{\text{Rate of control reaction} - \text{Rate of sample reaction}}{\text{Rate of control reaction}} \right) \times 100$$

Tyrosinase inhibitory activity

Tyrosinase inhibitory activity was determined spectrophotometrically (Vanni et al., 1990). Assay reaction mixtures were prepared by adding 40 U mushroom tyrosinase (20 μ L), the extract (20 μ L), 1.5 mM L-tyrosine (40 μ L) and 220 μ L of sodium phosphate buffer (0.1 M, pH 6.5). The resulting mixture was incubated for 10 min at 37°C and absorbance value was measured at 475 nm. Kojic acid was used as the standard compound. The percent inhibition of tyrosinase was determined according to the following equation:

$$\text{Tyrosinase inhibitory activity (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100$$

A_0 is the enzyme activity without an inhibitor.

A_1 is the enzyme activity in presence of an inhibitor.

RESULTS AND DISCUSSION

α -Amylase inhibitory activity

One of the approaches to treating type 2 diabetes is to reduce the rate of blood glucose absorption from the small intestine by slowing the digestion of starch. α -Amylases are hydrolytic enzymes that are secreted in the mouth and small intestine. The function of α -amylases is to catalyze the hydrolysis

of starch and lead to the production of maltose, maltotriose, maltotetraose, maltodextrins and glucose (Papoutsis et al., 2021). Inhibition of α -amylase activity helps to maintain circulating glucose at normal levels. In this study, α -amylase inhibitory activity of *A. lividus* was evaluated and rutin was used as a standard inhibitor. The results were expressed as IC_{50} value (the extract/standard concentration that inhibits enzyme activity by 50%) (Table 1). Low IC_{50} values indicate higher inhibitory potential of the tested samples. The water extract showed high α -amylase inhibitory activity compared to rutin. Rutin showed about 55% enzyme inhibition at 2.5 mg/mL, while the extract produced the same inhibition at a concentration of 1.5 mg/mL. According to our literature search, there has as yet been no study on the α -amylase inhibitory activity of *A. lividus*. However, there are a limited number of studies on the α -amylase inhibitory activity of other *Amaranthus* species. One such study reported that the methanol extract of *A. spinosus* significantly inhibited α -amylase activity (IC_{50} 46.02 μ g/mL) (Kumar et al., 2011). Kumar et al. (2012) showed that methanol extract of *A. viridis* exhibited α -amylase inhibitory activity (IC_{50} 10.19 μ g/mL). Another study reported that the water extracts of *A. gangeticus* and *A. inamoenus*, showed high α -amylase inhibitory activity at a concentration of 1 mg (Yang, Mong, Wu, Wang, & Yin, 2020). These findings are in agreement with the present data that *Amaranthus* extracts show good α -amylase inhibitory activity.

α -Glucosidase inhibitory activity

Oral antidiabetic agents, which act as competitive/reversible inhibitor on α -glucosidases, slow carbohydrate digestion and resulting in a decrease in postprandial blood glucose levels (Elbashir et al., 2018). In the present study, the water extract showed quite a good α -glucosidase inhibitory activity as compared to acarbose (Table 1). The water extract caused $73.28 \pm 0.14\%$ enzyme inhibition at 0.01 mg/mL concentration, while acarbose caused only $21.28 \pm 4.25\%$ at the same concentration. According to our literature search, there are two studies on the α -glucosidase inhibitory activity of *Amaranthus* species. A study by Mondal, Guria, & Maity, (2015) reported that methanol extract of *A. spinosus* showed significant α -glucosidase inhibitory activity (IC_{50} 8.49 μ M/mL) and that acarbose provides inhibition at IC_{50} values of 15.25 μ M/mL. Yang, Mong, Wu, Wang, & Yin (2020) reported that the water extracts of *A. gangeticus* and *A. inamoenus* showed high α -glucosidase inhibi-

Table 1. α -Amylase, α -glucosidase, acetylcholinesterase (AChE) and elastase inhibitory activities of water extract of *A. lividus*.

IC_{50} Values (mg/mL)*				
	α -Amylase	α -Glucosidase	AChE	Elastase
<i>A. lividus</i>	0.0103 \pm 0.00057	0.0068 \pm 0.0001	247.66 \pm 54.90	0.69 \pm 0.12
Standards	Rutin	Acarbose	Galantamine	Ursolic Acid
	0.96 \pm 0.15	0.069 \pm 0.005	0.0092 \pm 0.0002	0.0065 \pm 0.0001

*Values represent the means of three replicates \pm standard deviation. IC_{50} values indicate the extract/standard concentration that inhibits enzyme activity by 50%. IC_{50} values were calculated from dose-response curves (by plotting the percentage of inhibition against concentration) using Microsoft Excel software.

tory activity (80-50%) at a concentration of 1 mg. Considering the results of other studies, it can be said that the extracts of *Amaranthus* species are good α -glucosidase inhibitors.

Acetylcholinesterase (AChE) inhibitory activity

The use of AChE inhibitors is an effective therapeutic approach to alleviate the symptoms of Alzheimer’s disease (Silva et al., 2019). In this study, the water extract showed weak AChE inhibitory activity compared to galantamine (Table 1). According to our literature search, there has been no study as yet on AChE inhibitory activity of *A. lividus*. However, there are a few studies on AChE inhibitory activity of other *Amaranthus* species. As a result of the study conducted by Lee, Song, & Lee (2010), it was found that the extracts of water, 80% methanol and 70% ethanol (1 mg/mL) obtained from *A. mangostanus* did not cause AChE inhibition. Another study reported that the water extracts of *A. gangeticus* and *A. inamoenus*, showed moderate anti-AChE activity (50-60%) at a concentration of 1 mg (Yang, Mong, Wu, Wang, & Yin, 2020). In a study on *A. tricolor*, traditionally used as a memory enhancer in Anatolia (Orhan & Aslan, 2009), it was reported that the extract of the aerial parts (0.5 mg/mL) of *A. tricolor* showed 3% AChE inhibitory activity (Lobbens, Vissing, Jorgensen, van de Weert, & Jäger, 2017). However, another study by Hupparage et al. (2020) reported that the ethanolic extract of *A. tricolor* (leaves) showed good AChE inhibition (IC₅₀ value 193.9 µg/mL). Nuria, Suganda, Sukandar, & Insanu (2020) reported that ethanol extract of *A. cruentus* (leaves) at 1 mg/mL exhibited AChE inhibitory activity of 22.6±0.7%. A study by Ishtiaq et al. (2017) reported that methanol extract of *A. graecizans* subsp. *silvestris* showed mild AChE inhibition (24.29±0.57%). In agreement with our findings, other *Amaranthus* species have also been reported to no significant AChE inhibitory activity.

Elastase inhibitory activity

The increase in elastase expression due to excessive UV irradiation and oxidative damage can lead to decreased skin elasticity and induce wrinkling and sagging. Plants contain many natural substances that can be used as anti-aging agents, and also to treat dermatological conditions. It has been suggested that skin aging and anti-wrinkle effects are directly related to the breakdown of elastin, thus many studies have investigated the elastase inhibitory activity of plants (Liyanaarachchi, Samarasekera, Mahanama, & Hemalal, 2018). In this study, the water extract of *A. lividus* showed good elastase inhibitory activity,

although not as good as ursolic acid (Table 1). While the water extract at 2 mg/mL showed inhibition of 71.93±0.25%, ursolic acid at 0.001 mg/mL showed inhibition of 72.78±1.02%. According to our literature search, there has not as yet any study on elastase inhibitory activity of *Amaranthus* species.

Lipase inhibitory activity

Lipase inhibitors work by reducing the absorption of dietary fats and are thus effective therapeutics for obesity and hyperlipidemia (Elbashir et al., 2018). In the present study, the water extract showed good lipase inhibitory activity, although not as good as orlistat (Table 2). According to our literature search, there has as yet been no study on lipase inhibitory activity of *A. lividus*. However, there is a single study on the lipase inhibitory activity of other *Amaranthus* species. Yang, Mong, Wu, Wang, & Yin (2020) reported that the water extracts of *A. gangeticus* and *A. inamoenus*, showed good anti-lipase activity (70-50%) at a concentration of 1 mg. The results of the study are in agreement with our findings, namely that the water extract of *A. lividus* at 1 mg/mL caused 60.38±0.91% lipase inhibition.

Neuraminidase inhibitory activity

Neuraminidase inhibitors such as zanamivir and oseltamivir have been used as antiviral drugs to treat influenza. However, due to the development of resistance to these agents, new, natural antiviral product compounds based on neuraminidase inhibitory activity are needed for the treatment of influenza (Kwak et al., 2018). In the present study, the water extract of *A. lividus* showed quite low neuraminidase inhibitory activity compared to quercetin (Table 2). According to our literature search, there has not any study as yet on the neuraminidase inhibitory activity of *Amaranthus* species.

Tyrosinase inhibitory activity

Tyrosinase inhibitors are significant in cosmetics and pharmaceuticals as whitening agents and for the therapy of pigmentary disorders (Liyanaarachchi, Samarasekera, Mahanama, & Hemalal, 2018). In this study, the water extract of *A. lividus* showed good tyrosinase inhibitory activity, although not as good as kojic acid (Table 2). According to our literature search, there has as yet been no study on tyrosinase inhibitory activity of *A. lividus*. Moreover, there are only three studies on tyrosinase inhibitory activity of other *Amaranthus* species. In one study, an 80% methanol extract of *A. mangostanus* (aerial parts) showed 8% tyrosinase inhibition at a concentration of 100 µg/mL (Shin et al., 1997). Li & Wang (2014)’s study reported that methanol extract of *A.*

	IC ₅₀ Values (mg/mL)*		
	Lipase	Neuraminidase	Tyrosinase
<i>A. lividus</i>	0.56±0.06	1.48±0.23	2.27±0.08
Standards	Orlistat	Quercetin	Kojic Acid
	0.0009±0.00005	45.67x10 ⁻⁶ ±3.52x10 ⁻⁶	0.68±0.07

*Values represent the means of three replicates ± standard deviation. IC₅₀ values indicate the extract/standard concentration that inhibits enzyme activity by 50%. IC₅₀ values were calculated from dose-response curves (by plotting the percentage of inhibition against concentration) using Microsoft Excel software.

viridis (whole plant) has no α -tyrosinase inhibitory activity. In another study, the methanol extract of *A. viridis* showed higher anti-tyrosinase activity (IC_{50} 36.31 $\mu\text{g/mL}$) as compared to the extracts followed by chloroform extract (IC_{50} 57.12 $\mu\text{g/mL}$), water extract (IC_{50} 62.32 $\mu\text{g/mL}$) and hexane extract (IC_{50} 87.14 $\mu\text{g/mL}$), (Kumari, Elancheran, & Devi, 2018). In a study on tyrosinase inhibition of *A. albus* (Badem et al., 2018), the methanol extract was used at concentrations of 25, 50, 100 and 500 $\mu\text{g/mL}$, and the extract showed so low an inhibitory effect that the IC_{50} value could not be calculated.

In addition, there are a limited number of studies on tyrosinase inhibitory activity of *Amaranth* seeds. Seo et al. (2016) reported the inhibitory effect of *Amaranthus* spp. seed extract on tyrosinase activity in melan-a cells. The results showed that the water extract of a species crossed between *A. hybridus* and *A. hypocondriacus* showed significant inhibition of tyrosinase activity similar to that of kojic acid. A study by Yi, Kang, & Bu (2017) reported the tyrosinase inhibitory activities of 70% ethanol extract and subfractions of *Amaranth* seeds. The ethyl acetate fraction of the seeds showed stronger tyrosinase inhibitory activity than that of the positive control cynandione A at 50, 100 and 200 $\mu\text{g/mL}$. At a concentration of 50 $\mu\text{g/mL}$, cynandione A produced about 50% tyrosinase inhibition, while the ethyl acetate fraction produced about 65% inhibition. Finally, the study by Rocchetti et al. (2020) reported that *Amaranthus* microgreens have potential to inhibit tyrosinase activity.

CONCLUSIONS

Amaranthus spp. have been used in medicine from ancient times to the present for the treatment of various diseases, and the species also continues to be the subject of research. Although some activities of *A. lividus* have been reported in the literature, no study has been carried out on the inhibitory activity of α -amylase, α -glucosidase, AChE, elastase, lipase, neuraminidase and tyrosinase. In the present study, the inhibitory potential of *A. lividus* against some therapeutic target enzymes was investigated and brought to the literature for the first time. In conclusion, the water extract of *A. lividus* exhibited significant and promising α -glucosidase and α -amylase inhibitory activity compared to standard inhibitors. Also, the extract displayed good elastase, lipase and tyrosinase inhibitory activities. However, it exhibited low inhibitory activity on AChE and neuraminidase. In particular, due to antidiabetic activity of *A. lividus*, the plant and its active constituents can be used as a therapeutic enzyme inhibitor with future phytochemical constituent analysis.

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Evaluation of the medicinal potential of a traditionally important plant from Turkey: *Cerinth minor* L.

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ABSTRACT

Background and Aims: *Cerinth minor* L. has been used for both culinary and medicinal purposes in Turkey. Since the plant has remained uninvestigated pharmacognostically, this study aims to provide data on the phytochemical profile and *in vitro* biological activities of the ethanol extract prepared from the aerial and root parts of *Cerinth minor* L.

Methods: The phytochemical profile of the extract was determined by LC-MS/MS. Total phenolic and total flavonoid contents of *Cerinth minor* ethanol extract were determined as pyrocatechol, and quercetin equivalents, respectively. Antioxidant activity of the extract was investigated by DPPH, and ABTS cation radical scavenging activity, and CUPRAC activity assays. Enzyme inhibitory activity assays were used to investigate anticholinesterase, antityrosinase, and antiurease activities. Cytotoxic potential of the plant was clarified by using the XTT method, and antimicrobial activity of the extract was established by the microbroth dilution technique.

Results: According to the LC-MS/MS results, 11 different constituents were present in the extract, and the major compounds were, malic acid (5392.18±60.93 µg/g extract), fumaric acid (4730.99±58.66 µg/g extract), and rosmarinic acid (2470.07±176.12 µg/g extract). Total phenolic and flavonoid contents were found as 10.39±0.73 µg PEs/mg extract, and 0.75±0.07 µg QEs/mg extract, respectively. The extract demonstrated moderate antioxidant activity, whereas no enzyme inhibitory activity was exerted against the tested enzymes. No cytotoxic activity was observed on human renal (A498, UO-31) or human colon (COLO205, KM12) cancer cell lines. The extract was shown to possess low-moderate antimicrobial activity against *C. tropicalis* with a MIC value of 78.12 µg/mL.

Conclusion: Having several pharmacologically valuable compounds, and not causing any toxicity on studied cell lines, *Cerinth minor* is a plant that requires attention with its medicinal potential.

Keywords: *Cerinth minor*, LC-MS/MS, Antioxidant, Cytotoxicity, Medicinal potential

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INTRODUCTION

Since time immemorial, people have used medicinal plants for the prevention and/or treatment of various diseases and ailments. Although the popularity of herbal medicines had been dramatically decreased in the western world with the development of synthetic medications in the last 200 years, the recent huge demand for plant-based therapeutic agents as primary health care in well-developed countries, and the sharp upward trend for using traditional remedies and natural-based products indicate the importance of research on medicinal plants. The massively increased consumption of phytotherapeutic agents has resulted in an estimate by the WHO that more than 80% of the population relies solely or largely on medicinal plants for health care (Schippmann, Leaman, & Cunningham, 2002).

The Anatolian peninsula, where modern Turkey is located, is an extraordinary location being estimated to contain more than 11000 taxa currently, and more importantly, 1280 of them were reported to be used traditionally for medicinal purposes (Dalar, Mukemre, Unal, & Ozgokce, 2018). Plausibly arising from the outstanding historical background of Turkey, a great number of plants are still in use by local inhabitants, according to recent surveys about traditional medicine practices. A recent study reported that 59% of people using traditional and complementary medicine in Turkey use herbal mixtures (Şimşek et al., 2017).

In efforts to discover new drugs, indigenous medicinal plants have always been essential resources, and therefore an ethnopharmacological approach that includes chemical analyses and investigation of biological activities of the plants play a vital role in this context. It should also be emphasized that traditionally used medicinal plants must fulfill the requirements on quality, efficacy, and safety to be evaluated as therapeutic agents in today's era (Süntar, 2020). In this day and age, several advanced instrumental techniques are being used to provide scientific evidence supporting the traditional use of an ethnobotanically important plant (Boğa et al., 2021a). Safety concerns about natural agents are a major issue, considering that concurrent use of herbal products with synthetic medications can reach up to 88% in the elderly population (Taneri, Akis & Karaalp, 2021).

Cerinth minor L. (Boraginaceae) is among worth-mentioning traditionally used plants in Turkey. *C. minor* belongs to the tribe *Lithospermeae* Dumort, and genus *Cerinth* L., which is known as a small genus that includes approximately ten species distributed in the circum-Mediterranean region and Central Europe, more specifically, from the Atlantic region of Morocco to the western parts of the Irano-Turanian region (Selvi, Cecchi, & Coppi, 2009). As of today, four *Cerinth* species (*C. minor*, *C. retorta* Sm., *C. major* L., and *C. glabra* Mill.), and two *C. minor* subspecies (*C. minor* subsp. *auriculata*, and *C. minor* subsp. *minor*) have been identified in Turkey (Güner, Aslan, Ekim, Vural, & Babaç, 2012, Jetter & Riederer, 1999; Zengin et al., 2016). *C. minor* and *C. minor* subsp. *auriculata* (Ten.) Domacare known as "Hişhiş otu", and they are both most commonly consumed as food when cooked in different cities of Turkey, such as Tunceli, Batman and Kars (Doğan & Tuzlacı, 2015; Kadioğlu

et al., 2020; Yeşil & İnal, 2019). Furthermore leaves of *C. minor* are brewed as decoction and have been used to treat edema, and a mouthwash can be prepared with branches of *C. minor* subsp. *auriculata*, which has been used to treat gum wounds in folk medicine (Korkmaz & Karakuş, 2015; Kadioğlu, Kadioğlu & Sezer, 2021). Moreover, the traditional name of *C. minor* L. is "Cücegözü", in Iğdır province and "Gayebej, Gobel" in the Hasankeyf (Batman) district, where the plant has also been traditionally used for several ailments (Altundağ & Özhatay, 2009; Yeşil & İnal 2021).

Despite the fact that *C. minor* is a popular, commonly used medicinal plant in Turkey, interestingly enough, the plant has somehow remained "unexplored" pharmacognostically until now. To the authors' best knowledge, Zengin et al. (2016) conducted a study on *C. minor* subsp. *auriculata*, reporting the results of antioxidant, antityrosinase, anti- α -amylase, anticholinesterase, and anti- α -glucosidase activity assays of the plant, which is still the only report that provides data on the biological activities of the subspecies. Due to the constantly increasing popularity of herbal remedies, it has become unquestionably crucial and a primary task to shed more light on the chemistry and pharmacological activities of traditionally used medicinal plants which, for sure, requires extensive pharmacognostic research. With these efforts, it may be possible to provide solid scientific evidence of their efficacy, and over and above that, safety concerns related to these plants could be addressed. This is very important since the species have been shown to contain toxic alkaloids by previous studies (El-Shazly & Wink, 2014; Mroczek, Baj, Chrobok, & Glowinski, 2004). In efforts to discover natural and renewable resources to use in the pharmaceutical industry, gaining more knowledge about the toxicity potential of the plants is essential (Boğa et al., 2021a).

Bearing all this in mind, this study aimed to investigate the chemical constituents, and *in vitro* biological activities of *C. minor* collected from Kürecik-Malatya/Turkey. An LC-MS/MS analysis was conducted to determine the phytochemicals among 37 standards. Antioxidant, anticholinesterase, antityrosinase, antiurease, and antimicrobial activity assays were carried out on the extract obtained from aerial and root parts of the plant, also the cytotoxic potential of *C. minor* on COLO205 and KM12 colon, UO-31 and A498 renal, and high metastatic MG633, and low metastatic MG63 osteosarcoma cancer cell lines were detected by cell viability assay.

MATERIAL AND METHODS

Plant material

The aerial and root parts of *Cerinth minor* were collected from Kürecik-Malatya, Turkey in April 2014. The authenticity of the plant was confirmed by Assoc. Prof. Dr. Yeter Yeşil Cantürk (Istanbul University, Faculty of Pharmacy, Pharmaceutical Botany Department). A voucher herbarium specimen was preserved in the Herbarium of the Faculty of Pharmacy of Istanbul University (ISTE number: 116048).

Preparation of plant extract

The aerial and root parts of the plant (40.82 g) were combined and macerated in ethanol (100 mL) for 24 hours at room tem-

perature (25 ± 2 °C) and the macerate was filtered out by using a Whatman no 1 filter paper. The same extraction process was triplicated. Then all filtrates were concentrated in a vacuum at 35 °C by using a rotaevaporator. The extract yields were weighted (1.52 g), afterwards, and the obtained extract was stored at -20 °C until the assessments. The stored filtrates were diluted with ethanol (250 mg/L) before LC-MS/MS analysis, then filtered out by a 0.2 µm microfiber filter. The yield percentages were calculated using the following formula: Extract yield % = $R/S \times 100$ (where R; weight of dried plant and S; weight of plant raw sample).

Extraction yield (%) = $1.52 \text{ g} / 40.82 \text{ g} \times 100 = \%3.72$

Chemicals and instruments

All compounds which were used for all assays were purchased from Merck (Germany), Sigma (Germany); and Fluka (Germany). All solvents used for the assays were of analytical grade.

Quantitative analysis of 37 compounds by LC-MS/MS

Quantitative analysis of 37 compounds (Table 1) was conducted by LC-MS/MS, using a previously developed and validated method (Yilmaz et al., 2018). The LC-MS/MS system was a Shimadzu brand, Nexera model UHPLC, which was coupled to a tandem MS instrument. To process the data obtained from LC-ESI-MS/MS, Shimadzu Lab Solutions software was used

Total phenolic and flavonoid contents of the extracts

The total phenolic content of *C. minor* ethanol extract was calculated as micrograms of pyrocatechol equivalents (PEs) by applying the method developed by Boğa et al. (2021b). The total flavonoid content of the extract was determined by applying the method designed by Moreno, Isla, Sampietro & Vattuone (2000). The results of this assay were expressed as micrograms of quercetin equivalents (QEs).

To calculate the total phenolic and flavonoid contents of the extract, the equations below were used:

Absorbance = $0.0409 \text{ pyrocatechol } (\mu\text{g}) + 0.0495 (R^2 = 0.9975)$

Absorbance = $0.0347 \text{ quercetin } (\mu\text{g}) + 0.1174 (R^2 = 0.9992)$

Antioxidant activity

Antioxidant activity of the ethanol extract of *C. minor* was investigated by using DPPH free radical scavenging, ABTS cation radical scavenging, and CUPRAC activity methods.

DPPH radical scavenging activity

The DPPH free radical scavenging activity of *C. minor* was determined by using a previously applied method (Ersoy, Özkan, Boğa & Mat, 2020). After the incubation period, the percentage inhibition of absorbance at 517 nm was calculated for each concentration relative to ethanol.

The following equation was used to calculate the DPPH free radical scavenging potential:

DPPH scavenging effect (Inhibition %) = $(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$

ABTS cation radical decolorization (scavenging) assay

The ABTS cation radical decolorization (scavenging) method previously modified by Boğa et al. (2016) was used to estimate the ABTS cation scavenging activity of the *C. minor* ethanol extract. After the incubation period, the absorbance was read at 734 nm using the microplate reader.

ABTS cation radical scavenging effect (Inhibition %) = $(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$

Cupric reducing antioxidant capacity (CUPRAC)

Cupric reducing antioxidant capacity (CUPRAC) activity of the *C. minor* ethanol extract was determined according to the method specified before by Ersoy et al. (2020). After the incubation period, the change in absorbance was measured at 450 nm by the microplate spectrophotometer. The results of the cupric reducing antioxidant capacity (CUPRAC) of the extract and standards were presented as concentration giving 0.5 absorbance values

Enzyme inhibition activity assays

Anticholinesterase inhibitory activity

To determine the anticholinesterase activity of the *C. minor* ethanol extract, a previously described method by Ersoy, et al. (2020) was applied. Following the incubation period, the absorbance of the mixture was measured at 412 nm. A well-known cholinesterase inhibitor, galantamine, was used as the positive control.

Tyrosinase inhibitory activity

Determination of the tyrosinase inhibition activity of the *C. minor* ethanol extract was performed according to the previously used method by (Ersoy, Özkan, Boğa, Yılmaz & Mat, 2019). The change in absorbance of the mixture was measured at 475 nm at 37 °C. For the positive control, kojic acid was used.

Urease inhibitory activity

The urease inhibitory activity of the *C. minor* ethanol extract was determined according to the protocol reported by Zahid et al. (2015). The change in absorbance of the mixture was measured at 630 nm, and thiourea was used for the positive control.

The following equation was used to calculate the enzyme inhibition %:

$(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$

A_{control} = Absorbance of the control

A_{sample} = Absorbance of the samples

Cell viability assays

Cytotoxicity assay on renal and colon cell lines

The cytotoxic potential of the *C. minor* ethanol extract was tested on human renal cancer cell lines UO-31 and A498, and human colon cancer cell lines COLO205 and KM12. All details of the XTT cytotoxic activity assay were performed according to a previous study conducted by (Eroğlu Özkan, Boğa, Yılmaz, Kara & Yeşil, 2020).

Metastatic potential assay

The metastatic potential of the *C. minor* ethanol extract on high (MG63.3) and low (MG63) metastatic potential human osteosarcoma cell lines was identified by using XTT assay, according to a previously applied method by (Eroğlu Özkan, Boğa, Yılmaz, Kara & Yeşil, 2020).

Antimicrobial activity

In vitro antimicrobial activity of *C. minor* ethanol extract was investigated by one of the most well-known antimicrobial susceptibility testing techniques, which is the microbroth dilution method. Ten different human pathogenic strains including *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750 were applied in this study. The microbroth dilution technique was applied according to the protocols described by the Clinical and Laboratory Standards Institute (1997, 2006, 2010). Serial two-fold dilutions ranging from 2500 to 1,2 µg/mL were prepared in Mueller Hinton Broth for the tested bacteria and RPMI-1640 medium for the yeast, respectively. The stock solutions of molecules also prepared in DMSO. The inoculum was prepared using a 4–6 h broth culture of each bacteria and 24 h culture of yeast strains adjusted to a turbidity equivalent of a 0.5 McFarland standard, diluted in broth media to give a final concentration of 5×10^5 CFU/mL for bacteria and 0.5×10^3 to 2.5×10^3 CFU/mL for yeast in the test tray. The MIC was defined as the lowest concentration of tested extracts giving complete inhibition of visible growth. Experiments were performed in triplicate. The following standard antibacterial and antifungal agents were used as standard compounds: Cefuroxime-sodium, cefuroxime, ceftazidime, amikacin, and clotrimazole.

Statistical analysis

All measurements were performed in triplicate. The results were evaluated using a t-test with Microsoft Excel and expressed as mean \pm standard deviation. Differences for all tests were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

LC-MS/MS analysis

LC-MS/MS chromatograms of the standards (A) and the extract (B) were given in Figure 1. According to the results that are present in Table 1, 12 different constituents were present in the extract, and the major compounds were, malic acid (5392.18 ± 60.93 µg/g extract), fumaric acid (4730.99 ± 58.66 µg/g extract), rosmarinic acid (2470.07 ± 176.12 µg/g extract), and 4-OH-Benzoic acid (1101.90 ± 31.84 µg/g extract). The extract was also found to be rich in rutin (595.58 ± 9.47 µg/g extract), and nicotiflorin (343.92 ± 9.49 µg/g extract). Furthermore, *p*-coumaric acid (234.67 ± 12.11 µg/g extract), caffeic acid (192.12 ± 6.80 µg/g extract), isoquercitrin (193.07 ± 2.57 µg/g extract), salicylic acid (21.37 ± 0.70 µg/g extract), vanillin (2.23 ± 0.06 µg/g extract) and naringenin (0.73 ± 0.04 µg/g extract) were detected in the extract.

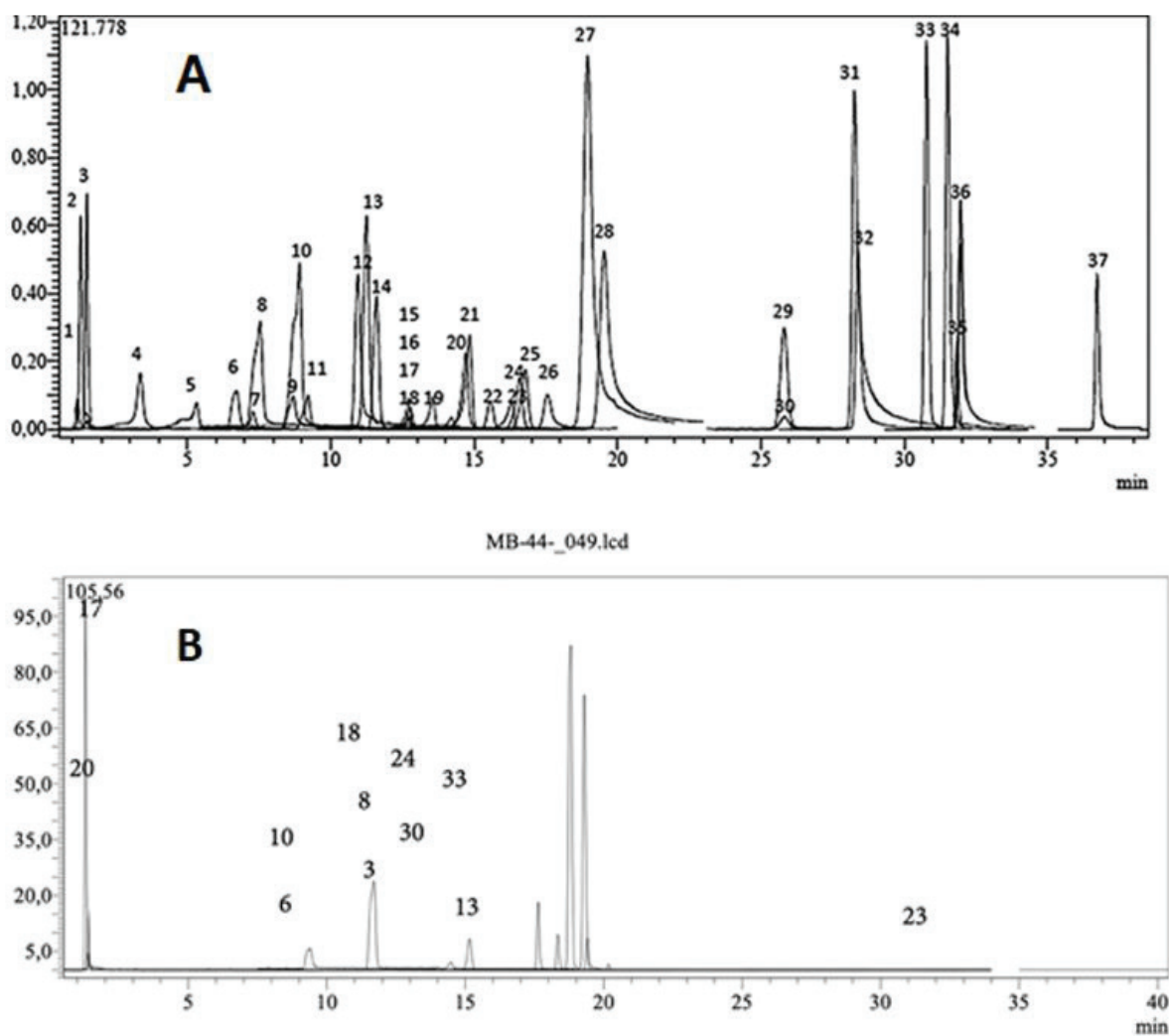
Since this is the first report of the determination of the phenolic content of *C. minor* aerial parts and roots extracts to the authors'

best knowledge, it is not possible to align all the research with the results of previous studies in comparison with the current one. However, there was a study carried out to investigate the secondary metabolites of the shoot and root extracts of the plant. Accordingly, allantoin, hydrocaffeic acid, and rosmarinic acid were found in the shoots extract, and allantoin and rosmarinic acid were found to be present in the roots extract of *C. minor* by high-performance capillary electrophoresis (Dresler, Szymczak, & Wójcik, 2017). Over and above that, there have been studies reporting the presence of pyrrolizidine alkaloids in *C. minor* extracts. Namely, 9-*viridifloryl*-retronecine monoester, 9-*angeloyl*-7-*viridifloryl*-retronecine, and 9-*angeloyl*-retronecine diester were identified in the leaves of *C. minor* (Mroczek, Baj, Chrobok, & Glowniak, 2004). Intermedine and lycopsamine are other pyrrolizidine alkaloids that were determined in *C. minor* extracts by further studies (El-Shazly & Wink, 2014). The cuticular wax of *C. minor* was also investigated by GC-mass spectrometry and GC-FTIR, whereas quantitation of individual compounds was revealed by GC-FID in a study performed by Jetter & Riederer (1999). A homologous series of 11 δ -lactones (1,5-alkanolides) was determined in cuticular waxes from the leaves of *C. minor*.

Malic acid, one of the major compounds determined in the studied extract, is a widely known organic acid that has many applications mainly in the food and beverage industry. It has been used as a taste enhancer and acidulant in many food products, also as a buffering and chelating agent in personal care formulations. Supplementarily, malic acid has further applications in the pharmaceutical industry. In particular, almotriptan malate is the active ingredient of a commonly used medication used for the treatment of migraines (Kövilein, Kubisch, Cai, & Ochsenreither, 2020). Besides, malic acid is effective against xerostomia by alleviating the dry mouth sensation of patients (Niklander et al., 2018). Last but not least, malic acid has been shown to possess protective effects on myocardial ischemia/reperfusion injuries, attributed to its anti-inflammatory and antiplatelet aggregation activities (Tang et al., 2013).

Fumaric acid was also found to be abundant in the *C. minor* extract. This finding can also be considered as worth mentioning since fumaric acid has important pharmacological activities. Speaking of which, fumaric acid esters have been licensed for the treatment of a chronic and progressive dermatological disease, psoriasis in Germany. Fumaric acid esters? Have been shown to be effective and safe for long-term clinical use as monotherapy or in combination (Atwan et al., 2015; Smith, 2017; Dickel, Bruckner & Altmeyer, 2018).

The studied *C. minor* extract was also shown to contain phenolic acids including rosmarinic and caffeic acids. Incidentally, there is an article pointing out the presence of rosmarinic acid in *Cerinth* species. Although it does not specify which species that is, stating that the compound had been isolated from these plants, this could be interpreted as *Cerinth* species are sources for rosmarinic acid (Khojasteh et al., 2020). A plethora of research has focused on the health benefits of phenolic acids, and accordingly, their properties such as antioxidant, antitumor, and anti-inflammatory activities are noteworthy (Al Jitan, Alkhoori, S, & Yousef, 2018).



3 – *p*-coumaric Acid, 6 – Caffeic Acid, 8- Salicylic Acid, 10- 4-OH-Benzoic Acid, 13- Rosmarinic Acid, 17- Fumaric Acid, 18- Vanillin, 20- Malic Acid, 23- Naringenin, 24- Rutin, 30- Isoquercitrin, 33- Nicotiflorin

Figure 1. LC-MS/MS chromatograms of the standards and the extract (A: standards, B: CM).

Determination of total phenolic and flavonoid contents

Total phenolic and flavonoid contents of the ethanol extract prepared from the whole plant aerial and root parts of *C. minor* were determined as pyrocatechol (PEs), and quercetin (QEs) equivalents, respectively. ($y = 0.0409$ pyrocatechol (mg) + 0.0495, $R^2=0.9975$ and $y = 0.0347$ quercetin (mg) + 0.1174 ($R^2=0.9992$).

The phenolic content of *C. minor* ethanol extract was calculated as 10.39 ± 0.73 μ g PEs/mg extract, and the flavonoid content of the extracts was calculated as 0.75 ± 0.07 μ g QEs/mg extract, which announces that the phenolic content of the extract is higher than its flavonoid content (Table 2). Considering the LC-MS/MS analysis results, it could be expected that the flavonoid content of the extract would not be high, therefore the results of the assays are consistent.

There has been only one study about the phenolic and flavonoid contents of subspecies, which is *C. minor* subsp. *auriculata*. Reportedly, the methanol extract of the plant was shown to contain 25.4 mg GAEs/g extract, the ethyl acetate extract contained 6.2 mg GAEs/g extract, and the aqueous extract of the plant had

the highest amount of total phenolics with 46.6 GAEs/g extract. Regarding the total flavonoid contents of the studied three extracts, the ethyl acetate extract was the richest among them with 52.4 mg REs/g extract. The methanol extract was shown to contain 27.8 mg REs/g extract, and the aqueous extract had 24.6 mg REs/g extract total flavonoids. As it stands, total phenolics were expressed as gallic acid equivalents, and total flavonoids were expressed as rutin equivalents in the study. These findings along with the data provided by the current study suggest that *C. minor* is not a flavonoid-rich plant, or more likely the ethanol extract of the plant cannot be considered as a flavonoid-rich extract.

Determination of antioxidant activity

With the purpose of evaluating the antioxidant activity of plant extracts, it is crucial not to make conclusions based on only one single antioxidant method, considering that there are various oxidation aspects in the systems (Ersoy et al., 2020). Bearing this in mind, the antioxidant activity of the ethanol extract prepared from the aerial and root parts whole plant material of *C. minor* was conducted by using three comparative methods, which were DPPH free radical scavenging, ABTS cation radical

Table 1. LC-MS/MS results of *Cerinth minor* extract.

	Analytes	RT ^a	Parent ion (m/z)	Daughter Ions	Ion. Mode	Quantification (µg analyte/g extract) ^b
						CM
1	Coumarin	17.40	147.05	91.0-103.2	Pos	N.D.
2	Hesperidin	12.67	610.90	303.1-465.1	Pos	N.D.
3	<i>p</i> -coumaric acid	11.53	162.95	119.25-93.25	Neg	234.67±12.11
4	<i>o</i> -coumaric acid	15.45	162.95	119.35-93.25	Neg	N.D.
5	Gallic acid	3.00	168.85	125.2-79.2	Neg	N.D.
6	Caffeic acid	8.80	178.95	135.2-134.3	Neg	192.12±6.80
7	Vanillic acid	8.57	166.90	152.25-108.25	Neg	N.D.
8	Salicylic acid	11.16	136.95	93.3-65.3	Neg	21.37±0.70
9	Quinic acid	1.13	190.95	85.3-93.3	Neg	N.D.
10	4-OH-Benzoic acid	7.39	136.95	93.3-65.3	Neg	1101.90±31.84
11	<i>tr</i> -Ferulic acid	12.62	192.95	178.3	Neg	N.D.
12	Chlorogenic acid	7.13	353.15	191.2	Neg	N.D.
13	Rosmarinic acid	14.54	359.00	161.2-197.2	Neg	2470.07±176.12
14	Protocatechuic acid	4.93	152.95	108.3	Neg	N.D.
15	Cinnamic acid	25.61	147.00	103.15-77.3	Neg	N.D.
16	Sinapinic acid	12.66	222.95	208.3-149.2	Neg	N.D.
17	Fumaric acid	1.48	115.00	71.4	Neg	4730.99±58.66
18	Vanillin	10.87	151.00	136.3-92.2	Neg	2.23±0.06
19	Pyrocatechol	6.48	109.00	108.35-91.25	Neg	N.D.
20	Malic acid	1.23	133.00	115.2-71.3	Neg	5392.18±60.93
21	Syringic acid	9.02	196.95	182.2-167.3	Neg	N.D.
22	Hesperetin	31.76	300.95	164.2-136.2	Neg	N.D.
23	Naringenin	30.68	270.95	151.2-119.3	Neg	0.73±0.04
24	Rutin	12.61	609.05	300.1-271.1	Neg	595.58±9.47
25	Quercetin	28.17	300.90	151.2-179.2	Neg	N.D.
26	Quercitrin	16.41	447.15	301.15-255.15	Neg	N.D.
27	Apigenin	31.43	268.95	117.3-151.2	Neg	N.D.
28	Chrysin	36.65	252.95	143.3-119.4	Neg	N.D.
29	Liquiritigenin	25.62	254.95	119.25-135.15	Neg	N.D.
30	Isoquercitrin	13.42	463.00	300.15-271.15	Neg	193.07±2.57
31	Cosmosiin	16.59	431.00	268.2-239.2	Neg	N.D.
32	Rhoifolin	16.11	577.05	269.2-211.15	Neg	N.D.
33	Nicotiflorin	14.68	593.05	285.1-255.2	Neg	343.92±9.49
34	Fisetin	19.30	284.95	135.2-121.25	Neg	N.D.
35	Luteolin	28.27	284.75	133.2-151.2	Neg	N.D.
36	Myricetin	18.72	317.00	179.15-151.25	Neg	N.D.
37	Kaempferol	31.88	284.75	255.1-117.3	Neg	N.D.

^aRT: Retention time, ^bValues in µg/g (w/w) of plant extracts. N.D.: not detected

scavenging, and CUPRAC activity methods. All samples were investigated by calculating the IC₅₀ (for DPPH and ABTS cation radical scavenging assays) and A_{0.5} (for CUPRAC activity assay) of antioxidant activity and comparing them by statistical analysis. The results are given in Table 2.

The extract showed moderate antioxidant activity according to the results of three conducted assays. The DPPH radical scavenging activity of the extract was calculated as IC₅₀: 155.17±0.41 µg/mL, whereas the ABTS cation radical scavenging activity of the extract, was found to be an IC₅₀ value of 72.44±1.20 µg/mL. An A_{0.5} value was calculated for the CUPRAC activity assay, which was 31.94±0.28 µg/mL.

Regarding the evaluation of the antioxidant capacity of medicinal plants, the typical approach has been based on analyzing correlations between antioxidant activity and the total phenolic content of an extract. It has been suggested that the extracts with higher total phenolic content are simply expected to be better antioxidants. However, this correlation is insufficient to reliably hand down a verdict, because the phenolic profiles, meaning the type of the phenolic compounds and also the relative amount of proportions of the phenolic compounds present in the extract may vary, and this affects the antioxidant activity directly (Jacobo-Velázquez & Cisneros-Zevallos, 2009). Not only that but also the extraction solvent along with the plant material, have been shown to have a huge influence on the antioxidant capacity. In a nutshell, there have been studies report-

Table 2. Total phenolic-flavonoid contents and antioxidant activities of *Cerithe minor* extract*.

Samples	Phenolic content ($\mu\text{g PEs/mg extract}$) ^a	Flavonoid content ($\mu\text{g QEs/mg extract}$) ^b	DPPH Free Radical IC_{50} ($\mu\text{g/mL}$)	ABTS Cation Radical IC_{50} ($\mu\text{g/mL}$)	CUPRAC $A_{0.5}$ ($\mu\text{g/mL}$)
CM	10.39 \pm 0.73	0.75 \pm 0.07	155.17 \pm 0.41	72.44 \pm 1.20	31.94 \pm 0.28
BHA ^c	-	-	7.88 \pm 0.20	2.74 \pm 0.03	0.63 \pm 0.02
α -TOC ^c	-	-	16.30 \pm 0.79	10.20 \pm 0.05	13.38 \pm 0.07
BHT ^c	-	-	58.86 \pm 0.50	3.16 \pm 0.06	2.02 \pm 0.01

CM: *Cerithe minor* extract; *Values expressed are means \pm standard deviation of three parallel measurements ($p < 0.05$); ^a PEs, pyrocatechol equivalents ($y = 0.0409x + 0.0495$, $R^2 = 0.9975$); ^b QEs, quercetin equivalents ($y = 0.0347x + 0.1174$, $R^2 = 0.9992$); ^c Standard compounds

ing that extracts with higher total phenolic content ended up having lesser antioxidant effects than less phenolic-rich extracts (Terpinc, Ćeh, Ulrih & Abramovič, 2012). *C. minor* may not be considered as a phenolic-rich, or more specifically a flavonoid-rich plant, according to the results of the current study. This may explain the comparatively moderate antioxidant activity results obtained from three antioxidant assays. Notwithstanding, since other parameters affect the total antioxidant capacity, further research on this issue is most definitely needed.

Enzyme inhibitory activities

The enzyme inhibitory potential of *C. minor* ethanol extract was evaluated by inhibitory activity on cholinesterase, tyrosinase, and urease enzymes. The results are given in Table 3.

Enzyme inhibitors decrease the bioactivity of the enzymes, which can play a vital role while correcting any metabolic imbalance, therefore there are many enzyme inhibitors available as medications on pharmacy shelves today (Boža et al., 2021b). Apart from a very low antityrosinase activity (4.22 \pm 0.08% at 200 $\mu\text{g/mL}$ concentration), *C. minor* ethanol extract did not exhibit any enzyme inhibitory activity according to the results of the current study.

The only study about the enzyme inhibitory activity of *C. minor* var. *auriculata* reported that only methanol and ethyl acetate

extracts demonstrated low anticholinesterase and antityrosinase effects, whereas the water extract did not exhibit any enzyme inhibition activity towards these enzymes (Zengin et al., 2016). This situation indicates that the extraction solvent is very important in this context. Regarding antityrosinase activity, quinic acid and gallic acid are two molecules that have been proven to exert significant tyrosinase inhibitory effects. Together with this, few flavonoids containing 3;4'-dihydroxyl substitution on ring B are known to inhibit the tyrosinase enzyme; (Şöhretoğlu, Sarı, Barut, & Özel, 2018; Ersoy et al., 2019). The absence of these compounds in the *C. minor* extract may illuminate the low antityrosinase activity. In connection therewith, quercetin, myricetin, myricitrin, and luteolin are among phenolic compounds with antiurease properties which were not present in the studied extract (Ersoy, et al., 2020). Expectedly, the extract did not exert any antiurease activity, either.

Cell viability assay

To investigate the cytotoxic properties of *C. minor* ethanol extract on COLO205 and KM12 colon, UO-31 and A498 renal, and high metastatic MG633, and low metastatic MG63 osteosarcoma cancer cell lines, a cell viability assay was carried out. The results are presented in Table 4. Accordingly, the studied extract did not cause any significant changes in the growth of cancer lines.

Table 3. Acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase and urease enzymes inhibition (%) values of *Cerithe minor* extract^a.

Samples	AChE	BChE	Tyrosinase	Urease
CM	NA	NA	4.22 \pm 0.08	NA
Gаланthamine ^b	83.31 \pm 0.09	86.38 \pm 0.10	-	-
Kojic acid ^b	-	-	95.26 \pm 0.23	-
Thiourea ^b	-	-	-	88.61 \pm 1.16

CM: *Cerithe minor* extract; ^a 200 $\mu\text{g/mL}$; ^b Standard compounds; NA: Not Active

Table 4. Cytotoxic activity results of *Cerithe minor* extract.

Extracts	2DAY-Renal (viability %)		2DAY-Colon (viability %)		META (viability %)	
	A498	U031	COLO205	KM12	MG633	MG63
CM	93.74	>100	81.5	>100	99.17	93.81

CM: *Cerithe minor* extract

Understanding the cytotoxic potential of *C. minor* can undoubtedly be considered as a crucial step in pharmacognostic research because the plant has been shown to contain pyrrolizidine alkaloids, as mentioned before (El-Shazly & Wink, 2014; Mroczek, Baj, Chrobok, & Glowniak, 2004). Plant-sourced pyrrolizidine alkaloids are known to be responsible for high hepatotoxic, genotoxic, cytotoxic, neurotoxic, and tumorigenic activities. Therefore, pyrrolizidine alkaloid-containing plants can be severely hazardous for humans and animals even through normal food intake, causing irreversible hepatic damage and cancer (Tamariz, Burgueño-Tapia, Vázquez, & Delgado, 2018). In the view of the foregoing, it was of utmost importance to investigate the cytotoxic potential of *C. minor*, since it has been consumed as food in several cities in Turkey. Our study has suggested that the plant can be comprehended as “safe” in terms of cytotoxicity on renal, colon, and osteosarcoma cell lines.

Antimicrobial activity

C. minor ethanol extract was screened for its antimicrobial activity against a standard panel of pathogenic bacteria and fungi, and the results are summarized in Table 5 (as MIC values). According to the obtained results, the extract was shown to possess low to moderate antimicrobial activity against *C. tropicalis* with a MIC value of 78.12 µg/mL, and low antimicrobial activity against *C. parapsilosis* with 312.5 µg/mL. This lack of high activity could be explained by the absence of an abundance of phenolic compounds in the extract, also the extract solvent could have affected the activity results.

Table 5. Antimicrobial activity results of *Cerinth minor* extract.

Microorganisms	MIC Values (µg/mL)
<i>Pseudomonas aeruginosa</i> ATCC 27853	NA
<i>Escherichia coli</i> ATCC 25922	NA
<i>Klebsiella pneumoniae</i> ATCC 4352	NA
<i>Proteus mirabilis</i> ATCC 14153	NA
<i>Staphylococcus aureus</i> ATCC 29213	625
<i>Staphylococcus epidermidis</i> ATCC 12228	1250
<i>Enterococcus faecalis</i> ATCC 29212	625
<i>Candida albicans</i> ATCC 10231	NA
<i>Candida parapsilosis</i> ATCC 22019	312.5
<i>Candida tropicalis</i> ATCC 750	78.12

CM: *Cerinth minor* extract; NA: No Activity. Standards; Cefuroxime-Na: 1.2 µg/ml for *S. aureus* ATCC 29213, Cefuroxime 9.8 µg/ml for *S. epidermidis* ATCC 12228, Amikacin 128 µg/ml for *E. faecalis* ATCC 29212, Ceftazidime 2.4 µg/ml for *P. aeruginosa* ATCC 27853, Cefuroxime-Na: 4.9 µg/ml for *E. coli* ATCC 25922 and *K. pneumoniae* 4352, Cefuroxime-Na 2.4 µg/ml for *P. mirabilis* ATCC 14153, Clotrimazole 4.9 µg/ml for *C. albicans* ATCC 10231, Clotrimazole 0.25 µg/ml for *C. parapsilosis* ATCC 22019, Clotrimazole 0.25 µg/ml for *C. tropicalis* ATCC 750 Amphotericin B 0.5 µg/ml for *C. parapsilosis* ATCC 22019, Amphotericin B 1 µg/ml for *C. tropicalis* ATCC 750, Amphotericin B 0.5 µg/ml for *C. albicans* ATCC 10231

CONCLUSION

Cerinth minor L. has a long history of being used in Turkey both for culinary and therapeutic purposes, traditionally. In spite of the fact that *C. minor* has been commonly consumed by people, the plant has remained uninvestigated pharmacognostically thus far. In order to evaluate the efficacy and safety of *C. minor*, it was crucial to identify the chemical constituents and to determine the pharmacologically important activities of the plant. In efforts to understand the properties of this plant in a better way, an LC-MS/MS analysis was performed, and the phytochemical profile of the plant was revealed by the current study. Antioxidant, anticholinesterase, antityrosinase, antiurease, and antimicrobial activity assays were carried out on the aerial and root parts whole plant ethanol extract, also the cytotoxic potential of *C. minor* on different cancer cell lines was detected by cell viability assay. In view of this, *C. minor* was reported to contain 11 different compounds, showed moderate antioxidant activity, whereas the extract did not demonstrate any enzyme inhibitory activities. More importantly, the extract was found to have no cytotoxic properties on the studied cancer cell lines, which was enlightening since the plant was shown to contain highly toxic pyrrolizidine alkaloids by previous studies. To conclude, *C. minor* was found to contain several biologically important compounds, a moderate antioxidant plant with no cytotoxicity on renal, colon or osteosarcoma cell lines. This study could be a major first step towards further research on *C. minor*, which is unquestionably required.

Peer-review: Externally peer-reviewed.

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Investigation of medication use patterns among pregnant women attending a tertiary referral hospital

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ABSTRACT

Background and Aims: Medication use during pregnancy presents a challenge and concern for pregnant women and health-care providers. The aim of this study was to explore patterns and factors associated with medication use by pregnant women.

Methods: This cross-sectional study was performed in a gynecology and obstetrics outpatient clinic of a tertiary referral hospital in Turkey. Data were collected by a questionnaire between October 2019 and January 2020. The questionnaire was consisted of 35 questions about participants' attitudes towards the use of medication. The sociodemographic features, medication and herbal product use during the current pregnancy, and participants' attitudes towards the use of medication were investigated.

Results: A total of 485 pregnant women were included in the study. The prevalence of using at least one medication during the current pregnancy was 45.6%, whereas herbal product use was 3.9%. Overall, 10.5% of participants used medication to treat chronic/long-term diseases before pregnancy. The most frequently used drugs were agents for nervous system (32.8%), followed by anti-infective drugs (20.8%) and agents for the alimentary tract and metabolism (19.2%). Participants with university degree or higher education, who had chronic disease before pregnancy, who had one or more previous pregnancies, who had a planned current pregnancy, who were in the second or third trimester, and who were unemployed were likely to use at least one medication.

Conclusion: Medication use is common in pregnancy and is associated with several maternal factors. The factors affecting medication use during pregnancy should be considered in order to incorporate them into clinical pharmacy practice when treating groups that need to be followed more closely in terms of drug use.

Keywords: Medication use, Pregnancy, Prevalence, Herbal product

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INTRODUCTION

Information about medication use during pregnancy and the attitudes of pregnant women about both drugs and over-the-counter (OTC) or herbal products are important. This information leads to the adoption of counseling services and the development of strategies to support pregnant women, which helps them make informed decisions. Identifying commonly used medications in pregnancy is also critical to determine research priorities regarding drug safety.

During pregnancy, the rapidly growing fetus is vulnerable at the cellular level, affecting cell growth and division. The potential unwanted effects of anything consumed present a challenge and concern (Bánhid, Lowry, & Czeizel, 2005). The most common concerns due to drug use by pregnant women were reported to be having a child with a birth defect, miscarriage, or their child developing an allergy (Mulder et al., 2017).

Medication use (both physician-prescribed and non-prescribed) and over the counter (OTC) drugs or herbal medication use among pregnant women vary all over the world. Women may use medication due to chronic disorders that need to be treated or due to pregnancy-related medical conditions that require pharmacological treatment (Florio, DeZorzi, Williams, Swearingen, & Magalski, 2021; McCarter-Spaulding, 2005).

Medication use during pregnancy is a concern because drug pharmacokinetics are altered, and drugs may cause harm to the fetus by passing through the placenta. In this context, proper management of medication use is crucial for public health. Therefore, pregnant women hesitate to use medication and exhibit attitudes and behaviors that can lead to different outcomes, such as termination of a desired pregnancy, unwillingness to use drugs for nausea, non-compliance with prescribed medication, preference for herbal products or OTC drugs, and other unspecified self-medication methods (Zafeiri, Mitchell, Hay, & Fowler, 2021; Baggley, Navioz, Maltepe, Koren, & Einarson, 2004; Coren, 2007; Erebara, Bozzo, Einarson, & Koren, 2008; Florio et al., 2021; Glover, Amonkar, Rybeck, & Tracy, 2003; Undela, Joy, Gurumurthy, & Sujatha, 2021; Holst, Wright, Haavik, & Nordeng, 2009).

It was reported that 81.2% of pregnant women used at least one medication, either prescribed or OTC drugs, and over 65% of pregnant women used self-medication with OTC drugs (Lupattelli et al., 2014). The use of drugs including OTC was reported in 88.8% of pregnancies in the USA, and the prevalence of prescribed medication use in pregnancy ranged from 26% to 93% in Europe, changing from country to country (Araujo et al., 2021; Lupattelli et al., 2014). The variability among countries may be due to the different designs and methodology of the studies.

This study aimed to determine medication use in pregnant women consulting in the outpatient gynecology and obstetrics clinic of a tertiary healthcare facility and to assess their attitudes according to sociodemographic and medical characteristics.

MATERIAL AND METHODS

Study design and participants

This cross-sectional study was conducted between October 2019 and March 2020. Pregnant women who visited the gynecology and obstetrics outpatient clinic of a university hospital for routine antenatal pregnancy care appointments or any symptomatic indications at any gestational week were eligible to take part in the study. Women who did not speak Turkish or were unable to complete the questionnaire were excluded.

The minimum sample size required for the study was calculated to be 461 pregnant women using Epi Info version 7.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA) applying the following assumptions: estimated prevalence of women who use at least one medication (excluding vitamin/mineral supplements) during pregnancy of 60%, confidence interval of 95%, margin of error of 5%, and additional non-response rate of 25%.

Data collection

The data of this study were collected between October 2019 and January 2020 through an anonymous self-completed questionnaire, consisting of three sections with 35 items. Pre-testing of the questionnaire was conducted with a smaller sample (50 subjects) to determine whether participants were interpreting questions as intended. After a few modifications to the phrasing of the items, the questionnaire was finalized with multiple choice and open-ended questions. The first section of the questionnaire included items investigating the sociodemographic (age, education level, employment status, residence, alcohol use, and smoking) and medical characteristics (presence of an illness, gravidity, gestational age [according to obstetric ultrasonography], and previous pregnancy history) of participants. The second section consisted of items concerning the use of medications with or without prescription (excluding vitamin and/or mineral supplements) and use of herbal products during the current pregnancy. Finally, the third section of the questionnaire was to explore the subjects' attitudes regarding medication use (i.e., consulting their physician regarding medication use, following the physician's advice, and informing their physician regarding chronic diseases and use of medications). Illiterate pregnant women completed the questionnaire with help from physicians.

Ethical considerations

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Clinical Trials Ethics Committee of S.B. Istanbul Medeniyet University Göztepe Research and Training Hospital (Date: 28.08.2019 / No. 2019/0329). The eligible pregnant women during the study period were informed and invited to participate in the study, and informed consent was obtained from those who agreed to participate.

Data analysis

The data analysis was carried out using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA). The sociodemographic characteristics of the participants were described with frequencies, proportions, and means and stan-

standard deviation values. Medication use during pregnancy was demonstrated without vitamin and/or mineral supplements. The percentages of the drugs were classified according to the Anatomical Therapeutic Chemical (ATC) classification system of the World Health Organization (WHO, 2020). Respondents were categorized as users if they used at least one medication in their current pregnancy, whereas others were categorized as non-users. Associations between medication use during pregnancy and maternal factors as independent variables (education, gravidity, presence of a chronic/long-term disease before pregnancy, planned pregnancy, age, gestational age, and working status) were investigated performing the binary logistic regression test (Backward LR method). Adjusted odds ratios, 95.0% confidence intervals, and p values of all independent variables included in the model were presented. P-value < 0.05 was considered statistically significant for all analyses.

RESULTS

The study population

A total of 520 pregnant women between October 2019 and March 2020 were invited to participate in our study; 485 agreed to participate (response rate: 93.3%). Sociodemographic characteristics of the sample are presented in Table 1. The mean age of the participants was 29.2 ± 5.15 years (ranged from 19 to 45 years). The majority were primary (35.4%) or secondary (28.8%) school graduates, unemployed (80.9%), and living in urban areas (98.9%). A total of 79 participants, 16.8% of whom had a chronic/long-term disease (diabetes %35.8, hypo/hyperthyroidism 20.9%, asthma 12.3%, cardiovascular disease 11.1%, rheumatologic disease 4.9%, and others 15.0%), and 12.8% of whom were current smokers. Most of the pregnant women were multigravida (73.4%), had a planned current pregnancy (66.7%), and did not use assisted reproductive techniques (98.1%). Of the 116 women (27.8%) who had a history of pregnancies not completed, 93.9% had had a miscarriage.

Behaviors and attitudes regarding medication use

After excluding vitamin and mineral supplements, 45.6% (n=213) of the participants used at least one medication during the current pregnancy. According to the ATC classification system, the most frequent classes of medications were anti-infective drugs for systemic use (J), agents for the nervous system (N), agents for the alimentary tract and metabolism (A), and systemic hormonal preparations (excluding reproductive hormones and insulin) (H) (32.8%, 20.8%, 19.2% and 8.4% respectively). Out of 469 respondents, 10.5% (n=49) used medication for the treatment of chronic/long-term diseases before pregnancy, and 37.5% (n=176) used medication for acute/short-term diseases (Table 2). The most frequently used medications for chronic/long-term diseases were levothyroxine (n=21, 31.3%), methyldopa (n=20, 29.8%), and metformin (n=18, 29.3%).

Among the participants, 97.5% of whom indicated that they would consult a physician regarding medication use, 90.1% of the participants informed their physician regarding the presence of a chronic disease and/or use of medications, 87.4% of whom would use medication in accordance with the physician's advice, 95.6% of whom would consult a physician in case

Table 1. Characteristics of the study participants.

		n	%
Age (years)	29.20 ± 5.15 (Mean ± SD)		
Education	Illiterate	13	2.8
	Literate (no formal education)	39	8.3
	Primary	166	35.4
	Secondary	135	28.8
	High school	46	9.8
	University	61	13.0
	Postgraduate	9	1.9
Working status	Unemployed	390	80.4
	Employed	95	19.6
Employment	Public-related sector	32	36.0
	Self-employed	14	14.0
	Non-public related sector	45	47.0
	Other	3	3.0
Residence	Urban	465	98.9
	Rural	5	1.1
Smoking	Yes	61	12.8
	No	366	80.9
	Quitted during pregnancy	30	6.3
Alcohol use	Yes	0	0.0
	No	460	98.1
	Quitted during pregnancy	9	1.9
Presence of a chronic/long-term disease before pregnancy	Yes	79	16.8
	No	390	83.2
Planned pregnancy	Yes	315	66.7
	No	157	33.3
Gravida	First pregnancy	127	26.6
	>1	351	73.4
Utilization of assisted reproductive techniques	Yes	9	1.9
	No	458	98.1
Gestational age	1 st trimester	36	7.8
	2 nd trimester	155	33.5
	3 rd trimester	271	58.7
History of pregnancies not completed	Yes	116	27.8
	No	301	72.2
Cause of pregnancies not completed	Dilatation and Curettage	7	6.1
	Miscarriage	108	93.9

Table 2. Use of medications[†] and herbal products.

		n	%
Any medication use	Yes	213	45.6
	No	256	54.4
Medication use for chronic/long-term diseases	Yes	49	10.5
	No	416	89.5
Medication use for acute/short-term diseases	Yes	176	37.5
	No	293	62.5
ATC class[‡] of medications	A	48	19.2
	B	14	5.6
	C	20	8.0
	G	9	3.6
	H	21	8.4
	J	52	20.8
	N	82	32.8
Use of herbal products	Yes	18	3.9
	No	445	96.1

[†]Vitamin/mineral supplements were not included.
[‡]ATC class A: Alimentary tract and metabolism - B: Blood and blood forming organs - C: Cardiovascular system - G: Genitourinary system and reproductive hormones - H: Systemic hormonal preparations (excluding reproductive hormones and insulin) - J: Anti-infective for systemic use - N: Nervous system - R: Respiratory system.

of drug-related side effects, and 81.3% of whom attended prenatal doctor visits regularly.

Table 3 shows the results of multiple logistic regression analysis investigating the associations between characteristics of the participants and medication use during the current pregnancy. The model was statistically significant (Log likelihood = -258.06, $\chi^2 = 84.38$ (10 df), $p < 0.001$) and explained 23.9% of variance in medication use (Nagelkerke $R^2 = 0.220$). The probability of using at least one medication was statistically higher in women who had a chronic disease before pregnancy, had one or more previous pregnancies, had a high level of education (university or more), had a planned current pregnancy, were in the second or third trimester, and were unemployed.

DISCUSSION

This study included a relatively large population of pregnant women with a high response rate to determine the prevalence of medication use and factors affecting their attitude regarding medication use in Turkey. However, to our knowledge, there is sparse information about the maternal characteristics associated with medication use in the Turkish pregnant population.

We found that 45.6% of the participants used at least one medication during the current pregnancy, excluding vitamin and mineral supplements. This ratio is low when compared to the prevalence of medication use in other studies. The rate

Table 3. Results of logistic regression models estimating the associations between maternal factors and medication use[†] during pregnancy.

Variables	AOR*	95.0% CI	p Value	
Age (years)	0.971	0.926 – 1.018	0.217	
Education	High school or less	Reference		
	University or more	2.360	1.313 – 4.243	0.004
Gravida	First pregnancy	Reference		
	>1	1.778	1.073 – 2.945	0.025
Presence of a chronic/long-term disease before pregnancy	Yes	7.543	3.835 – 14.838	<0.001
	No	Reference		
Planned pregnancy	Yes	1.790	1.058 – 2.737	0.015
	No	Reference		
Gestational age	1 st trimester	Reference		
	2 nd trimester	7.283	2.427 – 21.855	<0.001
	3 rd trimester	7.349	2.523 – 21.411	<0.001
Working status	Unemployed	2.162	1.164 – 4.016	0.015
	Employed	Reference		

[†]Vitamin/mineral supplements were not included.
*Binary logistic regression test (Backward LR method) was performed (Log likelihood = -207.14, $\chi^2 = 76.82$ (8 df), $p < 0.001$).
AOR= Adjusted Odds Ratio, CI=Confidence interval.

of medication use during pregnancy differs among the countries. While 88.8% of all pregnancies use medications in the USA (Mitchell et al., 2011), in Europe, prevalence estimates of prescribed medication use vary, ranging from 26% (Serbia) to 93% (France) (Araujo et al., 2021; Lacroix et al., 2009; Odalovic, Vezmar Kovacevic, Ilic, Sabo, & Tasic, 2012). A study conducted in an eastern Ethiopia tertiary hospital found a somewhat similar pattern of non-supplemental drug utilization and found the prevalence ratio to be 15.12% (Bedewi, Sisay, & Edessa, 2018).

Avoiding drug use during pregnancy may be dangerous and the medications used during pregnancy can prevent adverse outcomes not only for the mother but also for the fetus. Pregnant women underestimate the benefit of medicine use in some circumstances such as influenza, acute respiratory system or urogenital system infections, and hyperemesis gravidarum. During the pregnancy period, approximately 8% of pregnant

women need to use drugs due to chronic diseases and the most common chronic diseases accompanying pregnancy are epilepsy, diabetes mellitus, asthma, hypertension, thyroid diseases, migraine, and depression (Czeizel, 1999). In our study, the most commonly used medications for chronic/long-term diseases were related with their chronic health problems, such as disorder of the thyroid gland, hypertension, and diabetes mellitus, similar to the study conducted in India (Undela et al. 2021). It is pleasing that the majority of the patients use medication after consulting a physician. Our study also showed that anti-infective drugs and agents for the nervous system (such as paracetamol) and agents for the alimentary tract and metabolism (such as antacids) were the leading drugs used for acute/short-term diseases, as also shown by previous research. Analgesics, antacids, nasal decongestants/anti-allergic medications, and systemic antibiotics were reported to be the dominant medications (Czeizel et al., 2014; Navaro et al., 2018; Nordeng, Yström, & Einarson, 2010; Palmsten et al., 2015; Thorpe et al., 2013).

The study provided here will guide healthcare professionals regarding medication use during pregnancy. The determination of predictors for medication use in pregnancy could be useful in strategy development and to identify vulnerable groups of women who have a higher chance of being exposed to medications. Drug safety studies will also focus on the most prominent drug groups used during pregnancy. In our study we found medication use for health problems present before pregnancy to be the most important causative factor for medication use in pregnancy, similar to the study by Odalovic et al. (Odalovic et al., 2012) performed in Serbia.

The factors related to socio-demographic variables, such as level of education, history of previous miscarriage, and medical problems, are potential risks of using medication. Previous studies also reported that potential socio-economic and lifestyle predictors of unsafe medication use in pregnancy were place of residence, being single, being a smoker, being unemployed, or being nulliparous (Lee et al., 2006; Odalovic et al., 2013). Contrary to a previous study indicating an association between lower maternal education and more prevalent use of medication during pregnancy, in our study higher education was associated with a higher use of medication (Czeizel et al., 2014).

Interestingly, most women in this study were reluctant to use herbal products during pregnancy and this was reflected in the low percentage of herbal product users (3.9%). This shows a more conservative attitude than studies that reported the use of herbs during pregnancy in British, Italian, and Norwegian women as 57.8%, 50%, and 36%, respectively (Holst et al., 2009; Lapi et al., 2010; Nordeng & Havnen, 2004). This ratio is similar to results reported in Saudi Arabia (4.6%) (Zaki & Albarraq, 2014). In a recent study conducted in a city in the Central Black Sea region of Turkey, it was reported that almost half of women use at least one herbal product during pregnancy (Kissal, Çevik Güner, & Batkin Ertürk, 2017). The possible adverse effects of herbal products and the large disparity between different studies on the risk factors in pregnancy could result from a restrictive attitude, as reported before (Holst et al., 2009; Kebede, Gedif, & Getachew, 2009; Tiran, 2005). Baggley et al. reported that more than half of the pregnant

women who experience nausea and vomiting prefer to use herbs instead of medication (Baggley et al., 2004). Healthcare providers can improve the quality of life of pregnant woman by providing comprehensive information to those who have any disease.

Limitations

The limitations to this study were as follows: First, the information was collected by self-reporting, and patients' responses may have been subject to reporting bias. Response bias is also possible, as in all surveys, because respondents may answer questions as they are expected to rather than describing how they actually behave. However, as the data is collected through an anonymous survey, there is a low probability of this occurring. Second, the generalizability of our findings to other populations in other geographic areas may need to be established. We conducted a survey among participants living in a city. Their knowledge and attitudes about use of medications may be different from other pregnant women living in other parts of the country. However, our findings were in accordance with other findings reported.

The strengths of this study were that this is the first data regarding knowledge and attitudes about medication use among pregnant women in Turkey. Moreover, the high response rate and the inclusion of a representative sample of the population provide important insights into knowledge, attitudes, and practices regarding medication use.

CONCLUSION

In conclusion, several maternal characteristics were found to be associated with medication use during pregnancy. The pregnant women in our study seemed to prefer to consult their physician regarding medication use and adhere to treatment regimens. It is important to communicate with pregnant women about possible harms related to herbal products as well as medications.

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

Ethics Committee Approval: This study was approved by the Clinical Trials Ethics Committee of S.B. İstanbul Medeniyet University Göztepe Research and Training Hospital (Date: 28.08.2019 No: 2019/0329).

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Assessment of knowledge and attitude of undergraduate students' of Ahmadu Bello University Zaria towards depression

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ABSTRACT

Background and Aims: Depression is a leading cause of disability worldwide and was projected to become the second most burdensome disease by 2020. While there is growing literature on the mental health literacy of adults, there has not been a parallel interest in mental health awareness of young people in Nigeria. Thus, the objective of this study was to assess the knowledge of and attitudes towards depression in undergraduate students.

Methods: The study was a cross-sectional survey conducted from August to November 2019. Ethical approval for the study was sought from the University's research ethics committee. Consenting students across all levels were then sampled and recruited. Participants were presented with the 'friend in need' questionnaire designed to elicit the participants' recognition of mental health disorders depicted in the form of a vignette.

Results: Out of the 415 questionnaires distributed, only 365 were adequately filled indicating an 88% response rate. The majority of the participants were female (62.5%) and a total of 132 respondents (36.2%) correctly identified and labelled the depression vignette. Insomnia was the most identified symptom (29%) of depression by the participants. More than one-quarter (30%) of the participants reported that they would be extremely worried about the depressed character and believed it will take the character longer than a few months to recover (54%). Friends were the most recommended source of help (33.1%), followed by professionals (30.7%) and then others.

Conclusion: It was established that university undergraduate students do not have adequate knowledge about depression.

Keywords: Depression, Knowledge, Attitude, Undergraduate-students, Nigeria

INTRODUCTION

Depression and anxiety symptoms are reported to be common among university students in many regions of the world, which impacts their quality of life and ability to receive education; the extent of the problem of depression and anxiety among students in low- and middle-income countries (LMICs) is largely unknown (January, Madhombiro, & Chipamaunga, 2018). Depression is a leading cause of disability and was projected to become the second most burdensome disease by 2020 (Reddy, 2010). There's a well-established link between suicide and mental disorders, in particular, depression and alcohol use disorder in high-income countries, many suicides happen impulsively in moments of crisis with a breakdown in the ability to deal with life stresses, such as financial problems, relationship break-up or chronic pain and illness; Suicide was the second leading cause of death among

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young people aged 15-29 years, after road injury (WHO, 2019). Also, depression is a significant contributor to the global burden of disease and affects people in all communities across the world. It has been estimated to affect 350 million people: the world mental health survey conducted in 17 countries found that on average about 1 in 20 people reported having an episode of depression in the previous year (Marcus, Yasamy, Ommeren, Chisholm & Saxena, 2012). Stressful life events are well-known risk factors of depression and are suggested to be the major causative factor of depressive symptoms (Dawood, Mitsu, Ghadeer, & Alrabodh, 2017). Mental health literacy has been described as "knowledge and beliefs about mental disorders which aid their recognition, management or prevention." Although there is a large body of research examining mental health literacy of various populations, with depression extensively studied, most research focuses on developed countries, with inadequate examination of this area in the non-western developing world (Amarasuriya, Jorm, & Reavley, 2015). The prevalence of depression in LMICs is similar to that in developed countries; however, reliable data are unavailable in most countries in sub-Saharan Africa (Kutcher, 2015). In recent years few studies reported high prevalence of depression among health care students and the presence of depressive symptoms over their studying years (Dawood *et al.*, 2017). Exposure to stressful situations is a decisive factor that increases the risk of suffering from mental disorders, and in this sense, the pressure experienced during the years of university studies can be particularly stressful for many young people (Miron, Goldberg, Lopez-Sola, Nadal & Armario, 2019). Depression has long been recognized as an important target of intervention in psychology and psychiatry, but these fields have focused efforts primarily on treatment rather than prevention (Mc Laughlin, 2011). Although effective preventive intervention methods targeting high risk groups have been developed, they have thus far had poor reach and sustainability in the community (Tamez, Du & Shah, 2016). While, there is a growing literature on the mental health literacy of adults, there has not been a parallel interest in mental health awareness of young people in Nigeria (Aluh, Anyachebelu, Anosike & Anizoba, 2018). Thus, the objective of this study was to assess the knowledge of and attitudes towards depression of undergraduate students in a Nigerian University.

MATERIAL AND METHODS

The study was a cross sectional descriptive survey carried out among undergraduate students of Ahmadu Bello University in Zaria, Nigeria, from August to November 2019, after obtaining ethical approval from the University's Human Research and Ethics Committee. A sample size of 380 students was calculated for the study using the Rao soft online sample size calculator, maintaining a confidence interval of 95% and an estimated population of greater than 20,000. After allowing for attrition, a sample size of 415 was finally decided upon. A multi-stage sampling technique was used for the study. During the first stage, stratified sampling was used to calculate the number of participants required from each of the 13 faculties of the institution. This was followed by convenient sampling, which was then used to select participants from each faculty until

the required sample size was achieved from each stratum. Any undergraduate or diploma student of the university, who was willing to participate was eligible for inclusion.

A self-administered structured questionnaire adapted from an earlier study (Aluh *et al.*, 2018) was used to collect data. The questionnaire had two sections: the first section collected data on the students' demographic characteristics including age, gender, year of study etc. The second section assessed the participants' knowledge of and beliefs about depression through the use of both open and closed ended questions. To assess participants' knowledge, a case-vignette was used about a young male student suffering from fatigue, insomnia and weight loss, and whose academic performance had worsened over the past few months. After respondents had read the case, they were required to answer several open-ended questions about the case including what they thought was wrong with him, what gave them the strongest hints about it, how long it would take him to get better, whether he would require help to cope with the situation, and who could help him. The closed-ended questions, which were rated on a four-point likert scale enquired about respondents' degree of worry towards the character's emotional wellbeing. Possible answers were: not at all worried, a little bit worried, quite worried and extremely worried. To collect data, respondents were approached in the evenings while they were in the hostel after lectures were over. The data collector introduced herself and briefly explained the aim of the study, after which she invited them to participate. If they provided verbal consent, she gave them a copy of the questionnaire and returned after five days to retrieve the questionnaires.

Quantitative data collected was analyzed using Microsoft Excel 2016 software, and the results were presented as descriptive statistics using charts and tables. The open-ended responses were categorized based on similarity of thematic content and their frequencies/percentages that were reported. Responses to the open-ended question about what was wrong with the vignette character were coded according to the presence or absence of key words such as "depressed/depression", "emotional" or "psychological". Where participants were asked to state whose help they believed the vignette character required to cope with his problem, their responses were grouped into five categories. The first category covered professionals and included responses mentioning psychiatrists, psychologists, therapists etc. The second category was for family, which included parents, siblings, cousins, uncles and aunties. The friend category included friends, classmates and peers. A spiritual category was also created which included God, priests, pastors or imams etc. The "other" category contained responses that did not fall into any of the four previously listed categories.

RESULTS

Demographic characteristics of respondents

Out of the 415 questionnaires distributed, only 365 were adequately completed, indicating a response rate of 88%. The students were aged between 15 and 35 years of age, with a modal age of 19 years. Furthermore, only around 40% of them were male, with the majority being female. The majority of the respondents were in their second year (40.1%) and the small-

est group were 6th year students (1.4%), followed by others (1.7%) including those doing associate degrees, diplomas or other certificate courses as seen in Table (1) below:

Characteristics	Variables	n (%)
Gender	Female	228 (62.5)
	Male	137 (37.5)
Age	15-20	169 (46.3)
	21-25	151 (41.4)
	26-30	42 (11.5)
	Above 30	3 (0.8)
Faculty	Administration	27 (7.4)
	Law	20 (5.5)
	Medicine	62 (17.0)
	Pharmacy	31 (8.5)
	Veterinary medicine	24 (6.6)
	Engineering	31 (8.5)
	Environmental design	10 (2.7)
	Agriculture	26 (7.1)
	Physical sciences	25 (6.9)
	Life sciences	29 (7.9)
	Education	32 (8.8)
	Arts	28 (7.7)
	Social sciences	20 (5.5)
Year of Study	1 st Year	51 (14.0)
	2 nd Year	146 (40.1)
	3 rd Year	40 (11.0)
	4 th Year	66 (18.1)
	5 th Year	50 (13.7)
	6 th Year	5 (1.4)
	Other	6 (1.7)

Identification and labelling of the vignette

A total of 132 respondents (36.2%) correctly identified and labelled the vignette character as suffering from depression, while the other respondents labelled the vignette character as suffering from emotional distress (25.2%), psychological distress (5.4%), and others (33.2%)

Identification of symptoms presented in the vignette

The vignette used provided clear references to the most common symptoms of a Major Depressive Episode from the DSM-IV. Most of the respondents 219 (60%) were able to correctly identify some of the symptoms as a sign that something was wrong with the character depicted in the vignette, even when they did not know exactly what was wrong with him. Insomnia was the most identified symptom of distress (23.2%). Others were lack of concentration (17.2%) and fatigue (15%), while the least identified symptom was weight loss (4.1%) as shown in table (2) below:

Respondents estimated degree of concern and recovery times for the character in the vignette

Participants indicated their degree of worry for the character in the vignette using a four-point Likert-type scale, ranging from 1 (not at all worried) to 4 (extremely worried) as shown in figure 1.

Table 2. Identified symptoms by respondents (n=365).

Identified Symptom	n (%)
Constantly tired	54 (15)
Difficulty in sleeping	85 (23.2)
Lack of concentration	63 (17.2)
Weight loss	15 (4.1)
Lack of interest	51 (14)
Loss of appetite	25 (6.8)

Just a little over half of the respondents (54%) said that they would be quite or extremely worried for the character. When the respondents were asked how long they thought it would take for the character to ‘get better’ using a four-point Likert-type scale from 1 (a few days) to 4 (longer than a few months), a majority of them (54%) felt that the character in the vignette would require more than a few months to get better, as shown in figures 1 and 2 below:

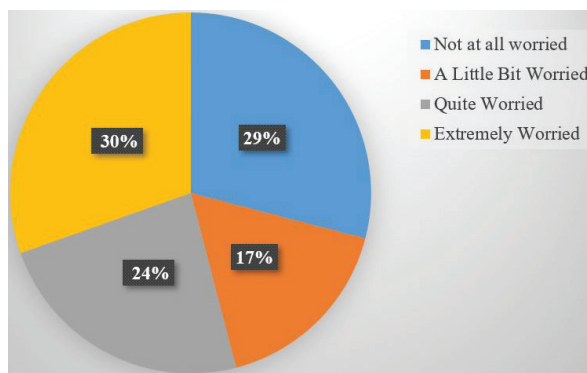


Figure 1. Respondents degree of concern for the vignette (n=365).

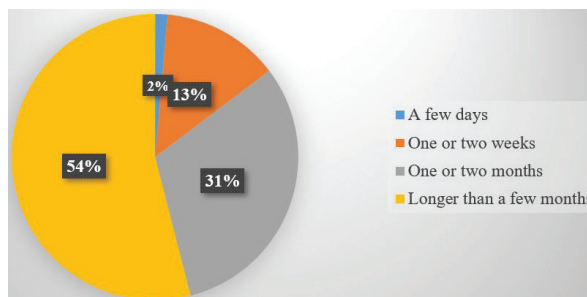


Figure 2. Respondents estimated recovery time for the character in the vignette (n=365).

Recommended sources of help for the vignette character

332 of the respondents (93.4%) answered in the affirmative that the character would need help to cope with the situation. Details of their answers can be seen below in table 3. Respondents’ identified friends as the most common source of help (33.1%), followed by professionals (30.7%) and then family (28.4%).

Table 3. Recommended sources of help by respondents for the vignette character (n=332).

Recommended Source	n (%)
Friends	110 (33.1)
Professionals	102 (30.7)
Family	94 (28.4)
Spiritual	9 (2.7)
Others	17 (5.1)

DISCUSSION

This study aimed to assess the knowledge and perceptions of undergraduate students towards depression, with a specific focus on their abilities to identify a depressed individual and their attitudes about seeking help. Findings from this study showed that the majority of respondents were unable to identify key symptoms of depression or label the character as suffering from depression. Previous studies have also reported that females are more likely to correctly label depression-based vignettes and endorse concern over a depressed peer than males (Burns & Rapee, 2006). Only a little over a third of this study's respondents were able to correctly label the vignette. This was relatively higher when compared to a previous study conducted among Nigerian secondary school students where only 10.4% of the respondents correctly identified the character as suffering from depression (Adeosun, 2016). This may be explained by the increased emotional and mental development of undergraduates through experiences, when compared to secondary school students. Other studies conducted in Sri Lanka and China have also reported that undergraduate students' ability to recognize depression is low: 9.5% and 12%, respectively (Amarasuriya *et al*, 2015; Lei, Xiao, Liu & Li, 2016). Some of the more common wrong labels for the character in the vignette were 'Emotional distress' and 'Worry'. This is in line with findings from a previous study where depression vignettes were mislabeled as 'emotional problems' and 'stress' (Adeosun, 2016; Gureje, Lasebikan, Ephraim-Oluwanuga, Olley & Kola 2005). The ability of adolescents to 'label' depression has been linked to their urgency in seeking help and who they seek help from (Aluh *et al*, 2018). Consequently, their inability to recognize depression leads to poor help-seeking attitudes. Proper diagnosis often leads to effective treatment, which in turn improves the patient's quality of life and decreases the morbidity and mortality associated with the condition (Yang *et al*, 2015). However, the fact that almost all of the undergraduates surveyed reported that the 'depressed' case needed to get help from another person indicated that they had some awareness of the severity of the symptoms portrayed. The most common source of help recommended by them in this study was friends. This is contrary to some reported instances where elders were the most recommended sources of help (Kutcher *et al*, 2015) This suggests that there may be a disconnect between the older generation and young adults. Thus, efforts should be made to improve relationships and communication between these populations so parents can be involved in improving mental health awareness and support

proper help seeking attitudes. However, only a very little percentage of surveyed respondents suggested God as a source of help for the 'depressed' case. This is inconsistent with the fact that in Nigeria, mental disorders are usually attributed to supernatural forces and spiritual healers are preferred sources of treatment over medical advice (Gureje *et al*, 2005; Egwuonwu, Okafor, Ogunyemi, Yusuf & Adeyemi, 2019). Almost a third of respondents recommended seeking professional help from psychiatrists, therapists, counsellors or psychologists. This was inconsistent with the findings from a similar study conducted in secondary school students where only four respondents recommended professional help from a psychiatrist or psychologist (Aluh *et al*, 2018).

Limitations of this study include the fact that the research relied on the use of a written case vignette. The extent to which such data can be translated into what actually is likely to happen in the real world is unclear. In addition, the questionnaire was administered in English, and this may have affected respondents' abilities to correctly label the vignette since all the respondents were non-native speakers of English.

CONCLUSION

The knowledge and perceptions of the respondents towards depression was found to be inadequate. This implies that the need to improve mental health awareness in undergraduates of the university is urgent because there has been a rise in the number of cases of suicides in the study area.

Friends were the most recommended source of help. As such, this group of people can be targets of interventions to increase mental health awareness. Schools are important settings for such intervention programs, because they are the places where young people spend most of their time. These programs should also target teachers and school counsellors who work with young people. They spend a significant amount of time with young people and should therefore be able to recognize first signs and symptoms and give first aid and support.

Abbreviations

LMICs= Low- and Middle-Income Countries

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

Ethics Committee Approval: This study was approved by the Ahmadu Bello University Human Research and Ethics Committee (No: ABUCUHSR/2019/UG/002).

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A review on hydrogen sulfide: Is it pro-nociceptive or anti-nociceptive?

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ABSTRACT

Pain is sensed by the activation of painful nociceptors in the periphery or by pain mediators, such as bradykinin, serotonin, histamine, and prostaglandin, released from the damaged tissue, afferent transmission to the medulla spinalis, and by transmission stages to the high centers over the dorsal horn. Pain, which was seen as only a warning sign in the past, is now accepted as a phenomenon in itself that needs to be treated and the search for new, stronger active substances with fewer side effects in the treatment of pain is in demand. Hydrogen sulfide (H₂S) is a modulator of T-type Ca²⁺ channels, especially in Cav3.2, which are known to play a critical role in the processing of pain. H₂S can also show an anti-nociceptive effect by opening K⁺ channels and blocking nociceptors. Exciting preclinical data has demonstrated that H₂S-derived Non-steroidal anti-inflammatory drugs (NSAIDs) and analgesic agents can be used to treat various types of pain. H₂S increases the resistance of gastric mucosa against injury occurred by drugs used for pain relief and accelerates its repair, so it provides an advantage to derivatized drugs. In addition, H₂S donors have also been shown to induce analgesia through μ -opioid receptors. Based on the studies, it is thought that the combination of H₂S with opioid receptor agonists may provide an additive or even synergistic analgesic effect. It is estimated that the modification of H₂S, with currently used analgesic drugs to prevent various side effects and increase analgesic effects, is a promising and wise approach.

Keywords: Hydrogen sulfide, Pain, Analgesic effect

INTRODUCTION

Gasotransmitters are molecules that regulate physiological and pathophysiological effects in mammalian tissues. Nitric oxide (NO) was the first molecule to emerge as an endogenous gas with biological activities, followed by carbon monoxide (CO), and H₂S (Cunha et al.; 2008; Shefa et al., 2017). In recent years, the number of studies aiming to understand the physiological effects and mechanism of action of H₂S has increased rapidly.

Previously, H₂S was defined as a colorless, poisonous gas with a rotten smell. However, it was later discovered that it was produced by enzymatic and non-enzymatic reactions in mammalian tissue and was responsible for many different biological activities (Li, Liu, Wang, Zhang, & Wang, 2020). H₂S is a molecule that is slightly larger than water and is similar to the water molecule. However, H₂S is less polar than water because the sulfur atom is less electronegative than the oxygen atom. Therefore, intramolecular forces are weaker and the melting and boiling point is lower than water (Caliendo, Cirino, Santagada, & Wallace, 2010). It also has acidic properties and has a high solubility in water. H₂S is found in two forms; mostly in neutral molecular form (H₂S) and mono ionized form (HS⁻) in physiological conditions (pH 7.4) (Zheng et al., 2017).

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H₂S biosynthesis

Cysteine is the main source of H₂S in mammals and it is synthesized by the desulfurization of L-cysteine primarily with cystathionine β-synthetase (CBS) and cystathionine lyase (CSE) (Wang, 2002; Predmore, Lefer, & Gojon, 2012; Donatti et al.; 2014). CBS enzyme is expressed in the liver, kidneys, brain (mostly Bergmann glial cells and astrocytes), ileum, uterus, placenta, and pancreatic islets. The expression of the CSE enzyme occurs in the liver, kidneys, thoracic aorta, ileum, portal vein, uterus, brain, pancreatic islets, and placenta (Kimura, 2011). 3-mercaptopyruvate sulfide transferase (3-MST) is another enzyme that is naturally responsible for the production of H₂S in the body (Huang & Moore, 2015, Kimura, 2011). 3-MST has been localized in the liver, kidneys, heart, lung, thymus, testicles, thoracic aorta, and brain (Kimura, 2011), and it is mostly located in the mitochondrial region, whereas CSE and CBS are mostly found in the cytosol. CBS and CSE enzymes produce H₂S using many different substrates. 3-MST catalyzes sulfur transfer reactions only from 3-mercaptopyruvate (3-MP) to various donors (Predmore et al., 2012). H₂S can also be obtained from glycolysis or direct reduction of glutathione and elemental sulfur (Kolluru, Shen, & Kevil, 2013).

Physiological roles and therapeutic targets of H₂S

The amount of available data on the physiological role of endogenous H₂S is increasing day by day. Many studies showed that H₂S performs physiological effects in a wide concentration range of 10 μM to 300 μM. The first described physiological effect of H₂S is the ability to relax smooth muscles (Cunha et al., 2008, Donatti et al., 2014). Endogenous H₂S has numerous physiological and pathophysiological roles in the cardiovascular, neuronal, gastrointestinal, urinary, and endocrine systems. H₂S has anti-inflammatory, antitumor, ion channel regulator, cardiovascular protective, and antioxidant effects (Fukami, Sekiguchi, & Kawabata, 2017). In addition, it has important roles in tissue repair and healing, apoptosis, cell cycle, mitochondrial function, energy metabolism, obesity, and aging (Rose, Moore, & Zhu, 2017). H₂S regulates insulin secretion from pancreatic beta cells, by activating the K⁺-ATP channel and suppressing L-type Ca²⁺ channel functions (Tang, Wu, & Wang, 2010). However, some studies suggest that H₂S inhibits insulin release, acts as part of a homeostatic mechanism that reduces glucose-induced cellular stress in pancreatic beta cells through its antioxidant properties, and it has been thought that this mechanism protects pancreatic beta cells from excessive-high glucose level-induced apoptotic cell death (Okamoto et al., 2014). Moreover, H₂S stimulates N-methyl-D-aspartic acid (NMDA) receptors, regulates the release of excitatory neurotransmitter and it is involved in the regulation of synaptic plasticity for NMDA receptor-mediated learning and memory (Tang et al., 2010).

H₂S has many therapeutic targets, including Alzheimer's, Parkinson's diseases, acute myocardial infarction, stroke, atherosclerosis, hypertension, erectile dysfunction, metabolic syndrome, diabetes, thrombosis, cancer, heart failure, organ transplantation, Huntington's disease, and peripheral arterial diseases (Predmore et al., 2012; Kimura, 2019). This study aims to draw attention to the role of H₂S in the process of pain formation and treatment.

H₂S and pain

Pain is the body's alarm system that creates reflexes to avoid the harmful agent and may lead to the treatment of damaged tissue. The Association for the Study of Pain (IASP) has described pain as 'an unpleasant feeling experience that occurs due to tissue damage that has occurred or will occur'. This definition emphasizes that pain occurs as a result of complex processes controlled by many different variables. Pain has continued to be one of the most investigated health problems for centuries (Orr, Shank & Black, 2017). Existing drugs do not provide adequate management of pain. Therefore, the search for novel therapeutic agents in the treatment of pain is still ongoing.

Various endogenous mediators such as serotonin, bradykinin, substance P, histamine, NO, and CGRP are known to play a role in the modulation of pain. Recent studies suggest that H₂S may play a role in the modulation as well and may be a new hope for pain management. H₂S has dual or even more complex roles in pain processing. It exhibits a pro-nociceptive effect, yet on the other hand, it produces an anti-nociceptive effect with different mechanisms.

The Goals of H₂S in the process of pain

The targets of H₂S in the anti-nociceptive effects

H₂S and K⁺-ATP channels

Studies have shown that potassium channels mediate the receptors (alfa2-adrenoceptor, opioid, GABA_B, muscarinic M₂, adenosine A₁, serotonin 5-HT_{1A}, cannabinoid, etc.) which play a role in pain modulation and the effects of other anti-nociceptive drugs (nonsteroidal anti-inflammatory and tricyclic anti-depressants, etc.). Many specific K⁺ channel subtypes are involved in the generation of the anti-nociceptive effect, but the most studied type is the K⁺-ATP channel. The opening of K⁺ channels in the peripheral and central nervous system is an important mechanism that mediates the anti-nociceptive effect of many drugs and natural products (Ocaña, Cendán, Cobos, Entrena, & Baeyens 2004; Tsantoulas & McMahon, 2014).

H₂S is thought to have a dual effect in inflammatory hypernociception: 1. It stimulates neutrophil migration and thus produces pro-nociceptive effect, 2. It modulates the K⁺-channels and shows the anti-nociceptive effect by directly blocking the nociceptor sensitivity (Cunha et al., 2008). In other words, H₂S acts as a negative regulator of visceral nociception by activating the K⁺-ATP channels and weakens the pain, while it also induces cytokine release (TNF-α) and produces a peripheral pro-nociceptive effect in relation to neutrophil migration (Tang et al., 2010).

Regarding the anti-nociceptive effect of H₂S, it is claimed that the anti-nociceptive effect induced by sodium hydrosulfide (NaHS), the H₂S donor, is reversed by the K⁺-ATP channel blocker glibenclamide. Also, it is reported that the K⁺-ATP channel opener, pinacidil, potentiates the NaHS-induced anti-nociceptive effect (Distrutti, 2006). These data indicate that K⁺ channels mediate the anti-nociceptive effect of H₂S (Distrutti, 2006; Lucarini et al.; 2018).

H₂S and NO

NO plays complex and different roles in the regulation of pain. Experiments with NO donors have revealed contradictory results because these molecules show both pro-nociceptive and anti-nociceptive effects (Schmidtko, Tegeder, & Geisslinger, 2009; Miculescu & Gordh, 2009). In the literature, NO is described as an important neurotransmitter that plays a role in the nociceptive process. Moreover, experimental data indicates that NO inhibits pain in the peripheral and central nervous systems. The analgesic effect of NO involves the activation of an intracellular signaling pathway including cyclic guanosine monophosphate (GMP) formation, protein kinase G (PKG) activation, and consequently the opening of K⁺ channels. Opening these channels increases the K⁺ current, which causes hyperpolarization of nociceptive neurons. It has also been shown that nitric oxide mediates the analgesic effect of drugs like opioids and NSAIDs (Cury, Picolo, Gutierrez, & Ferreira, 2011; Gomes, Cunha, & Cunha, 2020).

The H₂S donor NaHS-induced anti-nociceptive effect is reversed with NO synthase inhibitors. This data suggests that NO mediates the anti-nociceptive effect of H₂S (Distrutti, 2006; Xu et al., 2019).

H₂S and opioid receptors

Opioids have an inhibitory effect on pain by decreasing the Ca²⁺ influx from the voltage-dependent calcium channels in the neuron membrane, inhibition of adenyl cyclase (AC), and opening of K⁺ channels in the neuron membrane via the Gi protein (Distrutti et al., 2011; Przewłocki & Przewłocka, 2001).

Since H₂S has an effect on potassium channels, the role of opioid receptors in the analgesic effect of H₂S was evaluated with the visceral pain model induced with colorectal distension and a significant reduction in visceral sensitivity and pain was observed (Distrutti et al., 2011). In addition, the contribution of opioid receptors to analgesia was investigated by applying selective μ, κ, and δ opioid receptor antagonists to rats. Of these, CTAP, a selective antagonist of the μ receptor, was found to strongly inhibit H₂S induced analgesia, and hence, H₂S has been shown to induce analgesia via μ opioid receptors. (Distrutti et al.; 2011).

The targets of H₂S in the pro-nociceptive effects

H₂S and Ca²⁺ channels

Ca²⁺ channels play a critical role in the processing of somatic or visceral nociceptive information and pain control (Tang et al., 2010). H₂S is a modulator of T-type Ca²⁺ channels and distinguishes between the different subtypes of these channels (Elies, Scragg, Boyle, Gamper, & Peers, 2016; Fukami et al., 2017). It selectively regulates Cav3.2, but Cav3.1 and Cav3.3 are not affected. In nociceptor neurons, H₂S increases the function of Cav3.2 T-type calcium channels and TRPA1 channels and causes neuronal stimulation followed by pain or hyperalgesia/allodynia (Fukami et al., 2017).

In cystitis models induced on mice, it has been observed that H₂S facilitates the stimulation of sensory neurons through activation of Cav3.2 T-type Ca²⁺ channels in the later stages of the disease and leads to bladder pain. Pretreatment with

DL-propargylglycine, an inhibitor of the CSE enzyme involved in the synthesis of H₂S, eliminates nociceptive changes. This study suggests that targeting CSE or Cav3.2 T-type Ca²⁺ channels for the treatment of pain in patients with interstitial cystitis may be useful in developing a new therapeutic strategy (Matsunami et al., 2011).

H₂S increases the activity of Cav3.2 T-type calcium channels, leading to somatic pain and visceral nociception in the pancreas, colon, and bladder. It is suggested that H₂S mediates colonic nociception by activating Cav3.2 predominantly (Tsubota & Kawabata, 2019). Specifically, the role of Cav3.2 T-type Ca²⁺ channels in H₂S-mediated pain signals was investigated using the genetic deletion method and conclusive evidence is provided that Cav3.2 has an important role in HS-induced somatic and colonic pain (Matsui et al., 2019).

In another recent study, it was hypothesized that H₂S donor NaHS is effective in the treatment or prevention of migraine pain by decreasing in membrane currents through purinergic receptor P2X3 and suppression of ATP-induced calcium signals in trigeminal ganglion neurons (Koroleva et al., 2020).

H₂S and TRP channels

The transient receptor potential ankyrin-1 (TRPA1) is a member of the TRP channel family (Huang & Moore, 2015; Fukami et al., 2017). It is thought to be associated with the H₂S-induced pro-nociceptive process by working with Cav3.2. The transient receptor potential vanilloid 1 (TRPV1) also shows similar effects. It has been suggested that activation of TRPV1 and TRPA1 receptors with H₂S during neuroinflammation may lead to migraine pain by contributing to nociceptive stimulation in primary afferents (Koroleva, 2017). In a study using streptozotocin (STZ) induced diabetic rats, it was suggested that H₂S contributes to the formation of hyperalgesia and this is mediated by TRPV1, TRPA1, and TRPC channels (Roa-Coria et al., 2019). In summary, H₂S-induced hyperalgesia and pro-nociception are associated with the sensitization of both T-type Ca²⁺ channels and TRPA1 and TRPV1 channels (Tang et al., 2010).

However, in a recent study, it was emphasized that activation of primary sensory neurons with TRPA1 may have an analgesic effect, and somatostatin release, which has an inhibitory effect on pain, may be the source of this effect. This study presents new and original data on the analgesic effect of dimethyltrisulphite (DMTS), an organic trisulfite releasing H₂S, is realized by the activation of TRPA1-mediated somatostatin release and sst4 receptors (Pozsgai, Báta, & Pintér, 2019).

Also, it has been shown that polysulfide, an endogenous sulfur compound produced by the oxidation of hydrogen sulfide, has dual roles in the regulation of inflammatory pain through TRPA1 activation. It causes pain due to inflammation in the early stage but then relieves pain due to oxidative stress (Oguma, Takahashi, Okabe, & Ohta, 2021).

New approaches in the treatment of pain and H₂S

Recent studies have shown that H₂S modulates the inflammatory process. H₂S donors reduce edema, prevent leukocytes from adhering to endothelium and inhibit pro-inflammatory

cytokine synthesis. It also increases the resistance of the gastric mucosa to injury and accelerates its repair. Considering this information, it is thought that when anti-inflammatory drugs are modified to release H₂S, the efficiency will increase and the toxicity will decrease. Indeed, some NSAIDs have been modified to release H₂S, and preclinical data are promising (Wallace, 2007; Verma, Akhtar, & Singh, 2017).

NSAIDs are used to fight inflammation. However, they cause side effects by lowering the protective prostaglandin level. When H₂S is accompanied by NSAIDs, side effects will decrease and a better anti-inflammatory effect will be obtained (Verma et al; 2017).

A novel H₂S-releasing naproxen derivative, ATB-346 [2-(6-methoxynaphthalen-2-yl)-propionic acid 4-thiocarbamoyl phenyl ester] was developed. It inhibits COX activity and releases H₂S. The low dose of ATB-346 administered once a day was more effective than standard doses of naproxen or celecoxib and it significantly reduced pain in patients with osteoarthritis. It has been found to be safer for gastro-intestinal system (GI) compared to other NSAIDs (Wallace, 2007; De Cicco et al., 2016). In another recent study, sulindac was compared with NOSH-sulindac (AVT-18A) a nitric oxide and hydrogen sulfide donor, for its gastrointestinal safety, anti-inflammatory, and analgesic effects. The results show that NOSH-sulindac is safe for the GI and has a similar level of analgesic and anti-inflammatory effects to sulindac (Kashfi, Chattopadhyay, & Kodela, 2015).

NO and H₂S donor NOSH-aspirin dose-dependently reduced acetic acid-induced writhing responses and carrageenan-induced hyperalgesia and at the same dose, it was found more effective than aspirin. The potent effects of NOSH-aspirin have been associated with the reduction of the production of pro-nociceptive cytokines, such as IL-1b, and the direct activation of K⁺-ATP channels. Also, NOSH-aspirin is capable of reducing the neuronal sensitivity caused by PGE₂ by upregulation of K⁺-ATP channels. The anti-nociceptive effects of NOSH-aspirin on PGE₂-induced hyperalgesia were reversed with glibenclamide (Fonseca, Cunha, Kashfi, & Cunha, 2015).

An experimental study has shown that H₂S can increase the anti-nociceptive effect of dipyrone centrally and peripherally. It was also emphasized that the combination with H₂S donors can reduce the analgesic dose and thus side effects in this study (Erol et al., 2020). Furthermore, EV-34, a new H₂S-releasing ibuprofen derivative molecule, may be advantageous in patients with cardiovascular risk due to the cardioprotective effect of H₂S (Gyöngyösi et al., 2021). One of the most serious side effects of opioid drugs is that they cause addiction. In a study with morphine-dependent mice, it was stated that the use of opioids with H₂S prevented opioid dependence by inhibiting the cAMP pathway (Yang, Wu, Wood, Whiteman, & Bian, 2014). In another study, it was emphasized that H₂S synthase inhibitors such as CSE inhibitor propargylglycine and CBS inhibitor hydroxylamine may be beneficial in preventing morphine tolerance (Cetin et al., 2021).

In addition to NSAIDs, it has been suggested that the combination of H₂S with opioid receptor agonists such as trimebutin

can provide an additive and even synergistic analgesic effect. GIC-1001 (trimebutine 3-thiocarbonyl benzene-sulphate) is seen as a potential drug candidate because it strengthens the analgesic effects of trimebutine with H₂S release *in vivo*. This component shows the spasmolytic and peripheral opioid agonist effects of trimebutine as well as the anti-nociceptive effect of H₂S. Orally administered trimebutine only slightly reduced pain response in colorectal distention, however, GIC-1001 at the same doses significantly reduced nociceptive responses in mice (Cenac et al., 2015).

H₂S doses and administration methods may affect the pain response. It is hypothesized that systemic administration of enzymatic H₂S synthesis inhibitors and slow H₂S-releasing agents/low-dose H₂S-donors may be effective in reducing nociceptive and neuropathic pain (Guo, Li, & Yang, 2020). There are many studies showing that microglia activation mediates the pathogenesis of neuropathic pain. H₂S weakens the activation of microglia and central nervous system inflammation. For this reason, to investigate the effects of H₂S inhalation on neuropathic pain, a chronic constriction injury model of the sciatic nerve was established in mice. The results of this study show that inhaled H₂S inhibits the development of neuropathic pain in mice, possibly by suppressing microglial activation and attenuating the release of inflammatory cytokines. (Kida, Marutani, Nguyen, & Ichinose, 2015).

Oxaliplatin and paclitaxel, which are anti-cancer drugs, induce neuropathic pain in animal models. And it was observed that thiosulfocyanates (allyl-isothiocyanate and synthetic phenyl- and carboxyphenyl isothiocyanate) suppressed pain *via* H₂S release in neuropathic pain induced by anticancer drugs. The anti-neuropathic effect is largely thought to be mediated by the activation of Kv7 channels (Mannelli et al., 2017). GYY4137 is a novel, water-soluble, H₂S-releasing molecule, and it is thought to be an innovative approach in the treatment of resistant pain like neuropathic pain which is induced by chemotherapeutic drugs (Rose et al., 2017; Mannelli et al., 2017). Additionally, a recent study indicated that GYY4137 inhibits paclitaxel-induced neuropathic pain, possibly by blocking the reduction in paclitaxel-induced H₂S formation in tissues (Qabazard et al., 2020). Finally, another study suggested that H₂S could perhaps alleviate neuropathic pain using the NO / cGMP / PKG pathway and μ -opioid receptors (Li et al., 2020).

In another study, the effects of H₂S on bone cancer pain were investigated and a negative correlation was observed between H₂S level and pain scores. H₂S inhalation significantly reduced bone cancer pain by reducing thermal hyperalgesia and mechanical allodynia. This study suggests that H₂S may suppress the development of neuropathic pain in rats, by the deactivation of microglia and inhibition of inflammation in the spinal cord, in which the proliferator-activated receptor gamma/p38/Jun N-terminal kinase (PPAR γ /p38/JNK) pathway is involved (Zhuang et al., 2018).

Also, it has been shown that 4-methylbenzenecarbothioamide (4-MBC), an H₂S releasing thiobenzamide, reduced nociceptive response induced by formaldehyde and induced a long lasting inhibitory effect on carrageenan mechanical allodynia. The an-

ti-inflammatory and anti-nociceptive activities of 4-MBC may be induced by reducing neutrophils recruitment and cytokine and chemokine production (Melo et al., 2019).

More recent studies have investigated the effects of slow-release hydrogen-treated sulfur donors in neuropathic pain and osteoarthritis. Slow-release H₂S donors, allyl isothiocyanate (A-ITC), and phenyl isothiocyanate (P-ITC) have been shown to alleviate mechanical allodynia, grip strength deficits, and depressive-like behaviors accompanying osteoarthritis (Batalá, Cabarga, & Pol, 2020). It has also been suggested that A-ITC and P-ITC administration are effective in the treatment of neuropathic pain possibly by inhibiting inflammation and activating endogenous antioxidant responses (Cabarga et al., 2020).

RESULTS AND RECOMMENDATIONS

H₂S is a gas neurotransmitter that has attracted more attention in recent years. It is known that H₂S regulates physiological and pathophysiological events in many tissues. It has been reported in various studies that it also plays a role in pain modulation, but this role is complicated. H₂S plays dual or more complex roles in pain processes and it can exhibit pro-nociceptive or anti-nociceptive effects depending on the type of pain model and the different targets such as ion channels and receptors. However, recent studies provide exciting evidence that H₂S-derived analgesic agents can be used in the treatment of various types of pain. It is a well-known fact that existing analgesic drugs have various side effects, such as gastrointestinal damage and opioid addiction. Many studies have shown that the addition of H₂S to analgesic drugs reduces these side effects. However, due to the fact that the supraphysiological levels of H₂S have highly toxic effects, extreme care should be taken when developing H₂S-based therapeutic agents. Considering the information obtained, it is thought that H₂S may be the pioneer of a new therapeutic class in the future with its broad biological activity and predicted effects in current experimental studies.

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