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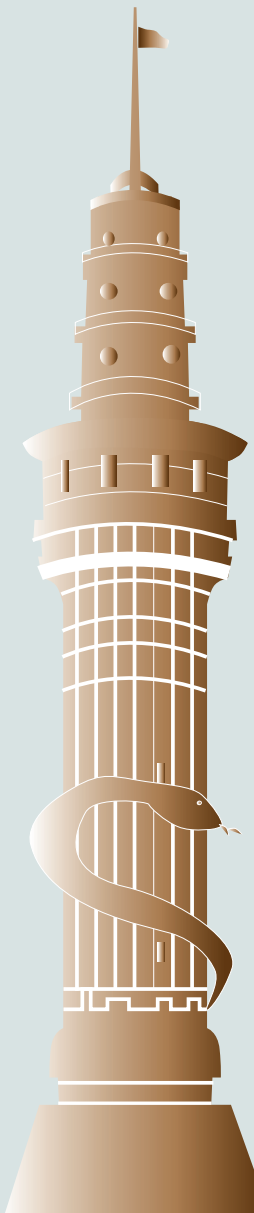

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İstanbul Journal of Pharmacy

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Web of Science - Emerging Sources Citation Index (ESCI)

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This is a scholarly, international, peer-reviewed and open-access journal published triannually in April, August and December.

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İstanbul Journal of Pharmacy

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İstanbul 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.

The target audience of the journal includes specialists and professionals working and interested in all disciplines of pharmaceutical, also medicinal, biological and chemical sciences.

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Reviewers' judgments must be objective. Reviewers' comments on the following aspects are expected while conducting the review.

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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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Statistical analysis to support conclusions is usually necessary. Statistical analyses must be conducted in accordance with international statistical reporting standards (Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J 1983: 7; 1489-93). Information on statistical analyses with specified statistical software and descriptive details of the chemical used should be provided with a separate subheading under the Materials and Methods section.

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Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

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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

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Short Paper	1000	200	No tables	10 or total of 20 images
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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.

REFERENCES

Reference Style and Format

İstanbul Journal of Pharmacy complies with APA (American Psychological Association) style 6th Edition for referencing and quoting. For more information:

- American Psychological Association. (2010). Publication manual of the American Psychological Association (6th ed.). Washington, DC: APA.
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Citation with two authors;

(Sayıner & Demirci, 2007)

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(Çavdar et al., 2003)

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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

Mucchielli, A. (1991). *Zihniyetler* [Mindsets] (A. Kotil, Trans.). İstanbul, Turkey: İletişim Yayınları.

c) Edited Book

Ören, T., Üney, T., & Çölkesen, R. (Eds.). (2006). *Türkiye bilişim ansiklopedisi* [Turkish Encyclopedia of Informatics]. İstanbul, Turkey: Papatya Yayıncılık.

d) Turkish Book with Multiple Authors

Tonta, Y., Bitirim, Y., & Sever, H. (2002). T*ürkçe arama motorlarında performans değerlendirme* [Performance evaluation in Turkish search engines]. Ankara, Turkey: Total Bilişim.

e) Book in English

Kamien R., & Kamien A. (2014). *Music: An appreciation.* New York, NY: McGraw-Hill Education.

f) Chapter in an Edited Book

Bassett, C. (2006). Cultural studies and new media. In G. Hall & C. Birchall (Eds.), *New cultural studies: Adventures in theory* (pp. 220–237). Edinburgh, UK: Edinburgh University Press.

g) Chapter in an Edited Book in Turkish

Erkmen, T. (2012). Örgüt kültürü: Fonksiyonları, öğeleri, işletme yönetimi ve liderlikteki önemi [Organization culture: Its functions, elements and importance in leadership and business management]. In M. Zencirkıran (Ed.), *Örgüt sosyolojisi* [Organization sociology] (pp. 233–263). Bursa, Turkey: Dora Basım Yayın.

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.

Article

a) Turkish Article

Mutlu, B., & Savaşer, S. (2007). Çocuğu ameliyat sonrası yoğun bakımda olan ebeveynlerde stres nedenleri ve azaltma girişimleri [Source and intervention reduction of stress for parents whose children are in intensive care unit after surgery]. *Istanbul University Florence Nightingale Journal of Nursing, 15*(60), 179–182.

b) English Article

de Cillia, R., Reisigl, M., & Wodak, R. (1999). The discursive construction of national identity. *Discourse and Society, 10*(2), 149–173. http://dx.doi.org/10.1177/0957926599010002002

c) Journal Article with DOI and More Than Seven Authors

Lal, H., Cunningham, A. L., Godeaux, O., Chlibek, R., Diez-Domingo, J., Hwang, S.-J. ... Heineman, T. C. (2015). Efficacy of an adjuvanted herpes zoster subunit vaccine in older adults. *New England Journal of Medicine, 372,* 2087–2096. http://dx.doi.org/10.1056/NEJMoa1501184

d) Journal Article from Web, without DOI

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

e) Journal Article wih DOI

Turner, S. J. (2010). Website statistics 2.0: Using Google Analytics to measure library website effectiveness. *Technical Services Quarterly, 27,* 261–278. http://dx.doi.org/10.1080/07317131003765910

f) Advance Online Publication

Smith, J. A. (2010). Citing advance online publication: A review. *Journal of Psychology.* Advance online publication. http://dx.doi.org/ 10.1037/a45d7867

g) Article in a Magazine

Henry, W. A., III. (1990, April 9). Making the grade in today's schools. *Time, 135,* 28–31.

Doctoral Dissertation, Master's Thesis, Presentation, Proceeding

a) Dissertation/Thesis from a Commercial Database

Van Brunt, D. (1997). *Networked consumer health information systems* (Doctoral dissertation). Available from ProQuest Dissertations and Theses database. (UMI No. 9943436)

b) Dissertation/Thesis from an Institutional Database

Yaylalı-Yıldız, B. (2014). *University campuses as places of potential publicness: Exploring the politicals, social and cultural practices in Ege University* (Doctoral dissertation). Retrieved from Retrieved from: http://library.iyte.edu.tr/tr/hizli-erisim/iyte-tez-portali

c) Dissertation/Thesis from Web

Tonta, Y. A. (1992). *An analysis of search failures in online library catalogs* (Doctoral dissertation, University of California, Berkeley). Retrieved from http://yunus.hacettepe.edu.tr/~tonta/yayinlar /phd/ickapak.html

d) Dissertation/Thesis abstracted in Dissertations Abstracts International

Appelbaum, L. G. (2005). Three studies of human information processing: Texture amplification, motion representation, and figure-ground segregation. *Dissertation Abstracts International: Section B. Sciences and Engineering, 65*(10), 5428.

e) Symposium Contribution

Krinsky-McHale, S. J., Zigman, W. B., & Silverman, W. (2012, August). Are neuropsychiatric symptoms markers of prodromal Alzheimer's disease in adults with Down syndrome? In W. B. Zigman (Chair), *Predictors of mild cognitive impairment, dementia, and mortality in adults with Down syndrome.* Symposium conducted at the meeting of the American Psychological Association, Orlando, FL.

f) Conference Paper Abstract Retrieved Online

Liu, S. (2005, May). *Defending against business crises with the help of intelligent agent based early warning solutions.* Paper presented at the Seventh

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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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Anti-proliferative effects of indomethacin, acemetacin and their tromethamine salts in HCT116 human colon cancer cells

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ABSTRACT

Background and Aims: Since 1980's, several preclinical studies have been published on the anti-colorectal cancer activity of the nonsteroidal anti-inflammatory drug indomethacin. The direct anti-proliferative effect of indomethacin seems to occur via a variety of reported COX-independent mechanisms. Acemetacin is a glycolic acid ester derivative of indomethacin and contrary to indomethacin, there is not much published research on anti-cancer effects of acemetacin. Herein, we compared the *in vitro* anti-proliferative properties of indomethacin, acemetacin, and their tromethamine salts in HCT116 colon cancer cells.
Methods: The tromethamine salts of indomethacin and acemetacin were synthesized and the structures were established by microanalysis, IR, ^{1}H-NMR, ^{13}C-NMR (APT) and 2D-NMR (HSQC and HMBC) spectrometry. Cell proliferation assays were performed using xCELLigence real-time cell analysis system.
Results: Indomethacin exhibited profound inhibitory effects with IC_{50} values at low micromolar ranges. Acemetacin exhibited far lower cytotoxic activity as compared to that of indomethacin. Surprisingly, indomethacin-tromethamine salt was 2-fold and 4.4-fold more potent than indomethacin at 48 and 72 h, respectively, while maintaining its activity at 24 h. The tromethamine salt of acemetacin was more potent than acemetacin at 24 h and 48 h post-treatment.
Conclusion: The anti-proliferative effect of indomethacin in HCT116 cells was found to be at low micro-molar levels. The esterification of indomethacin with glycolic acid caused a strong decrease in anti-proliferative effect. The salt formation caused a positive effect on the anti-proliferative activity of indomethacin and indomethacin-tromethamine salt may be a promising candidate for additional *in vivo* studies.

Keywords: Indomethacin, acemetacin, tromethamine, HCT116, anti-cancer

INTRODUCTION

Substantial evidence indicates that nonsteroidal anti-inflammatory drugs (NSAIDs) possess anti-colorectal cancer (CRC) activity. Most NSAIDs inhibit proliferation and reduce the growth of CRC cells *in vitro*, and many NSAIDs also slow tumor growth and suppress the formation of colorectal tumors in animal models and clinical studies (Ettarh, Cullen & Calamai, 2010). However, the molecular mechanisms underlying the anti-cancer effects of NSAIDs remain unclear, and they are a matter of ongoing debate.

In the early 1980's Waddell and co-workers reported that indomethacin, a member of NSAIDs, caused regression of colorectal polyps in patients with desmoid tumors (Waddell & Gerner, 1980; Waddell, Gerner & Reich, 1983). Since then, numerous *in*

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vitro and *in vivo* preclinical studies have established that indomethacin has anti-CRC activity (Hull, Gardner & Hawcroft, 2003; Hawcroft, Gardner & Hull, 2003; Seetha, Devaraj & Suthandiran 2020). The anti-proliferative effect of indomethacin may not be directly related to its ability to inhibit cyclooxygenase enzymes (COX-1 and COX-2) because the cell lines that do not express COX (e.g. HCT116, HCT15) have been also found to be sensitive to indomethacin (Ettarh et al., 2010). The direct anti-proliferative effect of indomethacin against cancer cells seems to occur *via* a variety of reported COX-independent mechanisms, including induction of apoptotic pathways (Jana, 2008; Cheng, Zhang, Li & Lin, 2013; Qin et al., 2015; Curry et al., 2019), inhibition of angiogenesis (Golab et al., 2000), effects on cell cycling (Smith, Hawcroft & Hull, 2000; Xu & Zhang, 2005) and suppression of reactive oxygen species (Giardina & Inan, 1998). The use of indomethacin is limited due to the risks of ulceration and bleeding in the gastrointestinal tract. The potentially serious adverse effects of indomethacin have prompted the researchers to develop new ester/amide derivatives with enhanced anti-infammatory efficacy and reduced gastrotoxicity (Hull et al., 2003). In 2013, Zhou et al. evaluated the anti-cancer efficacy of a new ester derivative of indomethacin, phospho-tyrosol-indomethacin. The esterification has been found to enhance the anti-cancer efficacy of indomethacin against colon, breast and lung cancer cells *in vitro* (Zhou et al., 2013).

Acemetacin is a glycolic acid ester derivative of indomethacin, used for the treatment of inflammation and pain in many countries. Its main advantage is to produce significantly less gastric damage than indomethacin. Acemetacin is regarded as the prodrug of indomethacin, and its pharmacological effects have been attributed to its hepatic conversion to indomethacin (Chávez-Piña et al., 2007). However, different experimental studies have demonstrated that acemetacin could exert pharmacological activities independent of biotransformation to indomethacin (Chávez-Piña et al., 2007; Tavares & Bennett, 1993). To our knowledge, contrary to indomethacin, there is not much published research on anti-cancer effects of acemetacin. In two reports published in 1993 and 1995, acemetacin was described to have anti-cancer activity in mice bearing colon 26 carcinoma and cause apoptosis in chicken embryo fibroblasts, respectively (Kisara et al., 1993; Lu et al.,1995).

Herein, we compared the *in vitro* cytotoxic properties of indomethacin (**I**) and acemetacin (**A**) in HCT116 colon cancer cells using xCELLigence real-time cell analysis system. The xCELLigence system allows monitoring cell viability and toxicity continuously, and thereby providing true monitoring of molecular and biochemical pathways regulating them. This system is precise and convenient to identify proliferation and cytotoxicity kinetics of HCT116 cells in real-time. It is very sensitive to determine time-dependent IC_{50} values. Further, we have prepared the tromethamine salts of these two drugs (**I-T** and **A-T**) and investigated their anti-cancer efficiencies on HCT116 colon cancer cells to see if the salt forms maintain the biological activity. Salt formation is the most common and efficient method to increase aqueous solubility and dissolution rates of pharmaceutically active small molecules. The modification of physical and chemical properties by salt formation may lead to changes in biological effects (Serajuddin, 2007). Tromethamine (**T**) is an amino sugar that has a widespread use in organic salt formation due to its excellent safety profile and tolerability. It is commonly found as an excipient in formulations for parenteral, oral, ophthalmic and topical drug administrations (Saal & Becker, 2013; Bookwala et al., 2018).

MATERIALS AND METHODS

Chemistry

Indomethacin (**I**) ($C_{19}H_{16}ClNO_4$, MW:357.79 g/mol) was provided by *Deva Pharmaceutical Company (Turkey)*. Acemetacin (**A**) ($C_{21}H_{18}ClNO_6$, MW: 415.82 g/mol) and tromethamine (**T**) ($C_4H_{12}NO_3$, MW:121.14 g/mol) were purchased from Sigma-Aldrich. Melting points were determined in open capillary tubes with a Buchi B-540 melting point apparatus and were not corrected. Microanalyses were performed on a Leco CHNS 932 (St. Joseph, MI, USA) elemental analyzer. IR spectra were recorded in KBr discs (wavenumber/cm^{-1}) on a Shimadzu IRAffinity-1 FTIR spectrophotometer. ^{1}H-NMR (DMSO-d_6), ^{13}CNMR (APT) (DMSO-d_6) and heteronuclearcorrelation ^{1}H-^{13}C (HSQC, HMBC) (DMSO-d_6) spectra were run on Bruker AVANCE (500 MHz) instrument. Chemical shifts are reported as δ (ppm) relative to TMS as internal standard and coupling constants (*J*) are given in hertz (Hz) (ar.:aromatic, al.: aliphatic, ind.:indole, tr.:tromethamine, ph.:phenyl).

*Indomethacin tromethamine salt (**I-T**).* To a solution of **I** (5.0 mmol) in hot ethanol (20 mL), **T** (5.0 mmol) was added and the mixture was heated at 80 °C for 30 min with stirring. Excess ethanol was evaporated and the resulting residue was recrystallized from ethanol: White powder (66%); mp 147-150°C; IR(KBr): υ_{max} 3345, 3273 (O-H), 3096, 3061, 3034 (ar. C-H), 2997, 2958, 2935, 2895, 2835 (al. C-H and NH_3^+(N-H)), 1670 (amide C=O), 1599 (carboxylate anion C=O); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 2.19 (3H, s, 2-CH_3-ind.), 3.37 (7H, s, CH_2-tr. with H_2O), 3.43 (2H, s, 3-CH_2COO^--ind.), 3.75 (3H, s, 5-OCH_3-ind.), 5.77 (1H, s, OH-tr.), 6.68 (1H, dd, J=9.0, 2.5, H6-ind.), 6.93 (1H, d, J=9.0, H7-ind.), 7.05 (1H, d, J=2.5, H4-ind.), 7.64 (2H, d, J=8.7, H3,5-ph.), 7.67 (2H, d, J=8.7, H2,6-ph.), 8.33 (1H, s, NH_3^+-tr.); ^{13}C-NMR (APT, HSQC, HMBC) (DMSO-d_6/125 MHz): δ 13.86 (2-CH_3-ind.), 32.73 (3-CH_2COO^--ind.), 55.78 (5-OCH_3-ind.), 59.98 (C-tr.), 61.09 (CH_2-tr.), 102.51 (C4-ind.), 111.46 (C6-ind.), 114.90 (C7-ind.), 116.72 (C3-ind.), 129.49 (C3,5-ph.), 130.69 (C7a-ind.), 131.53 (C2,6-ph.), 131.92 (C3a-ind.), 134.50 (C1-ph.), 134.91 (C2-ind.), 137.84 (C4-ph.), 155.88 (C5-ind.), 168.28 (N-CO), 174.30 (CO-O^-). Anal. Calcd for $C_{23}H_{27}ClN_2O_7$ (478.92): C, 57.68; H, 5.68; N, 5.85. Found: C, 57.79; H, 5.76; N, 6.10.

*Acemetacin tromethamine salt (**A-T**).* **T** (5.0 mmol) was added to a solution of **A** (5.0 mmol) in hot ethanol (20 mL) and the mixture was heated at 80 °C for 30 min with stirring. Excess ethanol was evaporated and the resulting residue was recrystallized from ethanol White powder (75%); mp 181-184 °C; IR(KBr): υ_{max} 3317, 3211 (O-H), 3088, 3074 (ar. C-H), 2945, 2922, 2887, 2839 (al. C-H and NH_3^+(N-H)), 1715 (ester C=O), 1697 (amide C=O), 1616 (carboxylate anion C=O); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 2.21 (3H, s, 2-CH_3-ind.), 3.44 (7H, s, CH_2-tr. with H_2O), 3.77, 3.78 (5H, 2s, 3-$CH_2COOCH_2COO^-$-ind. and 5-OCH_3-ind.), 4.26 (2H, s, 3-$CH_2COOCH_2COO^-$-ind.), 5.79 (1H, s,

OH-tr.), 6.70 (1H, dd, J=9.0, 2.5, H6-ind.), 6.95 (1H, d, J=9.0, H7-ind.), 7.10 (1H, d, J=2.5, H4-ind.), 7.65 (2H, d, J=8.6, H3,5-ph.), 7.69 (2H, d, J=8.6, H2,6-ph.), 8.32 (1H, s, NH_3^+-tr.); ^{13}C-NMR (APT, HSQC, HMBC) (DMSO-d_6/125 MHz): δ 13.77 (2-CH_3-ind.), 29.84 (3-CH_2COOCH_2-ind.), 55.87 (5-OCH_3-ind.), 60.12 (C-tr.), 61.02 (CH_2-tr.), 63.73 (3-CH_2COOCH_2-ind.), 102.11 (C4-ind.), 112.07 (C6-ind.), 113.55 (C3-ind.), 114.99 (C7-ind.), 129.51, 129.54 (C3,5-ph.), 130.60 (C7a-ind.), 131.14 (C3a-ind.), 131.65, 131.68 (C2,6-ph.), 134.62 (C1-ph.), 135.74 (C2-ind.), 138.06 (C4-ph.), 156.05 (C5-ind.), 168.35 (3-CH_2COOCH_2-ind.), 170.64 (N-CO), 170.80 (CO-O^-). Anal. Calcd for $C_{25}H_{29}ClN_2O_9$ (536.96): C, 55.92; H, 5.44; N, 5.22. Found: C, 55.86; H, 5.42; N, 5.46.

In vitro anti-proliferative assay using the xCELLigence DP system

HCT116 (human colon cancer) cell line was purchased from the American Type Culture Collection (ATCC). HCT116 cells were grown in DMEM (Gibco-Life Technologies) supplemented with 10% fetal bovine serum (Gibco-Life Technologies) and 1% Pen/Strep (Gibco-Life Technologies) at 37 °C, 5% CO_2 incubator.

Impedance-based real time detection of cell proliferation and cytotoxicity experiments were performed according to the instruction manual of the xCELLigence DP system (ACEA Biosciences Inc.). After determining the optimum HCT116 cell number from its proliferation pattern, 15000 HCT116 cells/well were seeded in E-Plate. Approximately 20 h after seeding, when the cells were in the log growth phase, HCT116 cells were treated with different concentrations of **I**, **I-T**, **A**, and **A-T** as indicated in the figure legends and monitored for every 30 min for 93 h. The cells were treated with a final concentration of 0.01% DMSO served as a vehicle control. The results were expressed by cell index (CI). The RTCA software was used to calculate IC_{50} values from dose response curve.

RESULTS AND DISCUSSION

Chemistry

Briefly, **I** and **A** were treated with tromethamine in hot ethanol, and the mixture was heated under reflux with stirring to generate the desired tromethamine salts (Figure 1). The structures of **I-T** and **A-T** were established by microanalysis, IR, ^{1}H-NMR, ^{13}C-NMR (APT) and 2D-NMR (HSQC and HMBC) spectrometry. **I-T** was previously synthesized in different studies, and the melting point and spectroscopic data were consistent with the findings of previous reports (Bookwala et al., 2018; Kahan, 1985). **A-T** was synthesized and characterized for the first time in this study, according to our knowledge.

In vitro anti-proliferative activity

Figure 2 shows the real time dynamic monitoring of HCT116 cell proliferation and the compound induced cytotoxicity with xCELLigence system. To explore the potential role of **I**, **A**, **I-T** and **A-T** on cell proliferation, HCT116 cells were seeded at 15000 cells *per* well of an E-plate (Roche, ACEA Biosciences) and treated with increasing concentrations of the tested compounds when the cells were in the log growth phase. Cell growth was measured every 30 minutes for up to 96 h (Real-Time and Dynamic Monitoring of Cell Proliferation and Viability for Adherent Cells. http://www.aceabio.com/wp-content/uploads/Monitoring-Cell-Proliferation-and-Viability-for-Adherent-Cells.pdf., 2013). As shown in Figure 2, treatment of HCT116 cells with the increasing concentration of **I**, **A**, **I-T** and **A-T** induced a dose-dependent cytotoxicity on HCT116

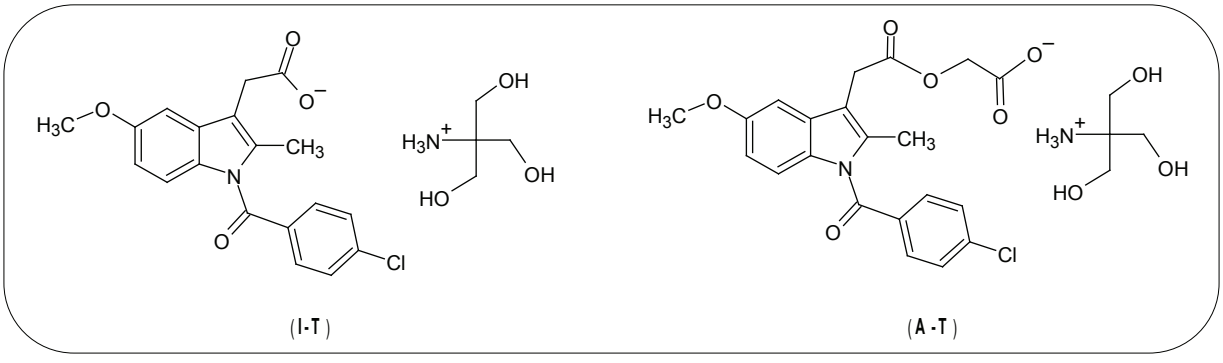

Figure 1. Chemical structures of tromethamine salts (**I-T** and **A-T**) of indomethacin (**I**) and acemetacin (**A**).

Table 1. IC_{50}[a] values of indomethacin (I), acemetacin (A) and their tromethamine salts (I-T and AT) determined with the RTCA[b] system after 24, 48 and 72 h treatments in HCT116 cells.

Compound	24 h IC_{50}	48 h IC_{50}	72 h IC_{50}
I	22.81 μM	133.55 μM	375.39 μM
I-T	27.52 μM	62.50 μM	85.68 μM
A	259.56 μM	110.00 mM	1.02 mM
A-T	183.50 μM	58.80 mM	329.00 mM

[a] The half maximal inhibitory concentration for dose-response curves, [b] Real time cell analyze, * The experiments were performed in triplicate.

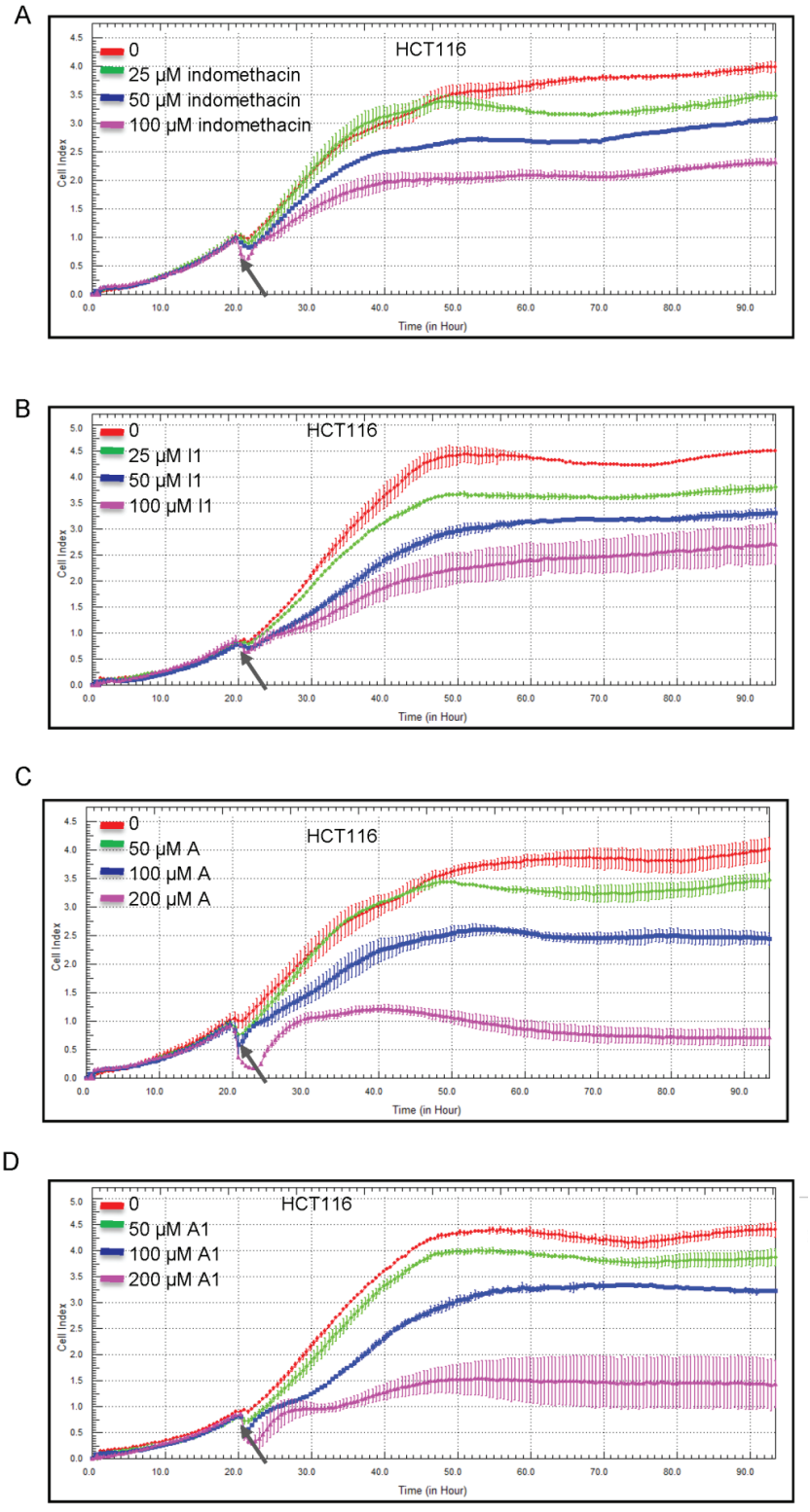

Figure 2. Cytotoxic effects of **I** (A), **I-T** (B), **A** (C) **A-T** (D) on HCT116 cells. Grey arrow indicates the time of addition of the compounds.

cells. Compound induced cytotoxicity in HCT116 cells was further quantified using the xCELLigence real time cell analyze (RTCA) software to determine IC_{50} values calculated at three specific post-treatment times, 24, 48 and 72 h (Table 1). It is expected to see time-dependent decrease in IC_{50} values, but it also depends on the mechanism of each compound in specific cell type. The time- dependent increase was observed on IC_{50} values of **I** and **I-T** whereas the time-dependent decrease was observed on IC_{50} values of **A** and **A-T** on HCT116 cells (except for compound **A-T** at 72 h) (Table 1).

The anti-proliferative effect of **I** on HCT116 cells was found to be at low (micro-molar) levels, as consistent with previous studies on anti-CRC activity of **I** (Hull et al., 2003). The esterification of **I** with glycolic acid (chain elongation) caused a strong decrease in anti-proliferative effect. The cytotoxic activity of **I** was approximately 10 to 1000-fold superior compared to the ester analogue. Surprisingly, salt formation caused a positive effect on activity. **I-T** was 2-fold and 4.4-fold more potent than **I** at 48 and 72 h, respectively, while maintaining activity at 24 h. **A-T** was found to induce cytotoxicity at millimolar concentrations at 48 and 72 h post-treatments as its precursor **A** (Figure 2 and Table 1). Comparing **I** and **I-T**, deprotonated form of carboxylic acid residue could lead to an increase in anti-proliferative and cytotoxic properties on HCT116 colon cancer cells.

CONCLUSIONS

We have compared the *in vitro* anti-CRC activity of **I** and its carboxymethyl ester **A** by real-time monitoring of HCT116 cells. **I** exhibited profound inhibitory activity with IC_{50} values at micromolar ranges. Ester formation with glycolic acid led to a dramatic decrease in biological activity, and **A** exhibited far lower cytotoxic activity as compared to that of **I**. We have further synthesized the tromethamine salts of **I** and **A** to investigate the effect of salt formation on the anti-CRC activity. The *in vitro* screening results revealed that salification could change the biological activity of **I**. **I-T** exhibited a significant anti-proliferative effect superior to **I** itself and may be a promising candidate for additional *in vivo* studies.

Peer-review: Externally peer-reviewed.

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Original Article

In vitro assessment of cytotoxic, apoptotic and genotoxic effects of metformin

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ABSTRACT

Background and Aims: Recent studies have shown the anticancer properties of metformin, which is widely used in diabetes mellitus. The possible mechanisms of anticancer effects of metformin have not been fully elucidated. We aimed to investigate the cytotoxic, genotoxic, and apoptotic effects of metformin in HepG2 and HeLa cells.
Methods: The cytotoxicity, genotoxicity, and apoptotoic effects were determined by MTT method, Comet assay, and FACS assay, respectively.
Results: Metformin significantly decreased cell viability above 4 and 32 mM in HepG2 and HeLa cells, respectively, for 48 h. The IC50 values were 57.3 mM (HepG2) and 76.9 mM (HeLa). Metformin (5-1000 µM) alone did not increase DNA damage in all cells. It did not change oxidative DNA damage in HepG2 cells but induced oxidative DNA damage in HeLa cells. HepG2 cells treated with only 32 mM metformin revealed 10% apoptosis. G0/G1 phase accumulation was statistically higher in the cells treated with 4, 8, and 64 mM metformin (91%, 99%, and 97% respectively) than in (-) control (80%). HeLa cells revealed apoptosis of 30%, 39%, 27% at 4, 32, and 64 mM concentrations, respectively. The results implicate that the inhibition of HepG2 cell viability may be due to the arrest of cell cycle in G0/G1 phase and apoptosis, whereas apoptotic response is mainly responsible for the cytotoxicity of metformin in HeLa cells.
Conclusion: Metformin may not induce DNA damage at non-cytotoxic high doses and lead to apoptosis, even if compatible with previous data. This study provides important information that metformin may play an essential role in apoptosis and cell cycle progression in carcinoma cell lines, which can explain the anticancer effects of metformin, but further studies are needed to support these results.

Keywords: Metformin, genotoxicity, apoptosis, HepG2 cells, HeLa cells

INTRODUCTION

Despite advancements in diagnosis and medical care, cancer remains as the leading reason for death worldwide. While the most common cancers in males are prostate, lung and bronchus and colorectal; the most common cancers in females are breast, lung and bronchus and colorectal (Siegel, Miller & Jemal, 2019).

Antidiabetic drugs are known to influence cancer progression, as high glucose level is a risk factor for both cancer and diabetes. Metformin (1,1- dimethylbiguanide) is a product of French lilac (*Galega officinalis* L.). It is an oral biguanide functioning as a hypoglycemic agent (Kamarudin, Sarker, Zhou, & Parhar, 2019). This drug lowering the blood glucose level is widely used for the treatment of type 2 diabetes. It is responsible for the activation of the energy sensor AMP-activated protein kinase (AMPK), and it has been associated with the inhibition of glucose production in primary hepatocytes (Kim et al., 2019). It is well-described in the literature that metformin suppresses

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carcinogenesis by inhibiting the transformative and hyperproliferative processes with anti-angiogenesis, radio-chemosensitizer and anti-metabolic effects (Salani, Del Rio, Marini, Sambuceti, Cordera, & Maggi, 2014; Leone, Di Gennaro, Bruzzese, Avallone, & Budillon, 2014; Jalving et al., 2010).

Cell checkpoints and apoptosis, which are important molecular pathways in anticancer effect, are well known for playing a vital role in regulating growth, development, and immune response, therefore removing cancerous cells. The avoidance of apoptosis is an important hallmark of cancer; thus, the ability to induce apoptosis and suppress cell growth is a promising therapeutic approach in cancer research. Although chemotherapeutics can be used to achieve this, their use is associated with high levels of toxicity. In several studies, the anticancer effect of metformin has been the focus of attention. Usefulness of metformin in reducing the risk for diabetes related cancers as well as breast cancer, cervix cancer, pancreas cancer, prostate cancer and colorectal cancer have been investigated in numerous studies (Kamarudin, Sarker, Zhou, & Parhar, 2019; Kim et al., 2019; Lopez-Bonet, et al., 2019; Donadon, Balbi, Casarin, Vario, & Alberti, 2008; Giovannucci & Michaud, 2007; Dombrowski, Mathieu, & Evert, 2006).

The antitumor effects of metformin on Hela and HepG2 cells and the mechanisms underlying apoptosis, and their cell cycle regulation remain elusive. There are conflicting results regarding the effects of metformin on genotoxicity. It seems that the mechanistic studies on anticancer effects are required to evaluate how metformin affects the apoptotic pathways and genotoxicity together. In this study, we aimed to investigate the cytotoxic, genotoxic and apoptotic effects of metformin on human hepatoma cells (HepG2) and cervical cancer cells (HeLa).

MATERIALS AND METHODS

Chemicals

Metformin was obtained from Sigma Aldrich (St. Louis, USA). Also 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), *Dulbecco's* modified Eagle's medium (DMEM), ethanol, ethidium bromide (EtBr), ethylenediamine tetra acetic acid disodium salt dihydrate (Na_2-EDTA), fetal bovine serum (FBS), hydrogen peroxide (35%) (H_2O_2), low melting point agarose (LMA), methanol, N-lauroyl sarcosinate, normal melting point agarose (NMA), penicillin-streptomycin, phosphate buffered saline (PBS), propidium iodide, Roswell Park Memorial Institute Medium (RPMI 1640), sodium chloride (NaCl), sodium hydroxide (NaOH), Tris, Triton X-100, and trypsin- EDTA were purchased from Sigma Aldrich (St Louis, USA). Cisplatin was acquired from Koçak Farma® (Istanbul, Turkey).

Cell culture

The human cell lines (HepG2 and HeLa) used in our study were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). The HeLa cells were cultured in 500 ml RPMI 1640 (RPMI 1640 medium containing L-Glutamine) with 50 mL FBS (10%) and 5 mL penicillin/streptomycin (1%). The HepG2 cells were grown in 500 mL DMEM (medium containing L-Glutamine and 5mM (1 g/L) glucose) supplemented with 50 mL of FBS (10%) and 5 mL of penicillin/streptomycin (1%).

The mediums were stored at +4°C and cells were removed by trypsinization. Cells were incubated in 5% CO_2 and 95% humidity at 37°C.

Cell viability assay

MTT assay was performed to determine the viability of HepG2 and HeLa cells treated with a wide range of doses of metformin. Non-cytotoxic doses and cytotoxic doses of metformin were selected for the evaluation of genotoxicity and apoptosis, respectively, after the determination of cytotoxicity. A 0.5 M metformin stock solution prepared freshly in medium was used after filtered through a 0.2 µM millipore filter. The MTT assay is a colorimetric assay by measuring colored formazan product to assessing cell metabolic activity. The cultured cells were plated into 96-well plates with $1x10^4$ cells/well. After 24 h, the cells were incubated with a wide range of doses of metformin (0.5-64 mM) for 48 h. Substance solutions were discarded at the end of the incubation period, 90 µL of medium and 10 µL of 5 mg/mL MTT stock solution were added to each well (final MTT concentration 0.5 mg/mL) and were allowed to incubate for 4 h. To dissolve the formazan crystals formed in the wells, 100 µL of DMSO was added to each well. The absorbance values of the samples at 570 nm wavelength were measured in the spectrophotometer.

Cytotoxicity was calculated by the percentage of the ratio between treated and untreated (control) cells (% cell viability) using eq. (1). A_{blank} and $A_{sample}/_{control}$ were indicating the absorbance of blank and absorbances of samples or control, respectively. IC_{50} values of the compounds and the concentration reducing the cell viability of treated cells by 50% with reference to the control (untreated cells), were determined from the dose-response curves. Four independent assays were performed. The medium was used as negative control and the medium containing 20 µM cisplatin as positive control.

Percentage of cell viability (% cell viability)

= (Asamples–Ablank) / (Acontrol–Ablank) x 100 (1)

Analyses of the cell cycle and apoptosis

Cells were planted in 12-well plates with $4x10^4$ cells in each well and incubated for 24 h to adapt to the culture environment in 5% CO_2 at 37°C. At the end of the incubation period, appropriate volumes of metformin solutions at 0.5-64 mM concentrations were added to each well and the cells were incubated for 48 h. Then the cells were washed 2 times with 2 ml of cold PBS and 400 µL trypsin-EDTA solution was added to each well. Then, 50 µL RNAse and 70 µL propidium iodide were added to HepG2 and HeLa cells, then incubated for 15 min in the dark at room temperature. Stained HepG2 and HeLa cells were analyzed by flow cytometry (BD FACSCalibur).

Analyses of genotoxicity (Comet assay)

HeLa and HepG2 cells ($4x10^4$ cells/well) were planted in 12-well plates. The cells were incubated with the non-cytotoxic doses of metformin (5-1000 µM) for 48 h. The Trypan Blue dye

exclusion test was applied to determine the viable cells and the cell viability was above 80% in the comet method. After the pretreatment of metformin for 48 h, oxidative damage was induced by replacing the medium with PBS containing 50 µM H_2O_2 and then incubating for 5 min on ice to assess the effect against oxidative DNA damage. At the end of the incubation period to examine the effects of DNA damage, the cells were trypsinized with trypsin-EDTA and washed with PBS. The cell suspension (50 µL) mixed with 100 µL 0.5% LMPA melted at 37°C±0.5°C was spread on agar-coated slides previously immersed in 1% NMPA solution and the coverslip was closed. Then they were placed into the electrophoresis solution for 20 min, at 25 V and 300 mA. After electrophoresis, the slides were stained with 50 µL ethidium bromide (20 µg/mL). 100 random cell analyzes were performed using fluorescence microscope (Leica) for each sample by using the comet computerized imaging system (Comet Analysis Software, version 3.0 Kinetic Imaging). The medium was used as a negative control and 50 µM H_2O_2 was used as a positive control.

Statistical analysis

All of the experiments were performed three or four times. The data are presented as means±standard deviation. Whether the data was normally distributed or not, it was evaluated using the Kolmogorov-Smirnov test and histograms. The differences between the groups were determined by one-way analysis of variance (ANOVA), LSD test. In all analyzes, statistical significance level was accepted as $p<0.05$.

RESULTS

Effects of metformin on cell viability

We used MTT assay to determine the cytotoxic effect of metformin on HepG2 and HeLa cells treated with a wide range doses of metformin.

It was observed that metformin did not have a significant cytotoxic effect in HepG2 cells at the concentration range of 0.5-2 mM after 48 h incubation when compared to negative control (PBS), but it produced a statistically significant decrease in cell viability at concentrations of 4 mM and above in a dose-dependent manner ($p<0.05$) (Figure 1). IC_{50} was found to be 57.3 mM in HepG2 cells exposed to metformin for 48 h.

Metformin did not produce a significant cytotoxic effect in HeLa cells at the concentration range of 0.5-16 mM after 48 h incubation when compared to negative control (PBS), but it significantly reduced cell viability at 32 mM and 64 mM concentrations in a dose-dependent manner ($p<0.05$) (Figure 2). IC_{50} value was found to be 76.9 mM in HeLa cells exposed to metformin for 48 h.

Effects of metformin on cell cycle and apoptosis

The changes in cell cycle progression were evaluated using the flow cytometry method to determine the growth inhibition in the metformin treated HepG2 cells.

In cell cycle analysis with HepG2 cells, 10% apoptosis was observed in the cell population at a concentration of only 32 mM (Figure 3). The statistically significant increases in the accumulation of G0/G1 phase at 4, 8, and 64 mM concentrations (91%,

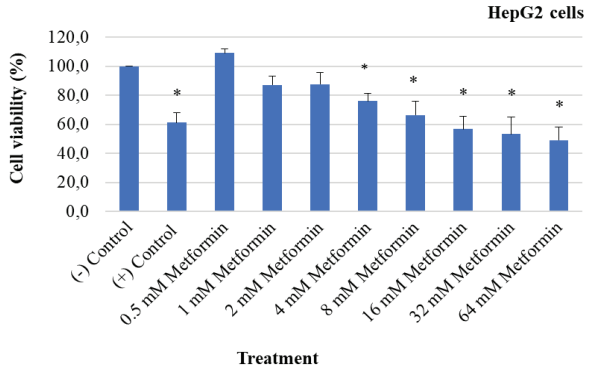

Figure 1. Effect of metformin on HepG2 cell viability at 24 h exposure. Values were given as the mean±standard deviation. *$p<0.05$, statistically different from negative control (PBS).

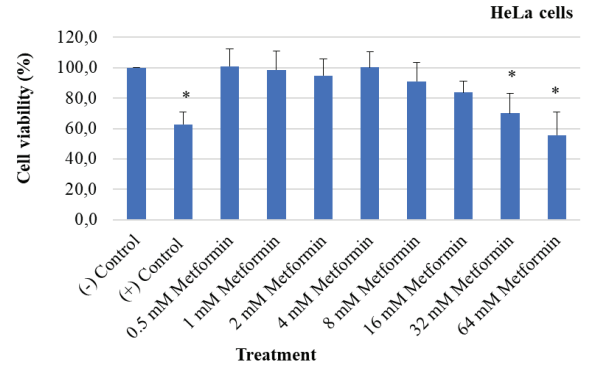

Figure 2. Effect of metformin on HeLa cell viability at 24 h exposure. Values were given as the mean±standard deviation. *$p<0.05$, statistically different from negative control (PBS).

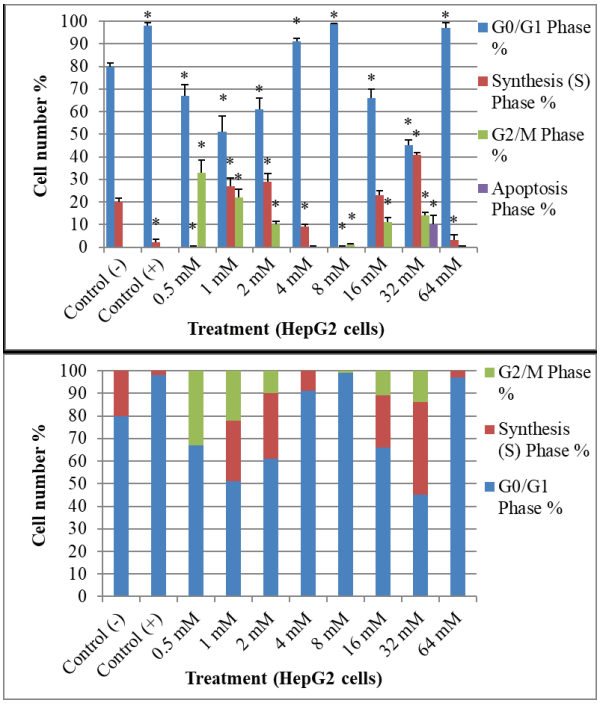

Figure 3. Effect of metformin on apoptosis and cell cycle in HepG2 cells. Values were given as the mean±standard deviation. *$p<0.05$, statistically different from negative control (PBS).

99%, and 97%, respectively) were found when compared to the (-) control (80%). Although the accumulation was detected in the G0/G1 phase, proliferation was observed in the cells. The accumulations in S phase at other concentrations of 1, 2, and 32 mM were found to be 27%, 29%, and 41%, respectively, which were statistically higher than (-) control (20%). The accumulation in the G2/M phase were found to be 33%, 22%, 10%, 11%, and 14% at the concentrations of 0.5, 1, 2, 16 and 32 mM, respectively, which were statistically higher than the (-) control (0%).

In the cell cycle analysis with HeLa cells, 30%, 39%, and 27% apoptosis were observed at 4, 32, and 64 mM concentrations of metformin, respectively. A statistical increase in the accumulation of G0/G1 phase was found at only 2 mM of metformin (88%) when compared to (-) control (78%), but proliferation occurred at this concentration. The accumulation in S phase at 16 and 32 mM concentrations (47% and 26%, respectively) were found to be statistically higher than the (-) control (22%). On the other hand, the accumulation in the G2/M phase was only observed at the concentration of 0.5 mM metformin (18%) (Figure 4).

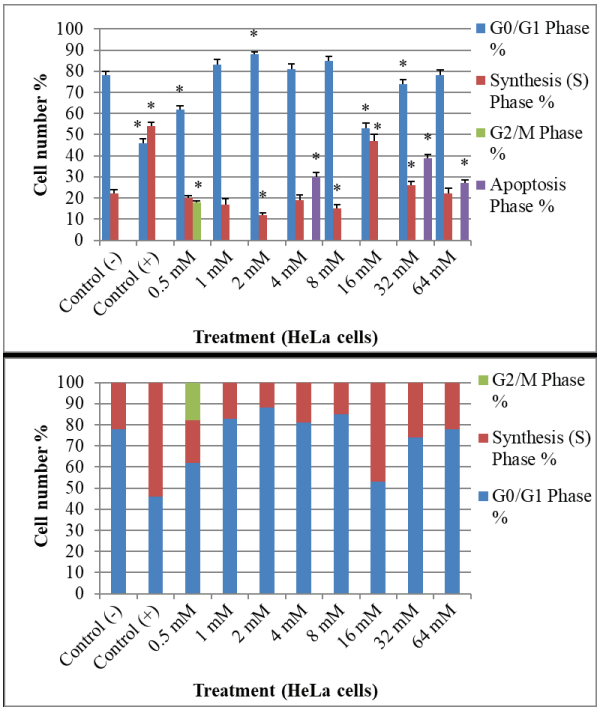

Figure 4. Effect of metformin on apoptosis and cell cycle in HeLa cells. Values were given as the mean±standard deviation. *p<0.05, statistically different from negative control (PBS).

Effects of metformin on the genotoxicity

The comet method was used to evaluate the genotoxic effect of metformin, and DNA tail intensity, DNA tail moment, and DNA tail migration were analyzed as DNA damage indicators. At the end of the 48 h incubation period, it was determined that metformin alone had no genotoxic effect on HepG2 and HeLa cells. Metformin also did not change H_2O_2-induced DNA damage in HepG2 cells, however it increased oxidative DNA damage in HeLa cells (Figures 5 and 6).

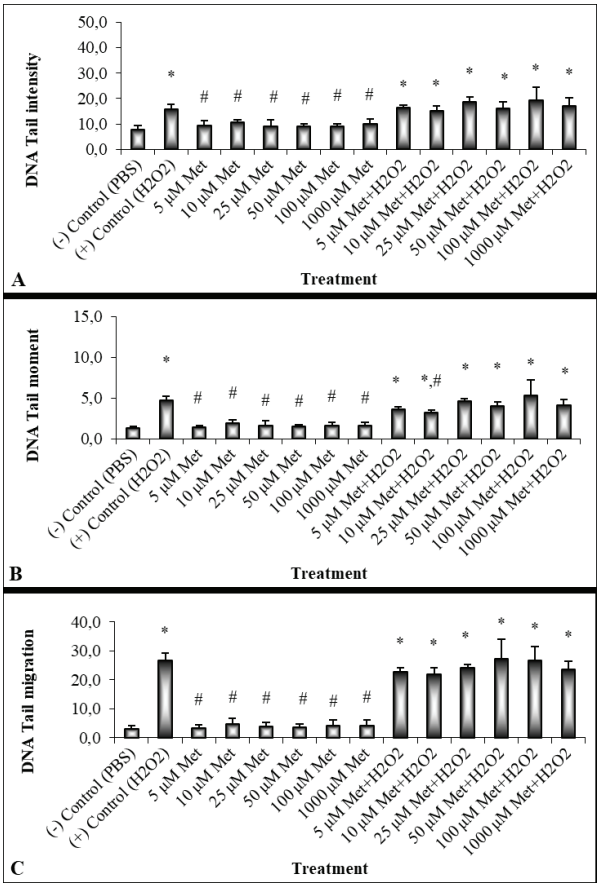

Figure 5. Genotoxicity of metformin in HepG2 cells. DNA damage expressed as DNA tail intensity (A), DNA tail moment (B), DNA tail migration (C) in HepG2 cells. Met= metformin. Values were given as the mean±standard deviation. *p<0.05, statistically different from negative control (PBS). #p<0.05, statistically different from positive control (50 µM H_2O_2).

DISCUSSION

Day by day cancer has become one of the most serious mortalities and morbidity causes in the world. Therefore, cancer treatment has begun to come to the fore. Current chemotherapeutic drugs cannot effectively control tumor progression. Resistance to drug inhibiting therapy is common, which increases the trend towards new approaches. New therapy initiatives are needed to increase the overall survival rate of cancer patients. Recently, some studies have provided preliminary evidence that metformin can reduce the risk of cancer and improve prognosis in diabetic patients. For this purpose, *in vitro* and *in vivo* studies have become widespread in various types of cancer related to metformin. The results are sometimes contradictory in the studies conducted. Some studies show that metformin has an inhibitory effect on the growth of various human cancer cells. However, data on whether these growth-inhibiting effects alone cause arrest of cell apoptosis or alter the cell cycle are not well known. Although some of these studies suggest several possible mechanisms, the detailed molecular basis is largely unknown. On the other hand, many studies have shown that metformin inhibits cell proliferation by causing apoptosis and can lead the cell to death (Will, Pa-

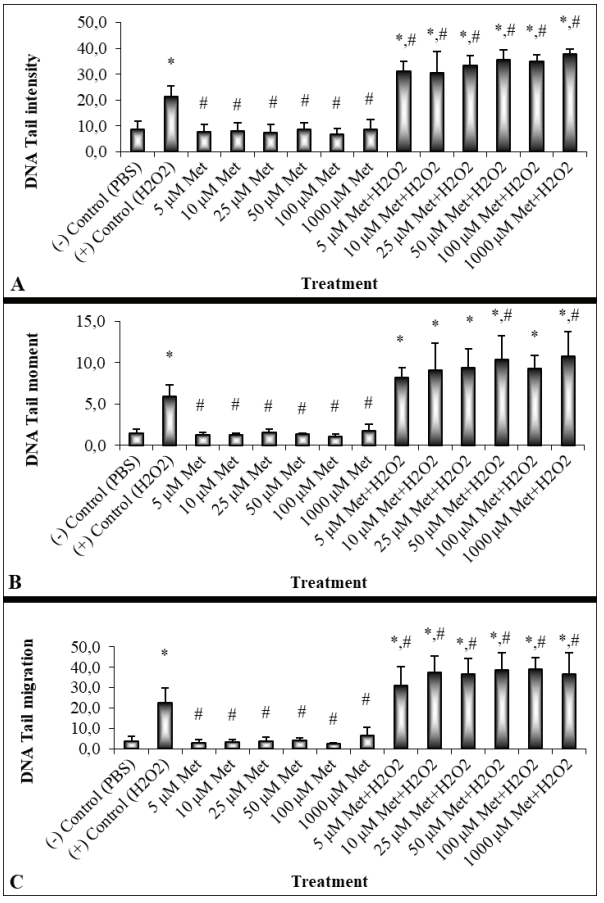

Figure 6. Genotoxicity of metformin in HeLa cells. DNA damage expressed as DNA tail intensity (A), DNA tail moment (B), DNA tail migration (C) in HeLa cells. Met= metformin. Values were given as the mean±standard deviation. *p<0.05, statistically different from negative control (PBS). #p<0.05, statistically different from positive control (50 µM H_2O_2).

laniappan, Peegel, Kayampilly, & Menon, 2012; Colquhoun et al., 2012; Zhuang & Miskimins, 2011).

Our data shows that the IC_{50} value was determined to be 57.3 mM for HepG2 cells at 48 h exposure. It was observed that metformin did not produce a significant cytotoxic effect in HepG2 cells in the concentration range of 0.5-2 mM, but it caused a statistically significant decrease in cell viability at the concentrations of 4 mM and above in a dose-dependent manner. Research on cell viability in HepG2 cells has also shown that metformin reduces dose-dependent cell viability, in accordance with our study (Zhang et al., 2018; Sun et al., 2016; Cai et al., 2013).

In our study, we found that metformin alone did not induce DNA damage and also did not change oxidative DNA damage in all studied non-cytotoxic concentrations (5-1000 µM) in HepG2 cells (p>0.05). These results suggest that metformin does not bring about DNA damage in HepG2 cells. In a study, metformin was shown to reduce ROS accumulation, DNA damage and mutations in experimental systems containing mitochondrial toxins (Algire et al., 2012). Although metformin causes a decrease in DNA damage, various mechanisms are emphasized, and the decrease in ROS level has been revealed in many studies conducted in different cell lines (Piro, Rabuazzo, Renis, & Purello, 2012; Kane et al., 2010; Ouslimani, Peynet, Bonnefont-Rousselot, Therond, Legrand, & Beaudeux, 2005).

DNA damage formation, inhibition of transcription and replication, and induction of apoptosis may produce different results that mediate cell death. Increased DNA damage and accumulation of these damages can lead to cell death or pause in different phases of the cell cycle. In the literature, it was observed that metformin induced apoptosis different cell lines including HepG2 cells. After longer exposures (3–14 days), metformin (≥40 µM) induced a dose-and time-dependent increase in the number of apoptotic β-cells (Kefas et al., 2004). It was found that metformin did not induce apoptosis but blocked cell cycle in G0/G1 in human prostate cancer cells (DU145, PC-3 and LNCaP cancer cells). This blockade was accompanied by a strong decrease of cyclin D1 protein level, pRb phosphorylation and an increase in p27kip protein expression (Ben Sahra et al., 2008). Metformin suppressed cell growth and induced apoptosis in a dose-dependent manner in hepatocellular carcinoma cells (Saito et al., 2013). In our study, metformin significantly reduced cell viability above 4 mM in HepG2 cells in a dose-dependent manner (p<0.05). We found that the accumulations in the G0/G1 phase at the concentrations of 4 mM, 8 mM and 64 mM metformin and the accumulations in the S phase statistically increased when compared to the negative control. Metformin caused 10% apoptosis in HepG2 cells at a concentration of 32 mM. It seems that metformin induced significant growth inhibition of HepG2 cells through the induction of G0/G1 phase and S phase cell-cycle arrest *(*Kefas et al., 2004; Ben Sahra et al., 2008; Saito et al., 2013).

In our results, it was observed that metformin did not have a significant cytotoxic effect in HeLa cells in a range of 0.5-16 mM concentration, but significantly reduced cell viability at 32 mM and 64 mM concentrations in a dose-dependent manner (p<0.05). We also found that metformin alone did not reveal DNA damage at the non-cytotoxic concentrations (5-1000 µM) (p>0.05), however it induced oxidative DNA damage in HeLa cells at all studied doses (p<0.05). In the cell cycle analysis with HeLa cells, 30%, 39%, 27% of apoptosis were observed in the cell population at concentrations of 4 mM, 32 mM and 64 mM, respectively. Few studies relating the effects of metformin on HeLa cells viability have similarly shown that metformin reduces cell viability and induces apoptosis (Xia et al., 2017; Tyszka-Czochara, Konieczny, & Majka, 2017; Tyszka-Czochara, Bukowska-Strakova, & Majka, 2017).

Different IC_{50} metformin levels reported in previous studies appear to be due to differences in cell types, cytotoxicity tests, and treatment times. The effects of metformin on the proliferation of esophageal squamous cell carcinoma cells (ESCC, EC109, and EC9706) treated with different concentrations were investigated for 24 h to 72 h using MTT assay. Cell viability decreased depending on the dose and time, consistent with our study. For 24 h, the significant decrease in cell viability was observed only at 20 mM metformin (about IC_{50}). A dramatic suppression in the growth of the EC109 cell lines

was observed after metformin (20 mM) treatment for 72 h (Cai et al., 2015).

Metformin inhibited the proliferation of esophageal carcinoma cell lines (T.T, KYSE30 and KYSE70 cells) as shown by WST-8 test, an MTT-like test. Metformin led to a dose-dependent and strong inhibition of cell proliferation. In KYSE70 cells, although 5 mM metformin did not affect the proliferation of cancer cells, treatment with 10 mM metformin inhibited the proliferation of cells. The highest dose (10 mM) of metformin significantly increased the cell proliferation for 24 h (Kobayashi et al., 2013).

Zhang et al. investigated the effects of metformin and curcumin on proliferation, apoptosis, invasion, metastasis and angiogenesis of hepatocellular carcinoma cells *in vitro* and *in vivo*. The IC_{50} values of metformin were 53.72 mM, 23.46 mM, 8.52 mM for 24 h, 48 h and 72h, respectively, in HepG2 cells by CCK-8 assay. 10 mM metformin also significantly increased the apoptotic effects of curcumin about two times in HepG2 cells. Metformin was also found to be involved in down-regulation of MMP2 and MMP9 (well-known proliferation/metastasis proteins) (Zhang et al., 2018).

Yudhani et al. reported that metformin enhanced the anti-proliferative effect of cisplatin in cervical carcinoma cell lines. Treatment of 10 mM metformin showed inhibition of HeLa cell proliferation and IC_{50} was reported to be 60 mM by MTT assay, which was like our result. Combination of 30 mM metformin and 5 μM cisplatin indicated the strongest anti-proliferative effect on HeLa cells (Yudhani, Pesik, & Indarto, 2016).

Wang et al. investigated the anti-myeloma effects of metformin in myeloma cells (RPMI8226 and U266). Cell viability was assessed with CCK8 cytotoxicity assay. The cell viability decreased with increasing concentrations of metformin and with increasing duration of treatment. The IC_{50} of metformin was reported to be 20.2 mM and 17.9 mM in RPMI8226 cells and U266 cells, respectively, for 48 h (Wang et al., 2018).

Xia et al. reported that metformin inhibited cervical carcinoma cells (HeLa and SiHa) proliferation, cervical cancer xenograft growth, expression of PCNA, p-PI3K and p-Akt. It induced apoptosis and caused cancer cell cycle arrest and also upregulated the expression of DDR-1 and p53. Metformin also regulated the mRNA and protein expression of MICA and HSP70 on the surface of human cervical cancer cells via the PI3K/Akt pathway, enhancing NK cell cytotoxicity. Metformin was reported to inhibit cervical carcinoma cells proliferation in a time-dependent manner for 24 h, 48 h and 72 h by using CCK-8 test, a MTT-like test. The IC_{50} values of metformin for 72 h were 25.13 mM and 19.43 mM in HeLa and SiHa cells, respectively, which were about three times lower than our results. The apoptosis ratio of the cells treated with 20 mM metformin for 48 h were found to be increased from 11.61 to 39.04% and 5.69 to 12.31% for HeLa cells and SiHa cells, respectively. The percentage of G0/G1 phase increased and the percentage of S phase cells decreased when HeLa and SiHa cells treated with 20 mM metformin for 48 h (Xia, Liu, He, Cai, & Chen, 2020).

Studies on the cell cycle analysis in its anticancer effect has shown that other different pathways may also be responsible for this effect. It was reported that metformin was effective in blocking the cell cycle in G0/G1, but not in the induction of apoptosis in human prostate cancer cells (DU145, PC-3 and LNCaP) treated with metformin (1 and 5 mM) (38-54% decrease in cell viability, in a dose-dependent manner). It inhibited cyclin D1 expression and pRb phosphorylation independently of the sensor pathway AMPK (Ben Sahra et al., 2008). It was observed that metformin (10 mM) blocked the cell cycle in G0/G1 for 24 h. This blockade was accompanied by a strong decrease of G1 cyclins, especially cyclin D1, as well as decreases in cyclin-dependent kinase (Cdk)4, Cdk6 and phosphorylated retinoblastoma protein (Rb). In addition, the expression of miRNAs was markedly altered with the treatment of metformin in vitro. Metformin inhibited the growth of esophageal carcinoma cells, and this inhibition may have involved reductions in cyclin D1, Cdk4 and Cdk6 (Kobayashi et al., 2013).

Wang et al. revealed the accumulation of cells in the G0/G1 phase, while the fraction of cells in the S phase decreased in the cells treated with 5 mM and 20 mM metformin for 24 h. They concluded that metformin effectively inhibited the cell proliferation, which is associated with the induction of autophagy and G0/G1 cell cycle arrest, but not apoptosis. They suggested that the molecular mechanism of metformin is also involved in AMPK activation (Wang et al., 2018). Kheirandish, Mahboobi, HYazdanparast, Kamal & Kamal (2018) stated that AMPK-dependent (decreases in folate level, c-Myc and NF- κB; increases in p53 phosphorylation) and AMPK-independent (decreases in ROS and cyclin D1; increases in mTORC1) pathways may be responsible for the anticancer effects of metformin. Metformin also decreases both pro-inflammatory cytokines and improves the immune response to cancer cells.

Cell cycle progression, involved in cell division and replication, can be restricted under conditions such as DNA replication error, nutrient depletion, DNA damage and low growth factor. Cell cycle regulatory functions are usually impaired in cancer cells. Therefore, the improvement in cell cycle progression might be an effective strategy for the treatment of carcinomas. Cyclin, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs) in the G1 phase interact with each other to regulate cell-cycle transitions and cell division. It was reported that the anti-proliferative action of metformin on esophageal squamous carcinoma cell lines (ESCC) was partially mediated by AMPK. Moreover, it was observed that metformin induced G0/G1 phase arrest accompanied by the up-regulation of $p21^{CIP1}$ and $p27^{KIP1}$. The results indicate that metformin may inhibit carcinoma cell growth via causing cell cycle arrest and delaying tumorigenesis (Cai et al., 2015).

In some reports it has been concluded that metformin might induce DNA damage**,** however some of reports indicated no genotoxic effects (Janjetovic et al., 2011; Attia, Helal, & Alhaider, 2009; Onaran, Guven, Ozdas, Kanigur, & Vehid, 2006). In one study, metformin was found to increase DNA damage at the dose of 114.4 μg/mL (882, 6 μM) in Chinese hamster ovary cells for 24 h using comet assay (Amador, Longo, Lacava, Dórea, &

Almeida Santos, 2012). In another study, it was reported that metformin did not reduce hydroperoxide-induced DNA damage at the concentrations ranging from 10 µM to 50 µM for 1 h treatment time in the human lymphocytes using comet assay (Onaran et al., 2006). However, metformin was found to reduce age- and oxidative stress-related accumulation of DNA damage marked by γH2AX foci and 8-oxo-dG in intestinal stem cells from *Drosophila* midgut treated with 5 mM metformin in food media for 6 days (Na et al., 2013).

In our study, metformin was found to be responsible for the changes in the cell cycle arrest, with differences between HepG2 and HeLa cells. In HepG2 cells, the G0/G1 phase accumulation may be mainly responsible for the regulation of cell proliferation. Moreover, G2/M phase accumulation was also shown, being greater at lower doses (33% and 22% for 0.5 and 1 mM, respectively). It is assumed that the G0/G1 phase and the G2/M phase play a role against cytotoxicity and maintaining cell viability, respectively, in HepG2 cells, whereas in HeLa cells, apoptosis rather than the G0/G1 and G2/M phases seems to be primarily responsible for the effect of metformin on cell proliferation.

CONCLUSION

In recent studies, metformin has been shown to be a promising anticancer drug. The number of studies on combinations and effects of metformin with various anticancer drugs is increasing. Our study showed that metformin decreased cell viability in a dose-dependent manner and it did not induce DNA damage in HepG2 and HeLa cells at non-cytotoxic doses, but did lead to a significant change in apoptosis at high doses. In conclusion, we suggest that metformin may not cause DNA damage but lead to apoptosis. This study provides important information that metformin may play an essential role in the apoptosis and cell cycle progression in carcinoma cell lines, which can explain the anticancer effect of metformin. Although there are various studies in the literature on which pathways metformin produce these effects, it is not certain yet. For this reason, further studies are needed to clarify these pathways.

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Original Article

Nickel oxide nanoparticles induced DNA damages in human liver cells

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ABSTRACT

Background and Aims: Nickel oxide nanoparticles (NiO-NPs) are one of the most used nanoparticles, especially as photosensitizers. Although some studies evaluate their toxicity in the liver, the information about their toxicity at the cellular and molecular levels is still controversial. In the present study, it was aimed to investigate the *in vitro* toxic potentials of NiO-NPs (average size 15.0 nm) in the liver (HepG2) cell line.

Methods: NiO-NPs were characterized by Transmission Electron Microscopy (TEM), the cellular uptake of NPs and the morphologic changes were evaluated by TEM and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), the cytotoxicity was evaluated by MTT and neutral red uptake (NRU) tests, comet assay was used for genotoxicity, Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was used for apoptosis/ necrosis evaluation and Enzyme-Linked Immune Sorbent Assays (ELISA) kits were used for the potential of oxidative damage.

Results: Our results showed that cellular uptake of NiO-NPs led to morphological changes in the cells, and caused cell death (IC50 was 146.7 µg/mL by MTT) mainly by apoptosis. Genotoxicity and oxidative damage were observed to be in a dose-dependent manner.

Conclusion: Results confirm previous data and draw attention to the toxic effects of NiO-NPs; further *in vivo* and *in vitro* studies need to be done to clarify the safety or toxicity of NiO-NPs.

Keywords: Nickel oxide, Nanoparticles, Genotoxicity, Oxidative stress, Apoptosis

INTRODUCTION

Humans are exposed to NPs through dermal absorption, ingestion, and inhalation due to their wide range of applications (Ahamed, Ali, Alhadlaq, & Akhtar, 2013; Ahmad, et al., 2013; Kim, Yu, Park, & Yang, 2010). In addition to direct exposure, indirect exposure to NPs is possible *via* the content in bulk material (Dhawan & Sharma, 2010). Studies have shown that NPs are more toxic than their bulk forms; different toxic effects like cytotoxicity, genotoxicity, oxidative damage and morphological changes in the exposed organ were observed after exposure to NPs (Oberdorster, 2001; Zhang, et al., 2012). However, the information concerning NPs' effects on humans and the environment systems are still insufficient (Arora, Rajwade, & Paknikar, 2012; Barillet et al., 2010; Brooking, Davis, & Illum, 2001; Chen et al., 2015). Different factors, such as the properties of NPs including the particles size, shape and surface charges, the route of exposure, the exposed cells' sensitivity and the assays used play an important role in the toxicity that will emerge and the results that will be obtained (Boverhof & David, 2010; Horev-Azaria et al., 2011; Horie et al., 2009; Napierska, Thomassen, Lison, Marten, & Hoet, 2010; Oberdorster, Oberdorster, & Oberdorster, 2005; Schrand et al., 2010).

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NiO-NPs are widely used as catalysts, pigments, and sensors in different medical and industrial applications because of their superior properties (Capasso, Marina, & Maurizio, 2014; Horie et al., 2009; Morimoto et al., 2011; Schrand et al., 2010). According to the International Agency for Research on Cancer (IARC), NiO and other Ni compounds have been classified as carcinogenic to humans (Group 1) (IARC, 1990; Dunnick et al., 1988). Additionally, the solubility of NiO-NPs is assumed to be higher than the fine particles, and so, it is thought that NiO-NPs release more Ni^{2+} than fine particles leading to more toxic effects (Horie et al., 2011). Researches have mainly focused on their pulmonary toxicity, and reported that Ni-based NPs induced oxidative stress and inflammatory responses in the respiratory system (Ahamed et al., 2013; Capasso, Marina, & Maurizio, 2014; Cho et al., 2010; Horie et al., 2011; Kang et al., 2011; Morimoto et al., 2011; Morimoto et al., 2010; Nishi et al., 2009; Ogami et al., 2009; Oyabu et al., 2007; Siddiqui et al., 2012). Additionally, it is well known that NiO-NPs are absorbed through the intestines and distributed with the blood to different organs like the liver; there are few controversial reports about NiO-NPs' toxic potential in the liver (Ahmad et al., 2013; Oberdorster, Oberdorster, & Oberdorster, 2005; Siddiqui et al., 2012).

In the present study, it was aimed to investigate the *in vitro* toxic effects of NiO-NPs on the liver (HepG2 hepatocarcinoma cell). Therefore, the NPs' size was characterized, and the cellular uptake and the cellular morphological changes were evaluated. Cytotoxicity, DNA damage, oxidative stress, and apoptosis were also studied using by *in vitro* methods. The preferred cell line and assays are widely used for NP toxicity studies. Many investigators select the highly differentiated human liver cell lines (HepG2) to study the apical uptake, metabolism, and absorption of nutrients and drugs, *etc.*, as models in *in vitro* conditions (Brand, Hannah, Mueller, Cetin, & Hamel, 2000; Martin, Failla, & Smith, 1997). O'Brien et al., (2006) observed the sensitivity of the cytotoxicity assay was 85% with a specificity of 98% when HepG2 cells were used as an *in vitro* model to predict the hepatotoxic potential of 243 drugs and chemicals.

MATERIALS AND METHODS

Chemicals

NiO-NPs, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), and neutral red dye were obtained from Sigma (MO, USA). Cell culture mediums, antibiotic solutions, fetal bovine serum (FBS), and phosphate buffer saline (PBS) were purchased from Multicell Wisent (Quebec, Canada). Oxidative stress detection ELISA kits (GSH, 8-OHdG, MDA and PC) were purchased from Yehua Biological Technology (Shanghai, China). Protein detection kits were obtained from Bio-rad (Munich, Germany); Annexin V-FITC/ PI apoptosis detection kits were obtained from Biolegend (CA, USA). The other chemicals were obtained from Merck (NJ, USA).

Particle size characterization

Dynamic light scattering (DLS) (ZetaSizer Nano-ZS, Malvern Instruments, Malvern, UK) was used to evaluate the average hydrodynamic size of NiO-NPs in the complete cell culture medium. Besides that, TEM (JEM-2100 HR, JEOL, USA) was used to estimate the size and distribution of NiO-NPs according to the method used by Abudayyak et al., (Abudayyak, Guzel, & Özhan, 2017; 2017b).

Cell cultures and exposure conditions

HepG2 human hepatocarcinoma cell line (HB-8065; American Type Culture Collection (ATCC), MD, USA) was used in this study. The cells were incubated at 37°C, 5% CO_2 and 90% humidity conditions, in a cell culture medium (Eagle's Minimum Essential Medium, EMEM) supplemented with FBS (10%), and antibiotics (100 units/mL of penicillin, 100 µg/mL of streptomycin). For cell death assays, the cell densities were $1x10^4$ cells/mL, for DNA damage evaluation (Comet assay) $1x10^5$ cells/mL, and $1x10^7$ cells/mL for both uptake by ICP-MS and morphology examinations by TEM. For apoptosis/necrosis and oxidative stress assays, the cell densities were adjusted to $1x10^6$ cells/mL.

The particle suspensions were freshly prepared before exposure. Therefore, 1 mg/mL NiO-NPs were suspended in 1 mL complete cell culture medium (containing 10% FBS) and sonicated at room temperature for 15 min and the different exposure suspensions were prepared by diluting with complete cell culture medium.

The cells were treated with different concentrations for 24 h (Abudayyak et al., 2017; 2017b), For the cytotoxicity assays the exposure concentrations were 50-500 µg/mL, for genotoxicity the concentrations were 15-120 µg/mL, and 100-700 µg/mL in the apoptosis/necrosis assay, for oxidative damage assays the exposure concentrations were 50-150 µg/mL, in the cellular uptake assay by ICP-MS (Thermo Elemental X series 2, USA) the concentrations were 50 and 100 µg/mL and they were 50 and 150 µg/mL in the morphology examination and cellular uptake by TEM. The exposure time of the particle suspensions was 24 h.

Cellular uptake by ICP-MS

The exposed cells were washed twice with the cell culture medium, trypsinized and counted by Luna cell counter (Virginia, USA). The cells were digested by 6 M for 2 h at room temperature and then stored at -20°C until analysis for Ni amount with ICP-MS. The unexposed cells were accepted as the negative control. The test was repeated four times.

Cellular uptake and morphology by TEM

Glutaraldehyde (2.5%) in Millonig's sodium phosphate buffer (pH 7.4) was used to fix the cells. The cells were centrifuged at 1200 rpm for 10 min, the pellets were washed in Millonig's buffer and centrifuged at 1200 rpm for 10 min. As post-fixation, 1% osmium tetroxide in Millonig's buffer (for 30 min, at room temperature) was used. Before embedding in araldite, agarose gel (2%) was used to block the fixed-cells, and a graded series of ethanol from 30% to absolute (96%) ethanol were used for dehydration. Toluidine blue was used in staining the semi-thin sections of the polymerized blocks, while an ultramicrotome (Reichert UM 3, Austria) was used in the preparation of the ultra-thin sections (50-60 nm). After that, the ultra-thin sections were placed on copper grids; uranyl acetate with lead citrate was used as the stain in this step. TEM (Jeol-1011, Tokyo, Japan) with an accelerating voltage of 80 kV and an attached digital camera (Olympus-Veleta TEM Camera, Tokyo, Japan) was used in photographing and the analysis of the cells.

Cytotoxicity assays

MTT and NRU assays were used to evaluate the cytotoxic effects of NiO-NPs (Repetto, del Peso, & Zurita, 2008; Van Meerloo, Kaspers, & Cloos, 2011). Therefore, cells ($1x10^4$ cells/well) were treated with freshly prepared NiO-NP suspension at concentrations of up to 500 µg/mL (Abudayyak et al., 2017; 2017b). The optical densities (ODs) were read using a microplate spectrophotometer system (Epoch, Germany). The unexposed (negative control) cells were used in the calculation of the half-maximal inhibitory concentration (IC_{50}) values.

Genotoxicity assay

Comet assay was used in the determination of the genotoxic potential of the NiO-NPs. The unexposed cells were evaluated as the negative control group, while the cells treated with Hydrogen peroxide (H_2O_2) (100 µM) were accepted as positive controls. A fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) and automated image analysis system were used in scoring the DNA breaks. At least 100 cells were evaluated for each sample, and the tail intensity (percentage of DNA in the comet tail) was used to investigate the DNA damage in the individual cells (Collins, 2004; Speit & Hartmann, 1999).

Oxidative damage assays

Human GSH, MDA, 8-OHdG, or PC ELISA kits were used to evaluate the oxidative damage in the cells exposed to NiO-NPs, while the Bradford method (1976) was used to measure the protein amount in 10^6 cells. ELISA Kits, which are based on biotin double antibody sandwich technology, were used according to the manufacturer's instructions (Abudayyak et al., 2017). A standard calibration curve was used in the calculation of GSH, MDA, 8-OHdG, and PC results, expressed as µmol, µmol, µg, and µg per g protein, respectively.

Apoptosis assay

Annexin V-FITC with PI apoptosis-necrosis detection kit was used to estimate the apoptotic/ necrotic effects of NiO-NPs. Therefore, the cells were treated with different final concentrations of NPs equivalent to 25, 50, and 75% cell death, respectively. The exposure concentration ranges were 100-200 µg/mL. The unexposed cells were accepted as a negative control. The cells were washed, collected and adjusted to be 10^6 cells in 1mL. Five µL of Annexin V-FITC and 10 µL of PI were added to the cells and incubated for 15 min in the dark, at room temperature. The stained cells were spread on microscopic slides and counted under a phase-contrast fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan). The percent of viable (non-fluorescent), apoptotic (green-fluorescent), and necrotic (red-fluorescent) cells were calculated.

Statistical analysis

The cytotoxicity assays were done in triplicate and repeated four times while genotoxicity, oxidative stress, and apoptosis assays were done in triplicate and each assay was repeated twice. Data were expressed as mean±standard deviation (SD). The significance of differences between the unexposed and exposed with NPs cells was calculated by one-way ANOVA Dunnett t-test using SPSS version 23 for Windows (SPSS Inc., Chicago, IL). *p* values of less than 0.05 were selected as the levels of significance.

RESULTS

Particle size and size distribution

The particle size and size distribution of NiO-NPs were analyzed with TEM images (Figures 1, and 2). In the water, the size of particles ranged from 4.2 to 38.1 nm (average 15.0 nm) with a narrow size distribution. A slight agglomeration/aggregation were noticed after the suspension of NPs in the cell culture medium (Figure 3), the NP sizes ranged from 7.2 to 60.5 nm with an average size of about 21.4 nm. The adsorption of proteins in the NP surfaces and the formation of protein-corona complexes could explain this increase in the size of NPs among water and medium suspensions (Walczyk, Bombelli, Monopoli, Lynch, & Dawson, 2010).

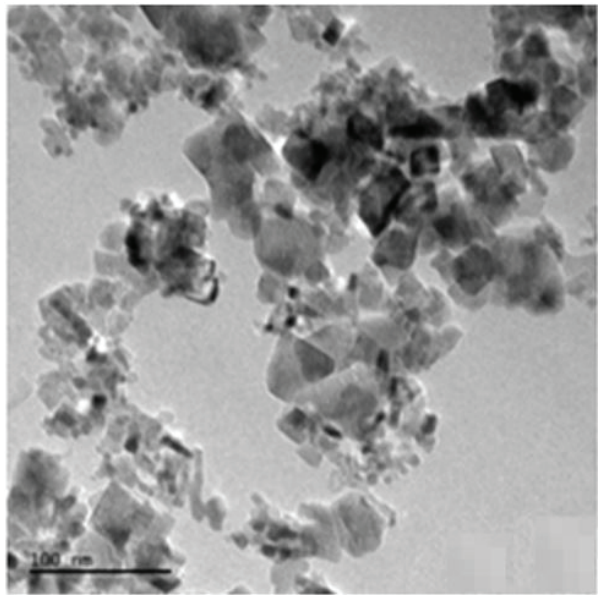


Figure 1. TEM images of NiO-NPs in water.

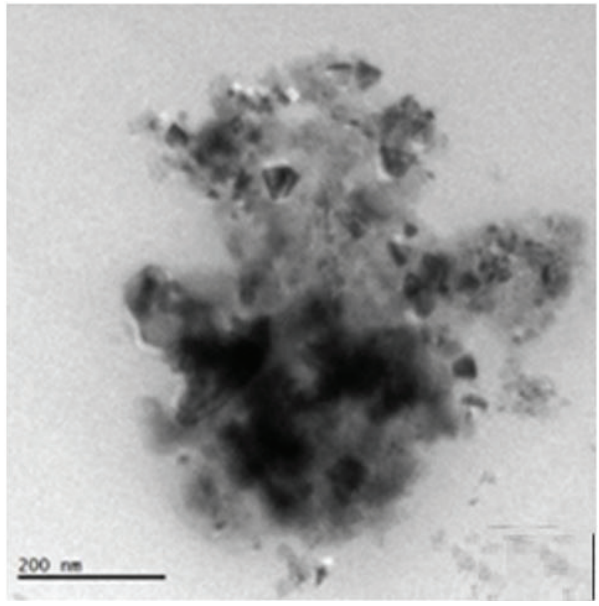


Figure 2. TEM images of NiO-NPs in cell culture medium.

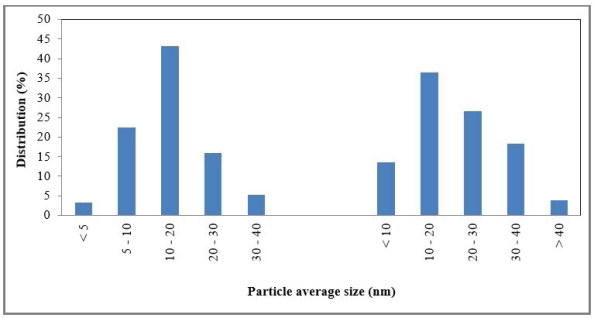


Figure 3. TEM images and the size distributions of NiO-NPs in water (a) and cell culture medium (b).

Cellular uptake

The results of ICP-MS indicate the cellular uptake of NPs after exposure to 50 and 100 µg/mL for 24 h (Table 1). The cellular uptake of NPs ranged from 1.87 to 7.92 mg/10^5 cells. The inclination of NPs to agglomerate and aggregate at the high concentrations could explain the decrease observed in the cellular uptake at higher exposure-concentration.

Table 1. The cellular uptake of NiO-NPs.

	Exposure concentration (µg/mL/10^5 cells)	Ni amount (mg/10^5 cells)
HepG2	Negative control	0.02±0.01
	50	7.92±0.26
	100	1.87±0.14

The cells were exposed to 50 and 100 mg/mL of NiO-NPs for 24 h. At the end of exposure time, their cellular uptake potentials were determined by ICP-MS, as described in the Methods section. Ni content of the unexposed cells (negative control) and the exposed cells were measured. The assay was repeated four times. The results were presented as mean ±SD.

Cellular morphology

Cellular morphological changes in the cells exposed to NiO-NPs at 50 and 150 µg/mL were evaluated using TEM photographs and compared to the morphology of the unexposed cells (negative control) (Figures 4-6). The NPs were observed in the cytoplasmic vacuoles of HepG2 cells at both 50 and 150 µg/mL. The size of the vacuoles was notably large, and particle agglomeration and aggregation within them were prominent at 50 µg/mL. The cytoplasmic vacuoles were observed to contain myelin figures as well as the NPs. The electron-lucent cytoplasmic vacuoles causing a complete degradation of the cytoplasm, and organelles were not evident. The nuclear and plasma membranes were intact. Although the size of the vacuoles and the NP amount within them were decreased in the cells exposed to 150 µg/mL of NiO-NPs, cellular damage was greater than with 50 µg/mL of NiO-NPs. In subjective assessment, according to the morphological changes in the cells including cytoskeleton, organelle shape, and size, *etc*., almost half of the cells were assessed as apoptotic or necrotic (Figures 4-6).

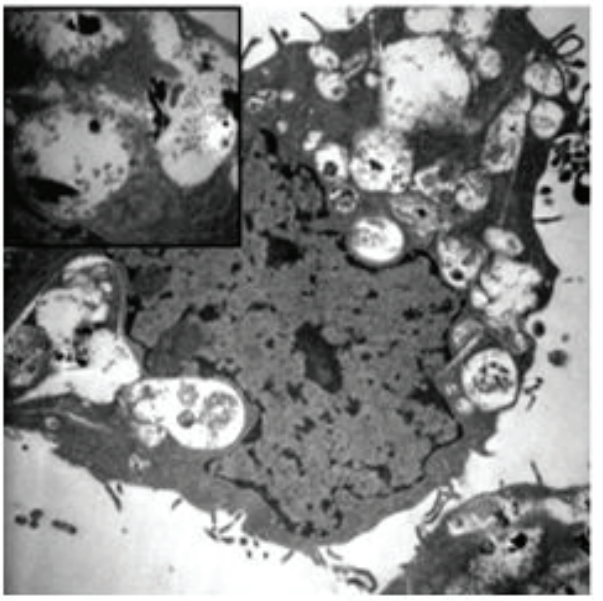


Figure 4. TEM observations of HepG2 cells after exposure to NiO-NPs for 24 h.

HepG2 cells exposed to NiO-NPs at 50 µg/mL

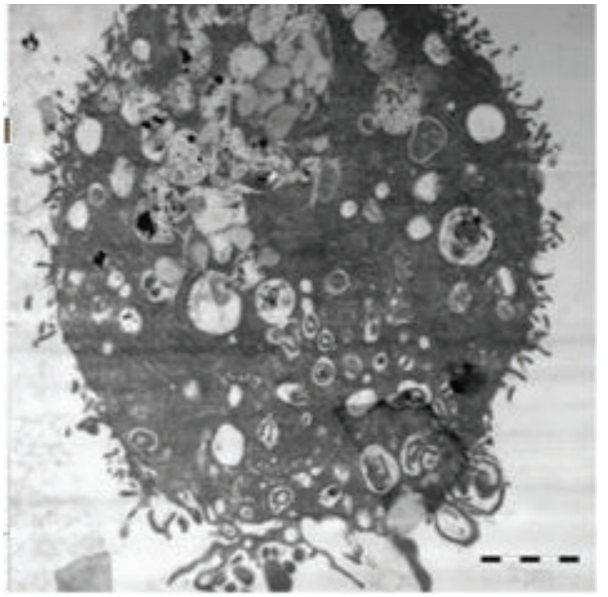


Figure 5. TEM observations of HepG2 cells after exposure to NiO-NPs for 24 h.

HepG2 cells exposed to NiO-NPs at 100 µg/mL

Lipid droplets were observed to increase with increasing NP concentrations, which could be an indicator or feature of the oxidative stress process (Khatchadourian & Maysinger, 2009; Lee, Homma, Kurahashi, Kang, & Fujii, 2015; Marmorato et al., 2011).

Cytotoxicity

The results of MTT and NRU assays showed that NiO-NPs decreased the cell metabolic activity after exposure to concentrations ranging from 50 to 500 µg/mL for 24 h (Figure 7). The dose-dependent reduction of cell metabolic activity and lysosomal functions could be related to an increase in cell death. The IC_{50} values were 146.7 and 203.8 µg/mL.

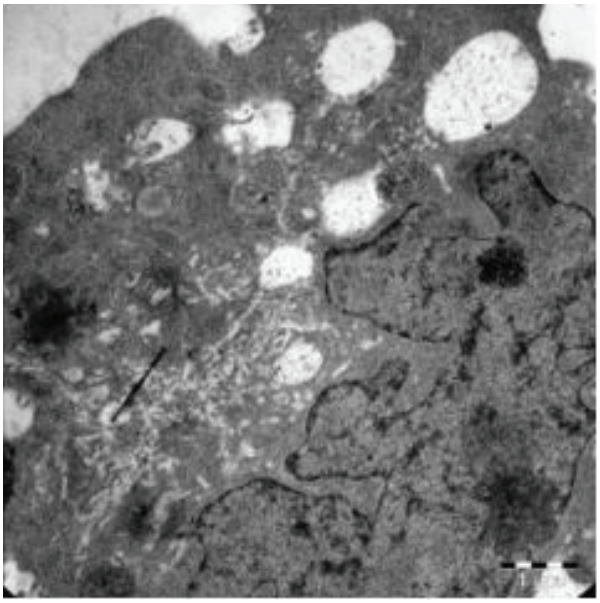
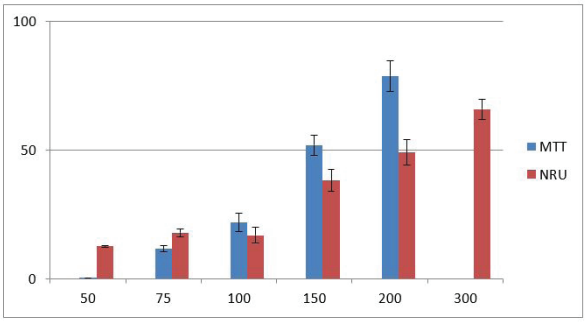


Figure 6. TEM observations of HepG2 cells after exposure to NiO-NPs for 24 h.

HepG2 unexposed cell (negative control)



	50	75	100	150	200	300	IC_{50}
MTT	0.33	11.63	21.86	51.89	78.74	-	146.7
NRU	12.59	17.8	16.9	38.17	49.06	65.8	203.8

Figure 7. NiO-NPs induced cytotoxicity to HepG2 cells.

The cells were exposed to different concentrations (50-300 mg/mL) of NiO-NPs for 24 h. At the end of the exposure time, cytotoxicity parameters were determined with mitochondrial function (MTT) and lysosomal activity (NRU) assays, as described in the Methods section. All experiments were done in triplicates and each assay as repeated four times. Data was expressed as mean±SD.

Genotoxicity

The genotoxicity of NiO-NPs was estimated by comet test at concentrations of 15-120 µg/mL (Figure 8). In positive controls (100 µM H_2O_2), the tail intensity was 22.6%. The results indicate that NiO-NPs significantly induced DNA damage in all cells (2.5 to 10.6-fold) in a concentration-dependent manner ($p \leq 0.05$). At a 120 µg/mL concentration (the highest; the cell deaths were ≤50%) the tail intensities were 22.6%.

Oxidative damage

The changes in the widely used oxidative damage parameters (i.e., GSH, MDA, 8-OHdG, and PC levels) were used to estimate the potential of NiO-NPs to induce oxidative damage to HepG2 cells after exposure to 50-150 µg/mL concentrations (Table 2).

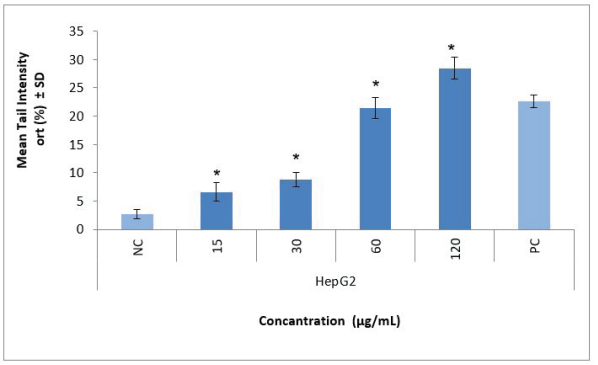


Figure 8. NiO-NPs induced genotoxicity to HepG2 cells.

The cells were exposed to different concentrations (15-120 mg/mL) of NiO-NPs for 24 h. At the end of the exposure time, their genotoxicity potential was determined by comet assay, as described in the Methods section. All experiments were done in triplicates and each assay as repeated twice. The results were presented as mean tail intensity (%) with ±SD. NC (PBS) and PC (100 µM H2O2) mean negative and positive controls, respectively. *p≤0.05 were selected as the levels of significance by one-way ANOVA Dunnett t-test

The NiO-NPs induced oxidative damage at all exposure concentrations. The levels of MDA were increased significantly (≤1.4-fold). A significant decrease in GSH levels was observed (≤34.2%). While the PC levels increased significantly (≤1.14-fold) at concentrations of 150 µg/mL, the levels were decreased significantly at 75 and 100 µg/mL (≤21.2%). No significant change in 8-OHdG levels was observed. In general, the potential of oxidative stress by NiO-NPs on HepG2 cells was statistically significant compared to the negative control ($p \leq 0.05$).

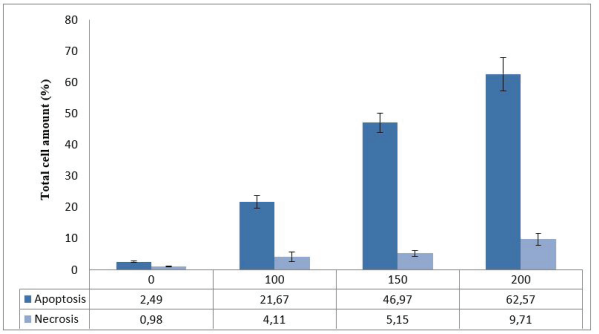
Table 2. Oxidative stress parameters that demonstrate NiO-NPs induced oxidative damage to HepG2 cells.

Cells	Exposure concentration (µg/mL)	8-OHdG (µg/g protein)	MDA (µmol/g protein)	GSH (µmol /g protein)	PC (µg/g protein)
HepG2	0	1.13±0.09	0.32±0.06	53.00±1.03	6.61±0.11
	50	1.10±0.15	0.44±0.04*	37.63±1.66*	6.48±0.49
	75	1.04±0.03	0.46±0.08*	39.72±1.98*	5.25±0.19*
	100	1.15±0.49	0.39±0.10*	34.90±2.09*	5.21±0.26*
	150	1.04±0.27	0.41±0.20	41.65±0.84*	7.46±0.20*

The cells were exposed to different concentrations (0-120 mg/mL) of NiO-NPs for 24 h. At the end of the exposure time, 8-OHdG, MDA, GSH and PC levels were determined by ELISA, as described in the Methods section.

Apoptosis

Test results indicate that NiO-NPs caused a concentration-dependent increase in the percentage of apoptotic cells (Figure 9). At the highest concentrations of NiO-NPs, equivalent to 75% cell death, the percentage of apoptotic cells was 86.6% whereas the necrotic cells were 13.4%.



	0	100	150	200
Apoptosis	2,49	21,67	46,97	62,57
Necrosis	0,98	4,11	5,15	9,71

Figure 9. NiO-NPs induced apoptosis/necrosis to HepG2 cells.

Cells were exposed to different concentrations of NiO-NPs, equivalent to 25%, 50% and 75% cell death (0-200 mg/mL) for 24 h; At the end of the exposure time, their apoptosis/necrosis inducing potentials were determined by Annexin V-FITC apoptosis detection assaywith PI, as described in the Methods section. All experiments were done in triplicates and each assay as repeated twice. The results were expressed as the percent of the total cell amount with±SD. *p≤0.05 were selected as the levels of significance by one-way ANOVA Dunnett t-test.

DISCUSSION

The previous studies showed that NPs could be absorbed through the gastrointestinal tract after accidental ingestion (as air pollution mixed with mucus and saliva) by industry and research laboratory workers or just by drinking and eating contaminated water and food (Ahmad et al., 2013). However, prior *in vitro* studies related to toxicity of NiO-NPs are controversial and mainly focused on the pulmonary system. In this work, it was aimed to evaluate the toxicity profile of NiO-NPs (average size of 15.0 nm) in HepG2 cells, which simulate one of the target organs for NPs after accidental exposure (Sadeghi, Tanwir, & Babadi, 2015; Sahu, Njoroge, Bryce, Yourick, & Sprando, 2014).

According to our results, NiO-NPs cause a dose-dependent decrease in the cellular metabolic activity (IC_{50}=146.7 – 203.8 µg/mL). However, the calculated IC_{50} values were higher than the values calculated by the previous studies. It is well known that free radicals and oxidative stress outcomes attack lipids, DNA, and proteins (Horie et al., 2011). The previous studies showed that NiO-NPs induce oxidative damage in different cell lines, such as HepG2, human neuroblastoma (SH-SY5Y), human lung carcinoma (A549), human colorectal carcinoma (Caco-2), and human breast epithelial (MCF-7) cells (Abudayyak et al., 2017; 2017b; Ahamed et al., 2011; Ahamed, et al., 2013; Ahmad et al., 2013; Horie et al., 2011; Siddiqui et al., 2012). The previous studies show that the induction of reactive oxygen species (ROS) after exposure to NiO-NPs plays an important role in lung inflammation (Cho et al., 2010; Horie et al., 2012; Jeong, Kim, Seok, & Cho 2016; Ogami et al., 2009). By the same token, Ahamed et al., (2013) reported that NiO-NPs caused cytotoxicity *via* ROS at 25-100 µg/mL. In this study, the significant changes in MDA, PC and GSH levels after exposure to 50-150 µg/mL concentrations could be an indication for the oxidative damage in the exposed cells.

Ahmed et al., (2013) reported that alteration of lysosomal and mitochondrial functions triggers cell death by apoptosis after exposure to NiO-NPs at 25-100 µg/mL. Similarly, the present results show that NiO-NPs altered the cell metabolic activity as assessed by the disruption of lysosome and mitochondrial functions; the IC_{50} values 146.7 and 203.8 µg/mL by MTT and NRU assays, respectively. In a study that investigated the cytotoxic effect of 24 NPs on human pulmonary cell lines (A549 and THP-1), IC_{50} values for NiO-NPs were 23.3 and 1613 µg/mL in two different labs (Lanone et al., 2009). They emphasized that differences in the sensitivity between cell types and cytotoxicity assays could be possible factors leading to differences in the obtained results and should be carefully considered while estimating NP toxicity.

Ahamed et al., (2013) indicated a significant increase in micronuclei induction in HepG2 cells after exposure to NiO-NPs; they reported up-regulation of mRNA expression levels of apoptotic genes Bax and caspase-3, and a down-regulation in the expression of bcl-2 an anti-apoptotic gene. Caspase-3 enzyme activity was also higher in exposed cells. An increase in caspase-3 enzyme activity was similarly reported by Siddiqui et al., (2012). They also reported a dose-dependent cytotoxicity, oxidative stress, apoptosis, and DNA fragmentation in HEp-2 and MCF-7 cells after exposure to NiO-NPs at 2-100 µg/mL. Capasso et al., (2014) indicated NiO-NPs at 20-100 µg/mL could be a potent activator of genotoxicity cascade in A549 and BEAS-2B human pulmonary cell lines. They also reported an alteration in the cell cycle of the exposed cells. Similarly, we observed NiO-NPs led to an increase in the percentage of apoptotic cells in a dose-dependent manner in HepG2 cells.

Horie et al., (2009) revealed the uptake of NiO-NPs into human keratinocyte (HaCaT) cells, and the particles were more cytotoxic to HaCaT cells than A549 cells. Duan et al., (2015) investigated the impacts of NiO-NPs on sirtuin 1, a NAD-dependent deacetylase involved in apoptosis in human bronchial epithelial cells (BEAS-2B). They reported an inhibition of the cell viability via the apoptotic process, and indicated that NiO-NP-induced apoptosis could occur *via* tumor protein p53 and bcl-2-associated X protein. As reported by the researchers, we observed NiO-NP-induced DNA damage and apoptotic/necrotic effects on the cells. The degree of apoptosis/necrosis corresponded with the severity of cytotoxicity (Figures 7 and 9). The apoptosis stage observed in HepG2 cells could depend on the damage of mitochondrial functions.

In conclusion, NiO-NPs are one of the widespread metal-based nanoparticles. The accidentally up taken NPs could attain the liver as one of the target organs. The previous studies emphasize the possible toxic effects of NiO-NPs on different target organs and cell lines. HepG2 was used previously to evaluate the toxicity of NiO-NPs. However, as different labs, the different methods and differently synthesized NPs could give different results, we have tried in this work to re-evaluate the same NPs using the same cell line but with different methods and assays. The results confirm the previous data; the DNA damage, apoptosis, and oxidative stress effects of NiO-NPs should attract attention. Further *in vivo* and *in vitro* studies using previously tested and new cell lines and animal models with different assays and protocols should be carried out to illuminate the safety associated with NiO-NPs and other NPs applications. Besides that, the supporting studies are needed to fully understand the mechanism of NiO-NP toxicity.

Peer-review: Externally peer-reviewed.

Author contributions: Conception/Design of Study- M.A., G.Ö.; Data Acquisition- M.A., G.Ö.; Data Analysis/Interpretation- M.A., G.Ö.,E.E.G.; Drafting Manuscript- M.A., G.Ö.,E.E.G.; Critical Revision of Manuscript- M.A., G.Ö.; Final Approval and Accountability- M.A., G.Ö.,E.E.G.

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

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Original Article

Effects of chlorpyrifos-methyl, chlormequat, deltamethrin, glyphosate, pirimiphos-methyl, tebuconazole and their mixture on oxidative stress and toxicity in HUVEC cell line

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ABSTRACT

Background and Aims: Humans and animals have daily contact with various chemicals, including food additives, pesticides, antibiotics, other veterinary drugs, and other xenobiotics. Pesticide exposure causes many health disorders. Mixed exposure to pesticides is an important issue for human and environmental health.
Methods: In this study, we have determined the cytotoxicity of chlormequat pirimiphos-methyl, glyphosate, tebuconazole, chlorpyrifos-methyl, deltamethrin, and the mixture of these six pesticides. We further investigated the role of oxidative stress, total oxidant status (TOS), lactate dehydrogenase (LDH) and antioxidant defense mechanism TOS, total glutathione (GSH) levels with the observed cytotoxicity.
Results: In this study, the mixtures of pesticides reduced total antioxidant status (TAS) and GSH level one by one and increased the reactive oxygen species (ROS) generation in HUVECs, respectively. The results also showed a significant contribution of oxidative stress on cytotoxicity during pesticide mixture exposure.
Conclusion: The findings are that pesticide mixture exposure might have an impact on human health risk at contaminated sites and under occupational exposure conditions.

Keywords: Chlorpyrifos-methyl, chlormequat, deltamethrin, glyphosate, pirimiphos-methyl, tebuconazole, oxidative stress, HUVECs

INTRODUCTION

Pesticides are commonly used compounds in farming that have a wide range of classes, such as insecticides, herbicides, fungicides, nematicides, acaricides, rodenticides, avicides, wood preservatives, and antifoulants. A remarkable amount of these pesticides spreads into the environment and this brings about immunotoxicity, carcinogenesis, and endocrine and developmental toxicity. Authorities such EFSA, EPA, and the Food, Drug, and Cosmetic Act, attaches importance to assessing pesticide compound mixture effects on the environment and living organisms (Laetz, Baldwin & Collier., 2009; Oyesola, Iranloye & Adegoke, 2019). Risk assessment of pesticide mixture is a highly important issue due to environmental and human exposure. People are always exposed to pesticide combinations due to protective strategies against pests in farms in farming. However, there is still no strict rule for risk assessment of pesticide compound mixture. Different combination risk assessment models

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can be seen in the literature. The most common risk assessment model is the dose addition model found in the European Food Safety Authority (EFSA) guidelines (Staal et al., 2018).

Conazole fungicides are widely used for agriculture and also treatment of mycosis and candida infections in humans and animals. Conazole fungicides cause disarrangement of fungal membranes via inhibition of cytochrome P450 enzyme lanosterol 14α-demethylase (CYP51). CYP51 is one of the main elements of ergosterol biosynthesis and normal fungal membrane integrity. Canozole fungicides have side effects such as endocrine disruption. These compounds exert their endocrine disrupting effects via CYP51 and CYP19 inhibition, which play a significant role as steroidogenic enzymes (Roelofs, Temming, Piersma, van der Beg & van Duursen, 2014). Tebuconazole is a widely used fungicide that has a sterol demethylation inhibitor effect. It has been reported that the Tebuconazole plasma half-life is approximately 600 days, and it causes toxic effects on the thyroid, liver, nervous system, and reproductive organs. In addition, it also exposes developmental and genetic toxicity (Li et al., 2020).

Chlormequat chloride is a quaternary ammonium compound which is used for floriculture as a plant growth regulator by reducing longitudinal shoot growth effect (Vijitharan, Warnasekare, Lokunarangoda, Farah & Siribaddana, 2016). Exposure to human beings is quite common. According to WHO, chlormequat is excreted 98% invariably and its acceptable daily intake (ADI) level is 0.05 mg/kg.bw. It is totally eliminated from the body within 46 h. It is important to investigate the potential toxic effects of chlormequat on cellular mechanisms due to the wide range of species that are exposed to it (Xiagedeer, Wu, Liu, & Hao, 2016). Organophosphorus pesticides (OP) are the most common pesticide type used worldwide. OPs are absorbed via inhalation, skin, eyes, and ingestion. The main acute toxic effect is inhibition of the acetylcholinesterase enzyme, which results in a cholinergic crisis. Pirimiphos-methyl belongs to the OP group of pesticides, which are non-cumulative and broad spectrum compounds. Even though pirimiphos-methyl is classified as "no reprotoxin, no teratogen" by OECD, its harmful effects on development and on the reproductive and immune systems have been shown in studies (Oyesola et al. 2019; Olsvik, Berntssen & Søfteland, 2017; Anogwih, 2014). Glyphosate is a non – selective organophosphorus herbicide and it has bioaccumulation potential in the environment. Glyphosate exposure causes toxic effects in mammalians by increasing oxidative stress levels. Due to its common usage, this pesticide residue can be found in food and environmental samples. The main toxicity mechanism of glyphosate increases the emergence of ROS and causes disruption on cellular macromolecules by ROS (Cai et al., 2020; Odetti et al., 2020; Zhang, Yang, Ma, Shi & Chen, 2020). Chlorpyrifos is another widely used broad-spectrum organophosphorus insecticide. It has been reported that chlorpyrifos might have some toxic effects on organs and systems. Its common toxic effect is on the nervous system by acetylcholinesterase (AChE) inhibition. Its main toxic effect mechanism is associated with cellular oxidative stress increment (Deng, Zhang, Lu, Zhao & Ren, 2016; Dokuyucu, et al., 2016). Deltamethrin is part of the potent pyrethroid class of insecticides and acaricides. Also, deltamethrin has been used for controlling human diseases caused by mosquitos that are vectors of Zika virus and Dengue virus. It has been reported that deltamethrin exerts its toxic effects by increasing cellular ROS and reactive nitrogen species (RNS). Oxidative stress remarkably increases the deltamethrin toxicity mechanism (Lu et al., 2019).

Oxidative stress is a cellular imbalance condition which is formed between free radical production and the antioxidant defense system. Several chemicals may cause free radical production and through this, these radicals interact with important cellular molecules and impair their functions pathologically (Abdollahi, Ranjbar, Shadnia, Nikfar & Rezaie, 2004). Pesticide exposure may cause reactive oxygen species production in the cell that exceeds antioxidant defenses system. The cellular antioxidant system includes several molecules and enzymes, including reduced glutathione (GSH), non-protein thiols, catalase (CAT), glutathione-S-transferases (GST), superoxide dismutase (SOD), and glutathione reductase (GR) (Ferreira, et al., 2010). The pesticide-induced oxidative stress molecular mechanism has still not been clarified and is under scrutiny by scientists seeking to evaluate risk factors and understand related pathologic diseases (Agrawal & Sharma, 2010).

There are limited studies in the literature for these six pesticides' effects on cellular oxidative stress in endothelial cells. HUVECs are cell models that are frequently used to investigate the underlying mechanisms of endothelial diseases (Medina-Leyte, Domínguez-Pérez, Mercado, Villarreal-Molina, & Jacobo-Albavera, 2020) In this study, we have evaluated the toxicity of singly and mixture In this study, we have evaluated the toxicity of chlorpyrifos-methyl, chlormequat, deltamethrin, glyphosate, pirimiphos-methyl, tebuconazole singly and as a mixture. Further oxidative stress-inducing potential was evaluated in HUVECs. This is the first study of the effects of this pesticide mixture on HUVECs.

MATERIALS AND METHODS

Cell culture and cell viability assays

The Human Umbilical Vein Endothelial (HUVEC) cell line was provided from American Type Culture Collection (ATCC® PCS-100-010™). Cells were grown in RPMI 1640 (GIBCO, Uxbridge, UK) including 10% fetal bovine serum (GIBCO, Uxbridge, UK), 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO, Uxbridge, UK), with incubation conditions of 37°C and a humidified atmosphere with 5% CO_2. Cell passage was performed with 0.025% trypsin-0.02% EDTA after cell co-fluency. Cells passage was performed twice a week. HUVEC cells are a sufficient model for investigating the molecular mechanisms of cardiovascular diseases under different chemical exposure conditions (Medina-Leyte et al., 2020).

Chlormequat chloride, pirimiphos-pethyl, glyphosate, tebuconazole, chlorpyrifos-methyl, deltamethrin, and their mixture's effects on the HUVECs viability were assessed by MTT test, which depends on measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) compound to formazan by the mitochondrial enzyme succinate dehydrogenase. After a 24 h exposure duration, cells were incubated with MTT for 3 h at 37°C in a humidified atmosphere (95%) with 5% CO_2. After incubation, cell media was discarded,

100 µL DMSO was added into each well, and absorbance of formazan compound was measured with spectrophotometry (Epoch, Biotek) at a wavelength of 595 nm. Pesticide exposure concentrations are shown in Table 1 (Roelofs et al. 2014).

Table 1. Pesticide exposure concentrations for cell viability assay.

Pesticide	Concentrations (mM)
Chlormequat chloride	25, 50, 100 and 200
Pirimiphos-Methyl	25, 50, 100 and 200
Glyphosate Potassium	25, 50, 100 and 200
Tebuconazole	25, 50, 100 and 200
Chlorpyrifos-methyl	25, 50, 100 and 200
Deltamethrin	2.5, 5, 10 and 20
Mix	50 and 200

Oxidative stress quantification assays

Lactat Dehydrogenase (LDH) Assay: LDH assay was performed with an LDH assay kit (ab102526-abcam). This assay was based on quantifying LDH activity *via* reducing NAD to NADH, then NADH interacts with a specific kit reagent that produces a color which can be detected at 450 nm. Cell lysates were mixed with reaction reagent and the colored sample was read at 450 nm with a spectrophotometer (Epoch, Biotek).

Total Antioxidant Status (TAS): TAS assay was performed via a TAS reagent kit (Rel Assay Diagnostics – Turkey) through the manufacturer's service bulletin. This assay was based on antioxidants reducing kit ABST component, and the absorbance change was detected by spectrophotometer at 660 nm. The assay is calibrated via trolox equivalent. Cell lysates and standards were mixed with reagent 1 and this mixture was measured spectrophotometrically. After reagent 2 was added into the mixture, the measurement was done at 5 min interval at 660 nm.

Total Oxidant Status (TOS): TOS assay was performed by TOS a Reagent kit (Rel Assay Diagnostics – Turkey) with the manufacturer's instruction. This assay was based on oxidants in the sample which oxidize the ferrous ions. Oxidized ferric ions make a colored complex that can be measured by spectrophotometer at 530 nm. The assay is calibrated via hydrogen peroxide. Cell lysates and standards were mixed with reagent 1 and the first spectrophotometric measurement wasdone with 530 nm. Then reagent 2 was added into the mixture and absorbance measurement was was done at 5 minutes interval.

Total Glutathione (GSH) Assay: GSH assay was performed by Glutathione Detection Assay Kit (Fluorometric) (Abcam-ab65322). This kit detects both reduced and oxidized glutathione. This assay is based on monochlorobimane (MCB), which is adducted with GSH that is catalyzed by GST. Bound MCB creates fluorescent blue light (Ex/Em=380/461 nm), which is detected by fluorometer (CARY ECLIPSE - USA).

RESULTS

Cell viability

According to MTT assay results, IC_{50} values of five pesticides and their combination exposure effects have been listed in Table 2 (Figure 1). 50 and 200 mM concentrations of each pesti-

Table 2. IC_{50} values of pesticides according to MTT results.

Pesticide	IC_{50} value (mM)
Chlorpyrifos-methyl	51.836
Pirimiphos-Methyl	52.729
Glyphosate	151.198
Chlormequat Chloride	54.232
Tebuconazole	25.837
Deltamethrin	8.567
Mix 50 and mix 200	38.060

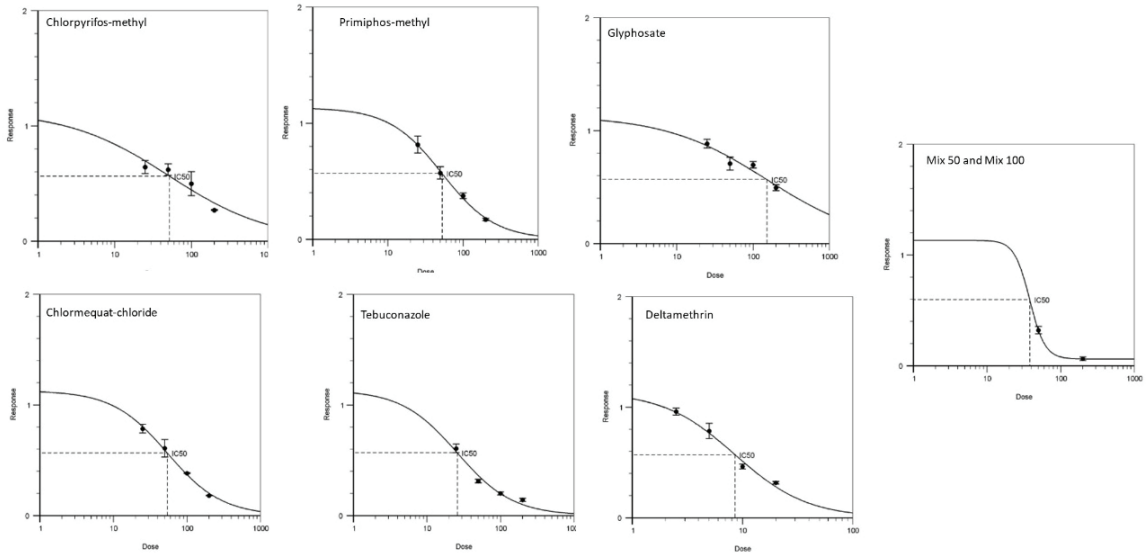

Figure 1. Cell viability assay graphs of six pesticided and their combination at 50 and 100 mM concentrations. IC_{50} values of each pesticide were calculated via graphs slope formulation.

cide were chosen for combination exposure. For LDH, TAS, TOS and GSH assays 25, 50, 100 and 200 mM concentrations were chosen for 24 h exposure. We used concentrations that were above the IC_{50} values to compare higher concentration effects on oxidative stress parameters.

LDH assay

LDH values significantly decreased at 25 mM concentration for all six pesticides compared to the control group. Chlormequat chloride exposure to all experimental concentrations decreased LDH level. Increased LDH levels were observed in higher concentrations of chlorpyrifos-methylp and pirimiphos-methyl groups. 50 and 200 mM mix groups showed a significant decrease in LDH levels compared to the control group (Figure 2). LDH levels after pesticide exposure are shown in Table 3.

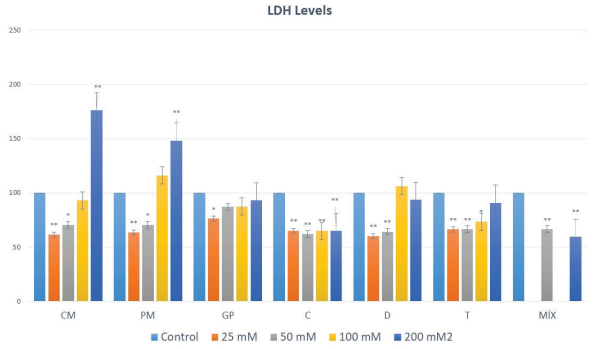

Figure 2. LDH assay results of six pesticides. *p<0,05; **p<0,001. CM: Chlorpyrifos-methyl, PM: Pirimiphos-Methyl, GP: Glyphosate, C: Chlormequat Chloride, D: Deltamethrin, T: Tebuconazole.

Total antioxidant status

TAS levels decreased in concentration dependent, but not significantly in the chlorpyrifos-methyl group. TAS levels decreased in pthe pirimiphos-methyl and deltamethrin groups. TAS levels decreased in the glyphosate, chlormequat chloride, and tebuconazole groups only in 25 and 200 mM exposure concentrations compared to the control group. TAS levels decreased in mixed groups compared to the control group (Figure 3). TAS levels after pesticide exposure are shown in Table 3.

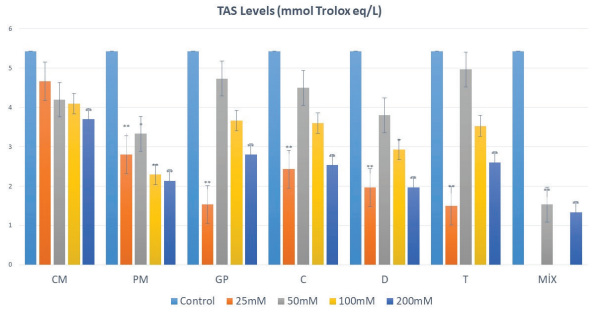

Figure 3. TAS assay results of six pesticides. *p<0,05; **p<0,001. CM: Chlorpyrifos-methyl, PM: Pirimiphos-Methyl, GP: Glyphosate, C: Chlormequat Chloride, D: Deltamethrin, T: Tebuconazole.

Total oxidant status

TOS levels increased significantly in pthe pirimiphos-m, glyphosate, chlormequat chloride, deltamethrin, and tebuconazole single exposures and in the mixture exposure compared to the control group (Figure 4). TOS levels after pesticide exposure are shown in Table 3.

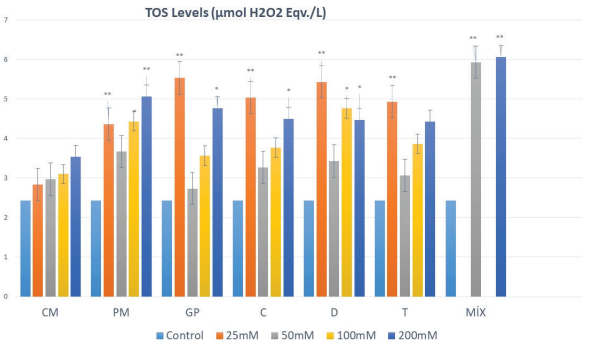

Figure 4. TOS assay results of six pesticides. *p<0,05; **p<0,001. CM: Chlorpyrifos-methyl, PM: Pirimiphos-Methyl, GP: Glyphosate, C: Chlormequat Chloride, D: Deltamethrin, T: Tebuconazole.

Glutathione levels

GSH levels decreased in all exposure concentrations significantly in the chlormequat chloride, deltamethrin, tebuconazole, and mix groups. In the glyphosate group 50, 100, and 200 mM concentrations, GSH levels decreased significantly compared to the control group. In pthe Pirimiphos-methyl 100 and 200 mM concentrations, GSH levels decreased significantly compared to the control group. However, for the 25 mM group, GSH level increased dramatically. For the chlorpyrifos-methyl group 100 and 200 mM concentrations, GSH levels decreased significantly compared to the control group (Figure 5). GSH levels after pesticide exposure are shown in Table 3.

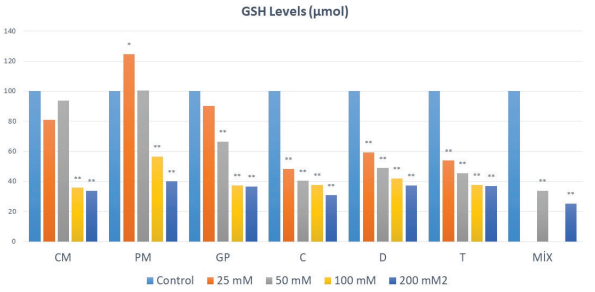

Figure 5. GSH assay results of six pesticides. *p<0,05; **p<0,001. CM: Chlorpyrifos-methyl, PM: Pirimiphos-Methyl, GP: Glyphosate, C: Chlormequat Chloride, D: Deltamethrin, T: Tebuconazole.

DISCUSSION

Pesticides are commonly used chemicals that protect agricultural products from weeds, insects, fungus, and rodents. However, pesticides are mostly toxic for the environment and living organisms. Also, pesticides are commonly used for controlling malaria and dengue disease vectors and controlling plant growth in public places, such as parks and gardens,

Table 3. LDH, TASS, TOS and GSH levels in HUVEC cells after 24h pesticide exposure duration.

Pesticides	LDH (%)	TAS (mmolTrolox eq/L)	TOS (μmol H_2O_2 eq/L)	GSH (% for control group)
Control	100	5.4±0.1	2.4±0.1	100
Chlormequat chloride (mM)				
25	65.1	2.4±0.1	5.1±0.2	48.4
50	62.2	4.5±0.2	3.2±0.2	40.3
100	65.1	3.6±0.2	3.7±0.1	37.6
200	61	2.5±0.1	4.5±0.1	30.7
Pirimiphos-Methyl (mM)				
25	63.6	2.8±0.1	4.3±0.1	100.5
50	70.6	3.3±0.2	3.6±0.1	100.3
100	116.8	2.3±0.2	4.4±0.05	56.6
200	140.1	2.1±0.3	5.1±0.2	40
Glyphosate Potassium (mM)				
25	76.2	1.5±0.2	5.5±0.1	90.3
50	87.4	4.7±0.2	2.7±0.1	66.5
100	87.5	3.6±0.3	3.5±0.2	37.3
200	93	2.8±0.1	4.7±0.1	36.5
Tebuconazole (mM)				
25	66.4	1.5±0.1	4.9±0.1	53.8
50	66.5	4.9±0.2	3.1±0.1	45.3
100	73.4	3.7±3.5	3.8±0.2	37.6
200	90.9	2.6±0.2	4.4±0.2	36.9
Chlorpyrifos-methyl (mM)				
25	61.5	4.6±0.1	2.8±0.2	81.1
50	70.6	4.2±0.3	2.9±0.2	93.8
100	93	4.1±0.3	3.1±0.1	35.7
200	170.1	3.7±0.4	3.5±0.1	33.8
Deltamethrin (mM)				
2.5	60.1	1.8±0.1	5.4±0.1	59.2
5	64.3	3.8±0.1	3.4±0.1	48.8
10	93.7	1.9±0.1	4.7±0.1	41.9
20	100	1.5±0.1	4.4±0.2	37.7
Mix 50	66.4	1.4±0.1	5.9±0.1	33.8
Mix 200	59.4	1.3±0.1	6.1±0.1	25.3

which trigger important risk factors for public health. Pesticide exposure has different acute and chronic effects, including cancer, asthma, diabetes, and neurodegeneration (Kim, Kabir & Jahan, 2017). One of the main mechanisms underlying reasons of pesticide toxicity is oxidative stress. Pesticides have induced oxidative stress mechanisms in relation with diseases, and this is still a question of debate. Oxidative stress is a cellular homeostatic imbalance between ROS and antioxidants. While ROS increases in the cell, ROS products interact with important cellular molecules and cause detrimental effects in the cell that bring about several different diseases. Antioxidants are very important cellular soldiers against ROS products. There are several different studies on the oxidative stress induction effects of pesticides in the literature (Agrawal &Sharma, 2010).

In our study, we have investigated the cytotoxicity and oxidative stress inducing effects of six different pesticides (chlorpyrifos-methyl, pirimiphos-methyl, glyphosate, chlormequat chloride, deltamethrin, and tebuconazole) and the exposure of the mixture of them on the HUVEC cell line. HUVECs are very important for analyzing endothelial markers and representing vascular physiology *in vitro*. It is a general model for endothelial cells with normal and pathological condition analyses. The HUVEC cell line is a good choice for analyzing xenobiotic toxic effects (Cao et al. 2017; Benachour & Séralini, 2009).

It has been reported that LDH levels increase during oxidative stress induction in the cells (Jovanovic et al., 2010). According to our results, LDH levels decrease in a lower concentration (25 mM) in all pesticide groups. LDH levels significantly decrease in all chlormequat chloride concentrations. Increased LDH levels are observed in a higher concentration of chlorpyrifos-methyl and pirimiphos-methyl groups. The increased LDH level of higher concentrations is associated with why these concentrations have exceeded IC_{50} values. Decreased LDH levels are observed significantly in 50 and 200 mM mix groups compared to the control group. In this study, TAS levels decrease and TOS levels increase in all groups, and this is balanced in itself. This is the first study to demonstrate chlormequat cloride effects on LDH levels and oxidative stress. It has been demonstrated that chlorpyrifos-methyl, pirimiphos-methyl, glyphosate, chlormequat chloride, deltamethrin, and tebuconazole increased LDH and oxidative stress levels in different studies (Banaee et al. 2019; Hatami, Banaee, Nematdoodt, & Haghi 2019; Nasr, El-Demerdash & El-Nagar, 2016; Yıldırım et al. 2013; Sai et al. 2014; Tilak, Veeraiah & Rao, 2005; Mansour & Mossa, 2011; Piatti, Marabini & Chiesara, 1994; Martínez et al 2020; Li et al. 2017; Gholami-Seyedkolaei, Mirvaghefi, Farahmand, & Kosari 2013; Gündüz et al. 2015; Abdel-Daim et al. 2016; Ncir et al. 2016; Berrouague et al. 2019; Ben Othmène et al.2020), and our result is in accordance with previous studies.

In our study, GSH levels significantly decreased concentration dependent for all exposure concentrations in the chlormequat chloride, deltamethrin, tebuconazole, and mixture groups. In the glyphosate group of 50, 100 and 200 mM concentrations, GSH levels decreased significantly compared to the control group. In pirimiphos-methyl 100 and 200 mM concentrations, GSH levels decreased significantly compared to the control group. For chlorpyrifos-methyl group 100 and 200 mM concentrations, GSH levels decreased significantly compared to the control group. This is the first study showing chlormequat chloride and Tebuconazole's effects on GSH level. This study is done in accordance with previous studies on decreasing GSH in the literature for deltamethrin (Zaki, Algaleel, Imam, Soliman & Ghoneim, 2020; Bhattacharjee, Borah & Das, 2020; Kumar, Sharma, Rana & Sharma, 2019; Yang et al. 2020; Ndonwi et al. 2019; Wang, Shen, Zhou, & Jin 2019; Velki &Hackenberger, 2013).

In conclusion, living organisms always encounter different types of pesticides and their combination in daily life through different exposure pathways. It is very important to clarify their toxic effect mechanisms and relations with diseases to make effective risk assessments and set regulations based on this information. Endothelial cells situate in the first phase of exposure to xenobiotics, and they are affected more. This study has confirmed that the oxidative stress inducing effects of exposure to different types of pesticides and their mixture (chlorpyrifos-methyl, pirimiphos-methyl, glyphosate, chlormequat chloride, deltamethrin, tand tebuconazole) and reducing antioxidant capacity of the cells. Further *in vitro* and *in vivo* studies need to clarify molecular interactions between pesticide inducing oxidative stress and endothelial cell related diseases. When these mechanisms are understood, protective and therapeutic strategies for the treatment of pesticide exposure's toxic effects can be developed.

Peer-review: Externally peer-reviewed.

Author contributions: Conception/Design of Study- Ç.S.; Data Acquisition- A.T.; Data Analysis/Interpretation-M.K.; Drafting Manuscript-M.K.; Critical Revision of Manuscript-Ç.S.; Final Approval and Accountability- Ç.S., A.T., M.K.

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

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Original Article

The effects of prochloraz on the levels of nuclear receptor genes expressions and global DNA methylation in human prostate carcinoma cells

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ABSTRACT

Background and Aims: Prochloraz (PCZ) is an imidazole fungicide which is used in agriculture and gardening. PCZ, with endocrine disrupting effect, disrupts reproductive and developmental functions. Previously, the effects of PCZ on the estrogen and androgen receptors have been shown *in vitro* and *in vivo*. Because of endocrine disrupting effects, PCZ could influence nuclear receptors which acted as ER and AR antagonists. Besides, PCZ has been thought to have no genotoxic effects. Therefore, we aimed to investigate possible effects of PCZ on nuclear receptor genes and epigenetic mechanisms in human prostate carcinoma (PC-3) cells.

Methods: In the present study, MTT and LDH tests were applied to evaluate the cell viability. Expression levels of nuclear receptor genes such as *AhR, PXR, PPARα, PPARγ* were studied on real-time quantitative PCR. For global DNA methylation analysis, the levels of 5-methylcytosine (5-mC%) were measured by elisa kit.

Results: According to MTT and LDH test results, IC50 value of PCZ has been determined as 144.19 and 116.65 μM, respectively. There were significant changes for the expression levels of *AhR, PPARα* and *PPARγ* genes after 5-50 μM of PCZ treatments. 5 and 50 μM of PCZ decreased the levels of 5-mC% in the rates of 22.6% and 26.9%, respectively.

Conclusion: It has been suggested that PCZ may cause alterations on the expressions of nuclear receptor genes which could be related to endocrine disrupting effects and may have implications on global DNA methylation.

Keywords: Cytotoxicity, DNA methylation, nuclear receptor genes, PC-3 cells, prochloraz

INTRODUCTION

Prochloraz (PCZ) (CAS no. 67747-09-5; N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]-*1H*-imidazole-1-carboxamide) is an imidazole fungicide used in gardening and agriculture. The action of imidazole fungicides is based on the blocking cytochrome P450-dependent enzyme activity, for the conversion of an essential component of fungal cell membranes (Henry & Sisler, 1984). Exposure to PCZ , mainly through consumption of fruits and vegetables such as wheat, barley, rice, cereal, cherries, has been important worldwide (Claeys et al., 2011). PCZ exhibited low acute toxicity and has been shown to display hepatotoxic effects (Goettel et al. 2015; Heise et al., 2015, 2018; Marx-Stoelting et al., 2017), induction of oxidative stress (Lundqvist, Hellman & Oskarsson, 2016; Alpertunga et al., 2014; Sanchez, Piccini & Porcher, 2008), endocrine disrupting effects (Vinggaard, Hnida, Breinholt & Larsen 2000; Vinggaard et al., 2002, 2006) and also tumorigenic effects following chronic exposure (EFSA, 2011). The modes mechanisms of action of PCZ

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were antagonism of the androgen and estrogen receptors and inhibition of steroidogenic enzymes which play a key role in biosynthesis and metabolism of steroid hormones as well as a potent aromatase inhibitor (Laier et al., 2006; Andersen, Vinggaard, Rasmussen, Gjermandsen & Bonefeld-Jørgensen, 2002; Vinggaard et al., 2002). PCZ also showed its effects by activating the aryl hydrocarbon receptor (AhR) through up-regulation of *CYP1A1* expression in different liver cell lines and rat liver (Marx-Stoelting et al., 2017; Halwachs, Wassermann, Lindner, Zizzadoro & Honscha, 2013; Long et al., 2003; Vinggaard et al., 2006).

DNA methylation, one of the most studied epigenetic modifications, plays crucial roles in aging, cell proliferation and various diseases such as cancer, diabetes (Anderson, Sant & Dolinoy, 2012; Kulis & Esteller, 2010; Jones & Baylin, 2007; Moggs, Goodman, Trosko, & Roberts, 2004; Baylin, 1997). Mutagenicity and genotoxicity of PCZ have been summarized by JMPR and EFSA that PCZ exerted in some test systems with negative results except in *in vitro* sister chromatid exchange assay (JMPR, 2001). Based on these results, JMPR (2001) and EFSA (2011) clarified that PCZ is improbable to be potentially genotoxic. In our previous study, PCZ also showed no evidence of mutagenicity and DNA damage in NRK-52E cells (Alpertunga et al., 2014). Controversially, Lundqvist et al. (2016) have indicated that non-toxic concentrations of PCZ triggered DNA damage in HepG2 cells by Comet assay. Therefore, non-gentoxic mechanisms may have a role in PCZ toxicity. Therefore, in present study we aimed to investigate global DNA modifications of PCZ for the first time. We studied potential toxic effects of PCZ on nuclear receptor genes and global DNA methylation as an epigenetic mechanism.

MATERIALS AND METHODS

Chemicals

PCZ, Pestenal, analytical standard (purity 99.2%), was obtained from Sigma-Aldrich Chemicals Co (St. Louis, Missouri, USA). A stock solution of PCZ was prepared by dissolving it in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, Missouri, USA). Cell culture media and all other supplements were purchased from Wisent Bioproducts (Saint-JeanBaptiste, QC, Canada), and sterile plastic materials were purchased from Nest Biotechnology (Jiangsu, China). Cytotox-LDH-XTT 2 Parameter Cytotoxicity kit was used for cytotoxicity (Xenometrix AG, Allschwil, Switzerland). DNA, RNA isolation kits and cDNA synthesis kits were obtained from Roche Life Sciences (Penzberg, Upper Bavaria, Germany). 5-methylcytosine (5-mC) DNA ELISA kit was purchased from Zymo Research (Irvine, CA, USA). Syber green master mix was obtained from Bioline (London, UK), and primers for gene expressions were obtained from Sentromer DNA Technologies (Istanbul, Turkey).

Cell culture and treatments

The human prostate adenocarcinoma cell line (PC-3 cells) was obtained from American Type Culture Collection (ATCC®CRL-1435™) and cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM-F12) under standard cell culture conditions as described previously (Senyildiz, Karaman, Baş, Pirinççi, Özden, 2016, 2017).

For gene expression and global DNA methylation analysis 1×10^6 cells were incubated in a 25 cm^2 culture flask for 24 h in CO_2 incubator prior to the treatment. The exposure concentrations of PCZ used in the experiments have been determined with our cytotoxicity studies and based on our previous study (Alpertunga et al., 2014).

Cell viability

Effects of PCZ on cell viability were assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, a tetrazole] and lactate dehydrogenase (LDH) cytotoxicity tests. Principle of the MTT test; yellow MTT is reduced to purple formazan in the mitochondria of living cells by the enzyme succinate dehydrogenase, and it measures cell viability and proliferation (Alley et al., 1988; Mossman, 1983). The LDH test is a cell viability test method, based on measuring membrane integrity via % LDH release (lactate dehydrogenase activity) (Decker & Lohmann-Matthes, 1988; Korzeniewski & Callewaert, 1983).

For cytotoxiciy assay, 1×10^4 cells (in 100 µL medium) were seeded in 96-well plates and were treated with PCZ in the concentration range of 7.81-500 µM and DMSO (1%, exposure concentration in culture media) as solvent control for 24 h. Then, cell viability and % LDH release were measured and assessed using the MTT and LDH test as described in our previous study (Karaman & Ozden, 2019; Demirel, Alpertunga & Ozden, 2015).

Gene expression analysis of nuclear receptor genes

After 5, 10 and 50 µM of PCZ treatments for 24 h, total RNA was isolated from PC-3 cells using High Pure RNA Isolation kit (Roche Life Science). Reverse transcription was performed by Transcriptor First Strand cDNA Synthesis kit from 500 ng of total RNA, and the mixture of anchored-oligo(dT) and random hexamer primers. 5 µL of the 1/10 diluted RT-reaction was used as the template in real-time quantitative PCR. Gene expressions of nuclear receptor genes such as *AhR* (aryl hydrocarbon receptor), *PXR* (pregnane-X receptor), *PPARα* (peroxisome proliferator-activated receptor alpha), *PPARγ* (peroxisome proliferator-activated receptor gamma) were measured using BioLine SensiFast™ Syber® No-Rox kit (London, UK) on LightCycler® 480 Instrument II (Roche Life Science). Primer sequences and their annealing temperatures of genes are illustrated in Table 1. Evaluations of results for all genes were performed as described previously (Karaman & Ozden, 2019).

Global DNA methylation

5, 10 and 50 µM of PCZ exposed to PC-3 cells for 24 h, then genomic DNA was isolated from cells using the High Pure PCR Template Preparation kit (Roche Life Sciences, Penzberg, Germany) according to the instructions provided by the manufacturer. To measure global levels of DNA methylation, 100 ng of DNA samples were applied to 5-mC DNA elisa kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions as previously described (Senyildiz, Karaman, Bas, Pirincci & Ozden, 2017). Also, gene expression analysis of DNA methyltransferase genes including *DNMT1* (DNA methyltransferase 1) and *MGMT* (O-6-methylguanine-DNA methyltransferase) were performed by real-time quantitative PCR analysis using BioLine SensiFast™ Syber® No-Rox kit (London, UK).

Table 1. Primers used real-time PCR analysis of nuclear receptor genes, DNA methyltransferase and the corresponding annealing temperatures.

Gene	Primer sequence (5'-3')	Tm (°C)	Reference
AhR	F: TGGACAAGGAATTGAAGAAGC R: AAAGGAGAGTTTTCTGGAGGAA	53	Ayed-Boussema et al., 2011
PPARα	F: CATTACGGAGTCCACGCGT R: ACCAGCTTGAGTCGAATCGTT	58	Rogue et al., 2011
PPARɣ	F: CTGAATGTGAAGCCCATTGAA R: GTGGAAGAAGGGAAATGTTGG	54	Harada et al., 2005
DNMT1	F: CCTCCAAAAACCCAGCCAAC R: TCCAGGACCCTGGGGATTTC	60	Ahmadnejad et al., 2017
MGMT	F: TGCACAGCCTGGCTGAATG R: GGTGAACGACTCTTGCTGGAA	58	Lai et al., 2008
β-actin	F: AACTACCTTCAACTCCAT R: TGATCTTGATCTTCATTGTG	48	Rosa et al., 2009

Statistical analysis

Global methylation levels (5-mC%) and cytotoxicity results were represented as mean±standard deviation (SD). Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test using "SPSS version 21.0 for Windows", statistical program (IBM Analytics, New York, USA). P values of less than 0.05 and 0.001 were selected as the levels of significance.

RESULTS

Effects of PCZ on the cell viability in PC-3 cells

We performed cytotoxicity of PCZ in the concentration range of 3.906-500 µM in PC-3 cells after 24 h exposure by using MTT and LDH assays. IC_{50} values of PCZ were determined as 144.19 and 116.65 µM, respectively. As shown in Figures 1a and 1b, treatments of PCZ for 24 h significantly decreased the cell viability ≥10.13% and ≥12.74% for MTT and LDH tests, respectively, in comparison to the vehicle control group.

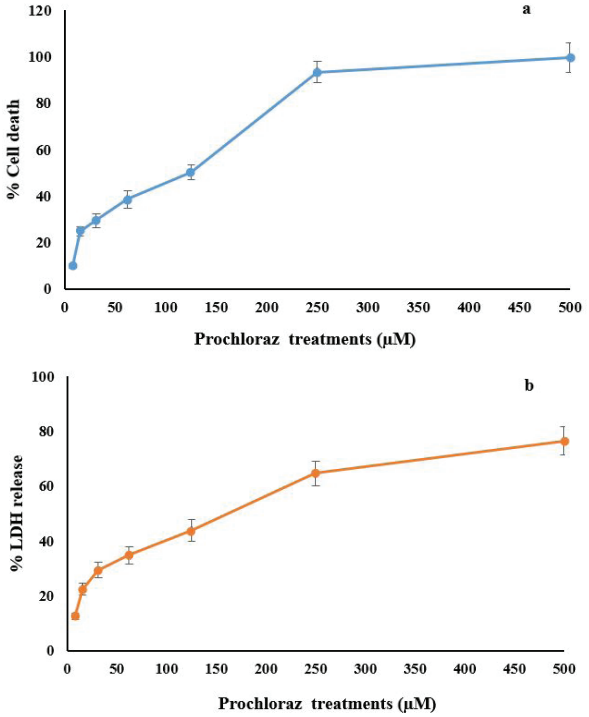

Figure 1. Effects of PCZ (7.81, 15.62, 31.25, 62.5, 125, 250 and 500 µM) on cell viability by MTT (a) and LDH (b) tests in PC-3 cells after 24 h exposure. Data are presented as mean±SD.

Effects of PCZ on global DNA methylation in PC-3 cells

Levels of 5-mC% were observed after 5, 10 and 50 µM treatments of PCZ for 24 h by elisa kit. Exposure to 10 and 50 µM of PCZ for 24 h resulted in a significant decrease in 5-mC% status (22.67%, $p<0.05$ and 26.97%, $p<0.05$, respectively) comparison with the control group (Figure 2a). Consistently, 10 µM (61.9%) and 50 µM (85.21%, $p<0.05$) of PCZ decreased the expression levels of *DNMT1* (Figure 2a) while non-significant decrease was observed for *MGMT* (Figure 2b).

Effects of PCZ on nuclear gene expression levels in PC-3 cells

We analysed the gene expression of selected nuclear receptor genes (*AhR*, *PPARα*, *PPARγ*, *PXR*) to investigate the potential effects of PCZ. In Figure 3, our data showed that 10 µM of PCZ significantly increased expression levels of *PPARα* (2.63 fold, $p<0.05$), 10 and 50 µM of PCZ significantly increased expression levels of *PPARγ* (1.73 fold, $p<0.05$ and 3.09, $p<0.05$, respectively), and 50 µM of PCZ significantly decreased expression levels of *AhR* (53.3%, $p<0.05$). However, no expression of *PXR* gene was observed at all experiment groups, even in control group.

DISCUSSION

PCZ has hepatotoxic properties, exerts tumorigenic effects following chronic exposure and induces gene expression and enzyme activity of CYP1A in animal studies (Goettel et al. 2015; Heise et al., 2015, 2018; Marx-Stoelting et al., 2017; Sturm, Cravedi, Perdu, Baradat & Segner 2001; Vinggaard et al., 2006). It has been reported that PCZ acts as endocrine disruptor through interfering with ER and AR as well as an aromatase inhibition, alterations in the several hormones levels and steroid biosynthesis (Andersen, Vinggaard, Rasmussen, Gjermandsen & Bonefeld-Jørgensen, 2002; Laignelet, Riviere & Lhuguenot, 1992; Ohlsson, Ullerås & Oskarsson, 2009; Robitaille, Rivest & Sanderson, 2015; Vinggaard et al., 2000, 2002, 2006). Moreover, several studies have shown that PCZ caused oxidative damage through induction of lipid peroxidation and alterations in the

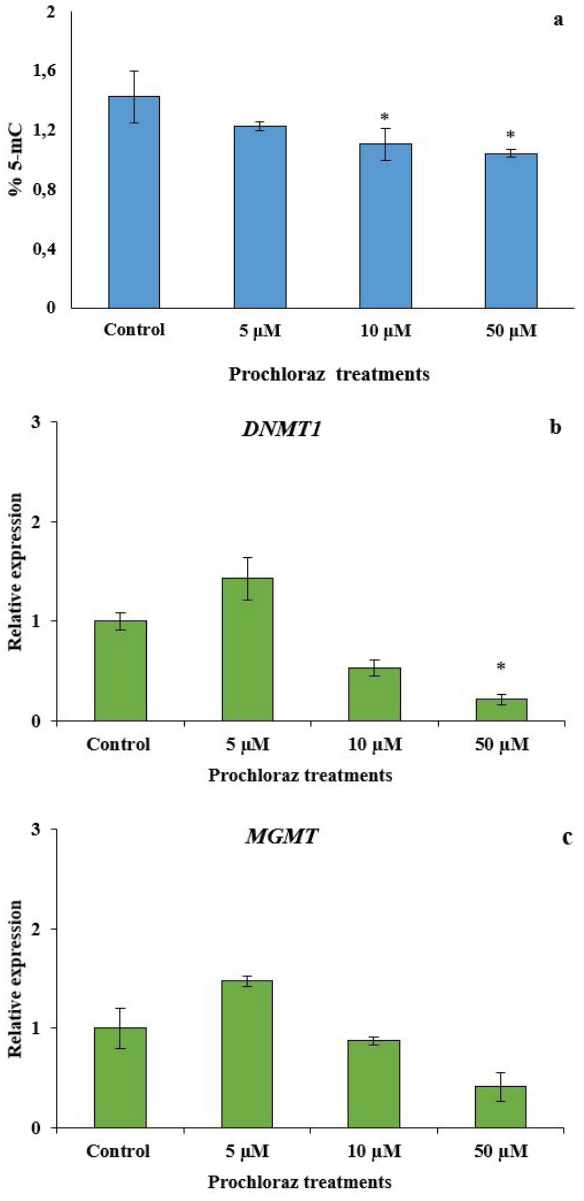

Figure 2. a) Effects of PCZ (0–50 µM) on levels of 5-mC% (a), relative gene expression levels of *DNMT1* (b) and *MGMT* (c) in PC-3 cells after 24 h exposure. Data are presented as mean ± SD. (Statistical analysis was performed by ANOVA + Dunnett post hoc test. Statistically significant changes are indicated by *p<0.05).

levels of antioxidant enzymes and glutathione *in vitro* (Alpertunga et al., 2014; Lundqvist et al., 2016; Sanchez et al., 2008).

In present study, IC_{50} values of PCZ were determined as 144.19 and 116.65 µM in PC-3 cells for 24 h by MTT and LDH tests, respectively. Our cytotoxicty results were consistent with the previous studies (Alpertunga et al., 2014; Rudzok et al., 2011; Strum et al., 2001). It has been reported that IC_{50} values of PCZ were 140 µM in trout hepatocytes by using LDH cytotoxicity test (Strum et al., 2001); 117, 87 and 99 µM in HepG2 cells by using MTT, neutral red uptake and Alamar Blue tests, respectively (Rudzok et al., 2011) and 110.76 µM in NRK-52E rat kidney cells by using MTT test (Alpertunga et al., 2014).

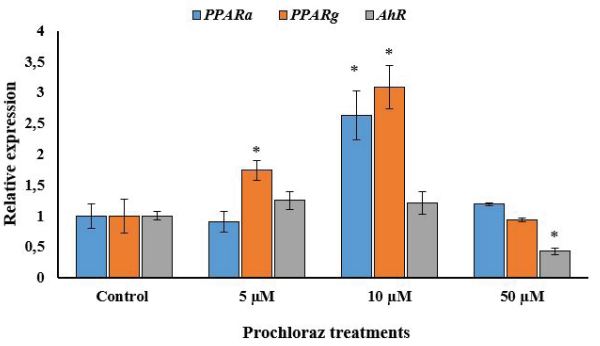

Figure 3. Effects of PCZ (0–50 µM) on relative gene expression levels of *AhR*, *PPARα*, *PPARγ* and *PXR* in PC-3 cells after 24 h exposure. Data are presented as mean ± SD. (Statistical analysis was performed by ANOVA + Dunnett post hoc test. Statistically significant changes are indicated by *p<0.05).

Investigating epigenetic alterations such as DNA methylation could be useful biomarkers for the toxicity assessment of endocrine disrupting chemicals (Casati, Sendra, Sibilia & Celotti, 2015; Greally & Jacobs, 2013; Maqbool, Mostafalou, Bahadar & Abdollahi, 2016). However, there has been any study on epigenetic modifications of PCZ toxicity. In present study, effects of PCZ on global DNA methylation, the most studied epigenetic alteration, have been investigated. PCZ decreased the global DNA methylation levels in a dose-dependent manner. As well as *DNMT* genes regulate DNA methylation in mammals. Especially, *DNMT1* provides regularity and continuity of methyltransferase activity that conserves the methylation state throughout DNA replication (Das & Singal, 2004; Pathania et al., 2015; Pradhan, Bacolla, Wells & Roberts, 1999). Consistent with our 5-mC% levels, expression of *DNMT1* was also decreased. We can suggest that PCZ could cause global DNA methylation which is a common feature in the oncogenesis of many tumor tissues leading to genomic instability (Gama-Sosa et al., 1983).

One of the sensors that enables cells to continually adapt and respond to the molecular changes by the environment, diet and host metabolism is the ligand-activated transcription factor AhR which perceives both endogenous factors and exogenous factors such as environmental toxins (McIntosh, Hogenesch & Bradfield, 2010). It has been suggested that PCZ exerts its effects by activating AhR which has an important role in PCZ-mediated hepatotoxicity (Marx-Stoelting et al., 2017; Rieke et al. 2014; Halwachs et al. 2013), and agonists of AhR could trigger tumor growth in rodent livers (Bock & Kohle 2005). Interestingly, our findings showed that *AhR* gene expression significantly decreased after 50 µM of PCZ in PC-3 cells. Evans et al. (2008) also have observed that higher concentrations of TCDD (2,3,7,8-tetrachlordibenzo-p-dioxin), other AhR agonist, caused a reduction of enzyme activity to 50% of the maximal response value which could reflect a negative feedback through the AhR repressor (AhRR) competition with AhR, similarly to our results.

Members of the PPAR subfamily, one of the nuclear receptors, including PPARα, PPARβ/δ, and PPARγ, are transcription

factors that regulate by activation of fatty acids and have essential roles in several biological processes including cellular differentiation, development, and metabolism (Derosa, Sahebkar & Maffioli, 2018; Michalik & Wahli, 2006). There have been no studies showing the effects of PCZ on *PPARs* gene expression. According to our findings, *PPARα* and *PPARγ* gene expression increased significantly after PCZ exposure. In the meantime, *PPARα* expression has been found among normal prostate and prostate cancer tissues while *PPARγ* expression was observed only in prostate cancer (Segawa et al., 2002). It has been also suggested that PPARγ acts as an oncogenic gene, and its activation promoted the development and progression of prostate cancer (Ahmad et al., 2016; Rogenhofer et al., 2012). Interestingly, it has been also reported that PPARγ signaling was regulated negatively by AR signaling (Olokpa, Bolden & Stewart, 2016). In the present study, while gene expressions of *PPARα* at 10 µM of PCZ and *PPARγ* at 5 and 10 µM of PCZ increased with the treatment, gene expression levels were decreased non-significantly after 50 µM exposure. It has been thought that these effects could be the result of non-monotonic dose responses due to the endocrine disrupting properties of PCZ.

In conclusion, our results, showing that PCZ is able to change nuclear receptor signaling and DNA methylation in cultured human prostate cells, suggests that PCZ might be one of the non-genotoxic promoters. Taken together, these findings are important for future assessment of endocrine disrupting pesticides, and we recommend that further studies should be needed to understand better the toxicity mechanisms of PCZ and especially, its role in epigenetic modifications.

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Original Article

Cytotoxicity and DNA protective effects of the *Terfezia* and *Picoa* species from the eastern region of Turkey

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ABSTRACT

Background and Aims: This study aims to investigate the cytotoxicity and DNA protective effects of semi-arid, arid, or desert truffles such as the *Terfezia* and *Picoa* species.
Methods: The DNA protective effects of the *Terfezia* and *Picoa* species was evaluated using plasmid pBR322 DNA treated with UV and H_2O_2. The cytotoxic effects of water and methanol extracts of the *Terfezia* and *Picoa* species on H1299 and HUVEC cells were evaluated using the MTT assay.
Results: Higher concentrations of *T. olbiensis* and *P. lefebvrei* extracts have demonstrated a DNA protective effect. The water extract of *P. juniperi* only at a 40 mg/mL concentration demonstrated a DNA-protective effect, whereas *T. boudieri* extract did not show any DNA-protective effect at all concentrations tested. H1299 cells showed more sensitivity to the water extract of *T. olbiensis* (4.66%), *P. juniperi* (12.04%), *P. lefebvrei* (21.93%), and the methanol extract of *T. boudieri* (20.93%). In general, the water and methanol extracts of the *Terfezia* and *Picoa* species demonstrated the least cytotoxic effects on HUVEC cells, except for the water extract of *P. juniperi* (38.46%).
Conclusions: In conclusion, results obtained from this study show that the *Terfezia* and *Picoa* truffle species have potential DNA protective and cancer prevention properties.

Keywords: arid-semi arid truffle, DNA protective activity, cytotoxicity effect, mushroom, *Terfezia, Picoa*

INTRODUCTION

Several studies have reported that food components and a balanced diet play a key role in maintaining human health integrity; however, when imbalanced, it can cause many chronic diseases (Ames, 2010). Despite extensive advances in treatment development over the past decade, the global prevalence of various diseases, cancer incidence, and the cancer-related mortality rate remain high (Ferlay et al., 2012; Torre et al., 2015). Genetic predisposition, sedentary lifestyle, poor dietary habits, diabetes, alcohol consumption, smoking, and other various external factors significantly increase the risk of developing various cancer types such as tumors of the liver, pancreas, lung, cervical, prostate, colorectal, skin, and breast (DeSantis et al., 2014; Siegel, Miller, & Jemal, 2016; Sheikh, Sarker, Kamarudin, & Ismail, 2017).

Some mushrooms species are edible for humans and can be used as dietary supplements. Not only the quality of their taste but also their biological properties are of significant value. Truffles are rich in many nutritional compounds such as protein,

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unsaturated fatty acids, carbohydrates, mineral substances, vitamins, amino acids, acids, dietary fibers, volatile organic compounds (Corrêa, Brugnari, Bracht, Peralta, & Ferreira, 2016; Ma et al., 2018). Mushrooms are also known as a significant source of biologically active compounds such as glycoproteins, beta-glucans, lectins, peptides, polysaccharides, vitamins, phenolic and polyphenolics, terpenoids, alcohols, and ergosterols. In the past thirty years, isolation of these compounds from various macrofungi has been performed and investigated as therapeutic agents in several studies (Aprotosoaie et al., 2017; Reis, Martins, Vasconcelos, Morales, & Ferreira, 2017). These biomolecules of epigeous mushrooms are thought to be responsible for the biological and medicinal activities such as antioxidant (Özyürek, Bener, Güçlü, & Apak, 2014; Souilem et al., 2017; Akyüz, Kireçci, Gökçe, Kirbağ, & Yilmaz, 2019), detoxification, antigenotoxic, anticancer, angiostatic, radical scavenging, DNA protective (Kim & Kim, 1999; Shameem, Kamili, Ahmad, Masoodi, & Parray, 2017), antiviral, antimicrobial, antiparasitic, antifungal, antiinflammatory, hypoglycemic, cardiovascular, hypocholesterolemic, antihypertensive, hepatoprotective and immunomodulatory, as well as cytotoxicity effects on cancer cells (Wasser & Weis 1999; Roupas, Keogh, Noakes, Margetts, & Taylor, 2012; Souilem et al., 2017; Kothari, Patel, & Kim, 2018). Furthermore, the most common uses of edible fungi are for the treatment of fatigue resistance, low immunity, microbial infection, heart disease, stroke, hypertension, diabetes, obesity, fatty liver, ageing, tumors, Parkinson and Alzheimer diseases (Valverde, Hernández-Pérez, & Paredes-López, 2015).

Among all edible mushrooms, truffles show some distinctive characteristics and recently received more attention than other epigeous mushrooms. Truffles are one of the oldest types of food and are consumed in large quantities because of their incredibly delicious taste and musky aroma (Wang & Marcone, 2011; Kagan-Zur, Roth Bejerano, Sitrit, & Morte, 2014; Dahham, Al-Rawi, Ibrahim, Majid, & Majid, 2018; Khalifa et al., 2019). The most commonly consumed truffles are the *Terfezia*, *Tuber*, and *Tirmania* species. They can be classified under two broad types: forest (*Tuber*) and arid, semi-arid, or desert truffles (*Terfezia, Tirmania,* and *Picoa*). Truffles are very rich in antioxidant compounds and can eliminate free radicals such as DPPH, ROS, and hydroxyl (OH) groups (Guo, Wei, Sun, Hou, & Fan, 2011; Beara et al., 2014; Ferlay et al., 2015; Patel, Rauf, Khan, Khalid, & Mubarak, 2017). Organisms have developed antioxidant defense and repair systems to protect their cellular environment from oxidative stress; however, these systems are insufficient to prevent damage completely (Wasser, 2011; Wasser, 2014; Sánchez, 2017). Therefore, antioxidant supplements or foods containing antioxidants can be used to help reduce the oxidative stress in the human body (Tadhani, Patel, & Subhash, 2007). Antioxidant compounds can eliminate free radicals. Various compounds such as vitamins, carotenoids, proteins, peptides, phenolic compounds, nucleotides, polysaccharides, alkaloids and organic acids have been reported to have antioxidant activities (Sarmadi & Ismail, 2010; Stajic, Vukojevic, Knezevic, Duletic Lausevic, & Milovanovic, 2013).

DNA protective properties and cytotoxicity effects of the Ascomycetes (*Terfezia* and *Picoa*) species grown in the eastern region of Turkey are not well investigated because truffles have received less attention than epigeous fungi. Our aim in the present study is to investigate the cytotoxic effects and DNA protective properties of the *Terfezia* and *Picoa* species.

MATERIALS AND METHODS

Truffle sample preparation

The samples used in this study were obtained from previous work (Akyüz, Kırbağ, Bircan, & Gürhan, 2015). The *Terfezia* and *Picoa* species were collected from Malatya (Central, Kale, Battalgazi, Arguvan Districts and theirs vicinity) and Elazığ (Baskil district and its vicinity), Turkey (N 38° 19'- 43' E 038° 19 '- 51' with an altitude of 690-1375 m, the beginning of March to the end of May (rarely continue until mid-July). The samples were dried and sieved at room temperature, weighed (30 g each), and placed in the cartridges of the Soxhlet instrument (Gerhardt EV 14). Extraction was performed for 3 h in 150 mL methanol (Merck) and dH_2O separately in the Soxhlet device. Then, the solvents were removed by an evaporator device. The extracts obtained were stored at +4 C. The day after extraction, studies were started with fresh extracts.

Evaluation of the DNA protective effect

The evaluation of the protective effect of the extracts on DNA damage was performed using the screening of plasmid DNA pBR322 (Vivantis) treated with UV and H_2O_2 in gel electrophoresis. Plasmid DNA's are widely used in DNA protective activity determination and give reliable results (Golla & Bhimathati, 2014; Verma, Shrivastava, & Kumar, 2015; Soumya et al., 2019). In the study, plasmid DNA samples were treated with the extracts, oxidized by H_2O_2 + UV treatment, and then, checked on 1.25% agarose gels, according to Russo et al. (2000). Each tube of the experimental samples contained 3 µL pBR322 plasmid DNA (172 ng/µL), 1 µL of 30% H_2O_2, and 5 µL of each extract at 40, 20, 10, and 5 mg/mL in a total volume of 10 µL. UV irradiation continued for five minutes on a UV transilluminator (DNR-IS) surface with an intensity of 8 mW/cm^2 at 302 nm at room temperature. After irradiation, 10 µL of the reaction mixture were mixed with loading dye (6×) and loaded on a 1.25% agarose gel for electrophoresis. Untreated pBR322 plasmid DNA and partially treated plasmids with only UV or only H_2O_2 were used as controls in each run of gel electrophoresis. Gels were stained with EtBr and photographed using the gel documentation system (DNR-IS, MiniBIS Pro) (Tepe, Degerli, Arslan, Malatyali, & Sarikurkcu, 2011).

Cell lines

H1299 (non-small cell lung cancer) and HUVEC (Human umbilical vein endothelial cell) cell lines were grown in the DMEM (Dulbecco's Modified Eagle Medium) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic solution (100 U/mL penicillin and 100 µg/mL streptomycin) and then incubated at 37ºC in 5% CO_2 humidified incubator (Jin, Zhang, Kang, Wang, & Zhao, 2010). Cells were counted with a hemocytometer and diluted to the cell density required for the MTT assay.

The Cytotoxic activity assay

The cell viabilities of H1299 and HUVEC cell lines treated with various concentrations of extracts were evaluated using the

MTT assay. The MTT assay is a highly preferred method for determining cytotoxic activity. The MTT dye is a yellow colored compound which is reduced by mitochondrial dehydrogenase produced by the live cells to water insoluble blue colored formazan crystals. When formazan crystals dissolve, it gives absorbance at 570 nm in the spectrophotometer (Mosmann, 1983). Firstly, $5x10^3$ cells/well were seeded in a 96 well-plate. After 24 hours, truffle extracts were added to cells at the concentrations of 6.25, 12.5, 25, 50, 100 µg/mL). Forty-eight hours later, the MTT solution was added and the cells were incubated at 37˚C and 5% CO_2 for four hours. Then, DMSO was added to each well to dissolve the formed formazan crystals. The color intensity was measured using a spectrophotometer at a wavelength of 570 nm. Viability (%) of cells were calculated using absorbance readings (Berridge, Herst, & Tan, 2005). A statistical calculation was made with the Graphpad Prism program with nonlinear regression analysis and graphs were created. Also, the relative cell viability was calculated using the following formula: Viable cells (%) = (As/Ac) *100 (As: Absorbance of extraction treated sample cells, Ac: Absorbance of the control cell group).

RESULTS

The DNA protective effects of water extracts obtained from desert truffle on pBR322 plasmid DNA were evaluated. The DNA protective activity of these extracts as shown in Figure 1. The DNA protective effects of five different concentrations of water extracts obtained from the *Terfezia* and *Picoa* species were evaluated using the plasmid pBR322 DNA screening method in gel electrophoresis. The DNA derived from plasmid pBR322 showed three bands in agarose gel electrophoresis. The faster-moving band on the gel is scDNA, and the slower-moving band is ocDNA and linDNA, which is the result of the separation of the open-circular DNA (Figure 1, Lane 1-3). These bands were stable in the presence of UV and H_2O_2 at the four different concentrations of water extracts obtained from the *Terfezia* and *Picoa* species (Figure 1, Lane 4-19).

According to our results, the water extract of *T. boudieri* did not show any protection of plasmid pBR322 DNA from damage induced by UV radiation and H_2O_2 in all concentrations tested (DNA could not maintain its stable structure) (Figure 1, Lane 4-7). Water extracts of *T. olbiensis* (40, 20, 10 and 5 mg/mL) showed a protective effect to plasmid pBR322 DNA form the damage induced by UV radiation and H_2O_2. Higher concentrations of this extract demonstrated remarkable protection effect on plasmid pBR322 DNA (Figure 1, Lane 8-11). Various concentrations of water extract obtained from *P. lefebvrei* have been found to have a DNA protective effect (Figure 1, Lane 12-15). The water extract of *P. juniperi* was found to prevent DNA damage induced by UV radiation and H_2O_2 at a concentration of 40 mg/mL (Figure 1, Lane 16), but the DNA could not maintain its stable structure at the concentrations of 20, 10, and 5 mg/mL (Figure 1, Lane 17-19).

Various concentrations of the methanol and water extracts of the *Terfezia* and *Picoa* species showed a cytotoxic effect on H1299 and HUVEC cell lines (Figure 2). The water and methanol extracts of *T. boudieri* had no effect on the viability of H1299 cells at concentrations of 6.25 and 12.5 µg/mL. However, the water extract showed cytotoxic effects of 92.84%, 63.32%, and 28.31% at 25, 50, and 100 µg/mL, respectively. The methanol extract of *T. boudieri* showed cytotoxic effects of 93.78%, 37.19%, and 20.93% at 25, 50 and 100 µg/mL, respectively on H1299 cell line. The water extract of *T. olbiensis* showed no cytotoxic effect on H1299 cell line at 6.25 and 12.5 µg/mL, whereas the cytotoxic effect on H1299 cell line at 25, 50, and 100 µg/mL were found to be 93.71%, 79.28%, and 4.66%, respectively. The cytotoxic effects of the methanol extract of the same species on H1299 cell line were found to be 83.02%, 70.68% and 47.15% at 25, 50 and 100 µg/mL, respectively. The water extract of *P. juniperi* showed no cytotoxicity on H1299 cell line at the concentrations of 6.25, 12.5, and 25 µg/mL, whereas it was found to be 56.72% and 12.04% at 50 and 100 µg/mL, respectively. The methanol extract of *P. juniperi* did not show any effect on cell viability at the concentrations of 6.25, and 12.5 µg/mL on the same cell line, however, it showed cytotoxic ef-

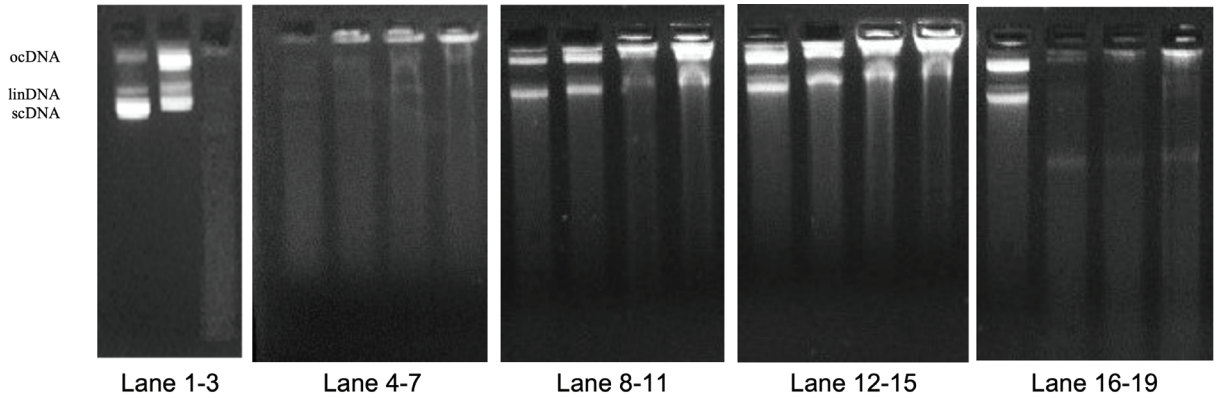

Figure 1. Electrophoretic pattern of pBR322 plasmid DNA after treatment with dH_2O, UV and H_2O_2 in the presence of water extracts. Lane 1: Plasmid DNA (3 µL) + dH_2O (6 µL), Lane 2: Plasmid DNA (3 µL) + dH_2O (6 µL) + UV, Lane 3: Plasmid DNA (3 µL) + dH_2O (6 µL) + H_2O_2 (1 µL) + UV, Lane 4-7: Plasmid DNA (3 µL) + 40, 20, 10 and 5 mg/mL of *T. boudieri* water extracts + UV + H_2O_2 (1 µL), Lane 8-11: Plasmid DNA (3 µL) + 40, 20, 10 and 5 mg/mL of *T. olbiensis* water extracts + UV + H_2O_2 (1 µL), Lane 12-15: Plasmid DNA (3 µL) + 40, 20, 10 and 5 mg/mL of *P. lefebvrei* water extracts + UV + H_2O_2 (1 µL), Lane 16-19: Plasmid DNA (3 µL) + 40, 20, 10 and 5 mg/mL of *P. juniperi* water extracts + UV + H_2O_2 (1 µL), respectively].

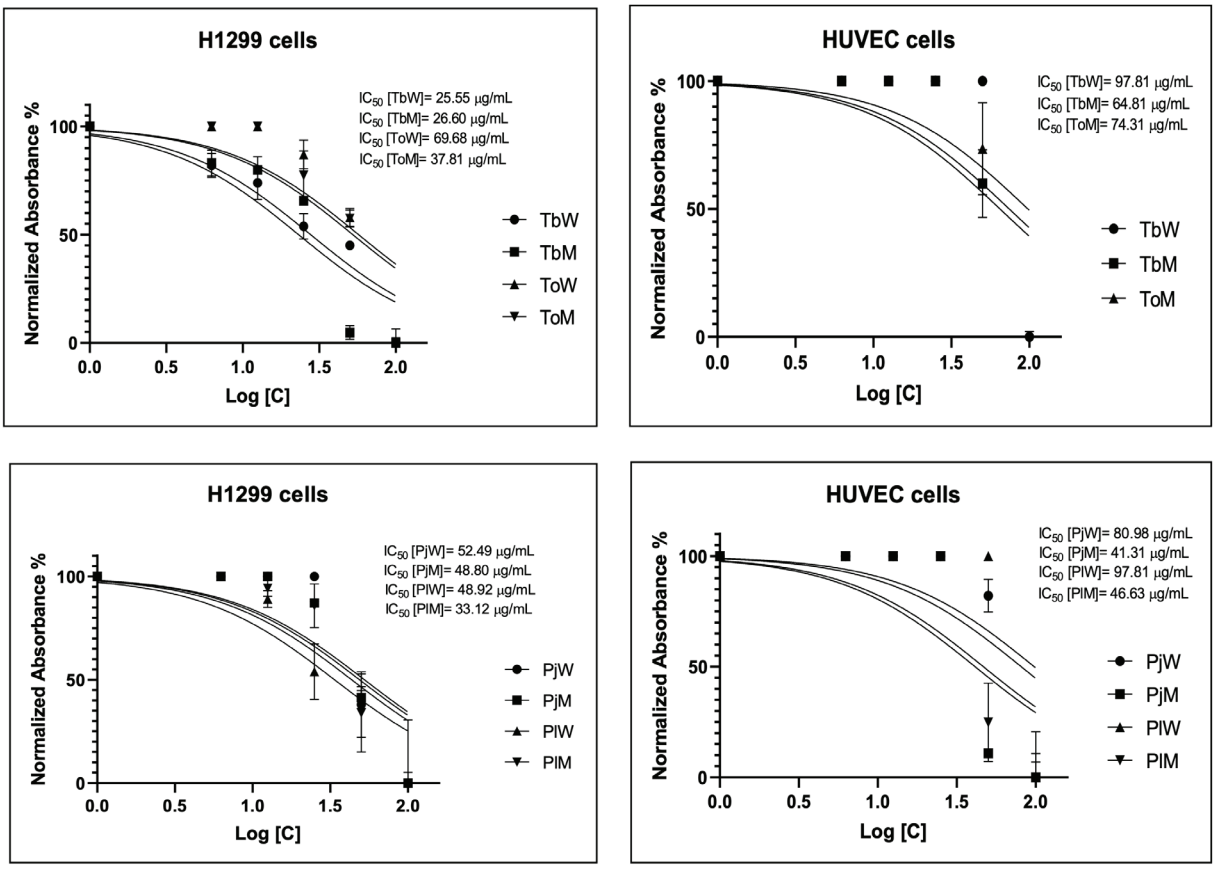

Figure 2. Normalized absorbances % of *Terfezia* and *Picoa* extracts on H1299 and HUVEC cell lines. (W: water, M: methanol, Pj: *P. juniperi*, Pl: *P. lefebvrei*, To: *T. olbiensis*, Tb: *T. boudieri*).

fects of 92.07%, 62.28%, and 36.09% at 25, 50, and 100 µg/mL, respectively. The water extract from *P. lefebvrei* did not show any cytotoxicity on H1299 cell line at a concentration of 6.25 µg/mL; however, it showed cytotoxic effects of 99.46% at 12.5 µg/mL, 64.32% at 25 and 50 µg/mL, and 21.93% at 100 µg/mL. The methanol extract of *P. lefebvrei* at a concentration of 6.25 µg/mL did not show any cytotoxic effect on the same cell line; however, it showed cytotoxic effects of 97.26%, 97.15% 55.06% and 42.41% at 12.5, 25, 50, and 100 µg/mL, respectively.

The water extract of *T. boudieri* showed no cytotoxic effect on HUVEC cell line at the concentrations of 6.25, 12.5, 25 and 50 µg/mL; however, it showed a cytotoxic effect 96.02% at 100 µg/mL. The methanol extract did not show any effect on cell viability at the concentrations of 6.25, 12.5 and 25 µg/mL on the same cell line; however, it showed a cytotoxic effect of 98.9% and 69.77% at 50, and 100 µg/mL, respectively. The water extract of *T. olbiensis* had no cytotoxic effect on HUVEC cell line at the concentrations of 6.25, 12.5, 25, 50 and 100 µg/mL. The methanol extract of *T. olbiensis* had no cytotoxic effect on HUVEC cell line at the concentrations of 6.25, 12.5 and 25 µg/mL; however, it showed cytotoxic effects of 95.82% and 67.31% at 50, and 100 µg/mL, respectively. The water extract of *P. juniperi* on HUVEC cell line did not show any effect on cell viability at concentrations of 6.25 and 12.5 µg/mL, however it showed cytotoxic effects of 98.89%, 96.39% and 38.46% at 25, 50, and 100 µg/mL. The methanol extract showed no cytotoxicity on the same cell line at 6.25, 12.5, and 25 µg/mL, while it showed cytotoxic effects of 83.02% and 62.28% at 50 and 100 µg/mL. There was no cytotoxic effect of water and methanol extracts obtained from *P. lefebvrei* on H1299 cell line at the concentrations of 6.25, 12.5, and 25 µg/mL. The water extract showed a cytotoxic effect of 87.22% and 64.32% at 50 and 100 µg/mL, respectively on the same cell line, while the methanol extract showed a cytotoxic effect of 85.17% and 55.14% at 50, and 100 µg/mL, respectively (Figure 2).

DISCUSSION

Several studies have demonstrated the antiangiogenic, antimetastatic, chemopreventive, antitumor, antiproliferative, and neo-adjuvant properties of traditional medicinal plants in vitro, in vivo and in various clinical cancer models (Roupas et al., 2012; Siegel et al., 2016; Kothari et al., 2018; Khalifa et al., 2019). Many diseases, such as neurodegenerative diseases and cancer, arise as a result of the imbalance between oxidant and antioxidant defense mechanisms. Previous studies (Kim & Kim 1999; Guo et al., 2011; Beara et al., 2014; Xiao et al., 2015; Aprotosoaie et al., 2017; Shameem et al., 2017) have reported that these diseases are responsible for oxidative DNA damage caused by ROS, such as superoxide anion, hydroxyl radical and hydrogen peroxide. In the present study, results showed that all concentrations of water extracts obtained from *T. olbiensis* and *P. lefebvrei*, and the 40 mg/mL concentration of *P. juniperi* protected the ocDNA band

of pBR322 plasmid DNA. Some mushrooms can protect cellular DNA from oxidative damage. It has been suggested that antioxidant properties of fungi can be identified by various oxidation process stages and by different mechanisms as mentioned by previous studies (Tadhani et al., 2007; Sarmadi & Ismail, 2010; Wasser, 2011; Stajic et al., 2013; Wasser, 2014; Sánchez, 2017; Živković et al., 2019). Edible mushrooms can be proposed as a dietary supplement or traditional drug to confer prevention and/or treatment of conditions arising from oxidative damage. These edible fungi are also able to protect DNA from damage induced by hydroxyl, superoxide anion, and hydrogen peroxide radicals as reported by the previously mentioned studies. As a result, some edible mushrooms represent a valuable source of bio compounds with the potential to protect cellular DNA from oxidative damage in contrast to other mushroom species (Roupas et al., 2012). According to the data mentioned above, the DNA protective effect of both the *Terfezia* and *Picoa* species is strongly dependent on the truffle type and their origins. In this regard, our study results support the findings of the previously published studies, as mentioned above.

For evaluating the cytotoxic effect of the extracts, H1299 and HUVEC cell lines were used, which are the most relevant to perform cytotoxicity studies. The water extracts of *T. olbiensis*, *P. juniperi*, *P. lefebvrei*, and the methanol extract of *T. boudieri* has remarkably decreased the percentage of viability in H1299 cells. Intriguingly, the water extract of *T. olbiensis* at 100 µg/mL, in particular, showed a selective cytotoxic effect. The water and methanol extracts of the same species showed no strong cytotoxic effect on HUVEC cell line, however, the water extract of *P. juniperi* showed strong cytotoxic effect at 100 µg/mL. These results suggest that various species of *Terfezia* and *Picoa* have potent cytotoxicity effects on H1299 and HUVEC cell lines. Various solvent extracts such as aqueous, methanol, ethanol, hexane, ethyl acetate, and bioactive molecules such as polysaccharides, glucans, agaritine, cordycepin, ergosterol, triterpenes, polysaccharopeptides, lectin, lentinan, are extracted using multiple assays and techniques from the fruit bodies of various mushrooms such as *Agaricus* spp., *G. lucidum*, *L. edodes*, *G. frondosa*, *Pleurotus* spp., *H. erinaceus*, *C. militaris*, *Tuber magnatum*, *T. aestivum*, *Terfezia claveryi* and showed cytotoxic activities on MCF-7, SK-Hep1, A549, HepG2, L929, HeLa, HT-29, U-87 MG, PC3, T24 cells in a dose-dependent manner (Roupas et al., 2012; Beara et al., 2014; Souilem et al., 2017; Kothari et al., 2018; Dahham et al., 2018; Elsayed, Alsahli, El Enshasy, & Wadaan, 2019). Therefore, dietary supplementation of edible mushroom extracts may serve as a potential antioxidant or immunity-boosting agent that helps in cancer prevention. Some Ascomycetes and Basidiomycetes are clinically used for the prevention and treatment of several diseases in some countries. The activity of these mushrooms is generally related to the presence of biomolecules, which are considered as biological response modifiers, as reported by the previously published studies. Various assays and techniques have been used to investigate the efficacy of the biologically active compounds in vitro, in vivo and in clinical trials. The results of the studies performed on fungi species in various cancer cell lines are quite different from each other as stated in the aforementioned studies. We think that these different results may depend on the species of fungi tested, sample preparation methods, the solvent used for extraction and the cancer cell line tested, various assays, techniques and analysis methods used, as shown in the above mentioned studies.

CONCLUSION

In conclusion, our findings suggest that the truffle (*Terfezia* and *Picoa*) species might contain some cytotoxic substances related to these metabolites. Given these results, the truffle (*Terfezia* and *Picoa*) species demonstrate potential DNA protective and cytotoxic activities, which may be responsible for their therapeutic effects. This study provides a useful basis for future studies on the DNA-protective and cancer-preventive properties of truffles.

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Original Article

Central possible antinociceptive mechanism of naringin

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ABSTRACT

Background and Aims: The object of this study was the investigation of the central antinociceptive effects of naringin as well as the association of stimulation of opioidergic, serotonergic, adrenergic, and cholinergic (muscarinic and nicotinic) receptors to the central analgesia of mice due to naringin.
Methods: Several intraperitoneal doses (20, 40, and 80 mg/kg) were injected into mice models and analyzed via hot-plate (integrated supraspinal response) and tail-immersion (spinal reflex) for the possible antinociceptive effects of naringin. Moreover, the involved action mechanism was investigated using 80 mg/kg naringin (i.p.) administered to the mice which were previously pre-treated with opioid antagonist naloxone (5 mg/kg, i.p.), serotonin 5-HT2A/2C receptor antagonist ketanserin (1 mg/kg, i.p.), α2-adrenoceptor antagonist yohimbine (1 mg/kg, i.p.) and muscarinic antagonist atropine (5 mg/kg, i.p.), as well as nicotinic antagonist mecamylamine (1 mg/kg, i.p.).
Results: It can be claimed that a dose-dependant antinociceptive effect of naringin was noticed for 40 and 80 mg/kg doses in tail-immersion and hot-plate tests, respectively. Furthermore, the improvement of inactivity of naringin-induced response to thermal stimuli was counteracted by mecamylamine and naloxone when tested with the tail-immersion test, and hot-plate analyses.
Conclusion: From the data, it was confirmed that naringin presents central antinociceptive effects which may be coordinated by supraspinal/spinal mediated opioidergic and nicotinic (cholinergic) inflection. Nevertheless, it is unclear how naringin organizes the interactions of the aforementioned modulatory systems. To conclude, naringin could be a possible candidate for pain relief management.

Keywords: Antinociception, opioidergic receptors, nicotinic antagonist, pain, central mechanisms, naringin

INTRODUCTION

Pain, in accordance with the definition by the International Association for the Study of Pain, can be described as an 'unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Dziechciaż, Balicka-Adamik, & Filip, 2013). This phenomenon affects the central nervous system (CNS) and peripheral nervous system (PNS) in such a manner that sensory input is first transferred to the spinal cord via the PNS and then transmitted to higher centers in the CNS for perception and the final analysis (Mahmoudvand et al., 2020). During pain chronicity, a variety of activity-dependent and disease-related alterations might take place over the peripheral and central components of the somatosensory nociceptive pathway. Nociceptive pathways are subject to modulation from a plethora of hormonal and neurotransmitter systems, along with dopaminergic, serotonergic, adrenergic, and cholinergic pathways (Naser & Kuner, 2018).

The management of pain is very important for any clinician since both acute and chronic pains are significant health problems that influence patients' quality of life . Currently, numerous analgesic drugs are available in clinical practice; some of them present low analgesic activity leading the researchers to develop innovative compounds that effectively manage pain-

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ful situations, especially chronic pains. Therefore, researchers have directed their attention to natural substances that present encouraging analgesic or other pharmacological properties (Araújo et al., 2017).

Flavonoids, an important group of polyphenolic substances, are secondary metabolites and a source of bioactive molecules in plants. Their widespread availability along with their low toxicity has presented them as potent therapeutic candidates (Hui et al., 2017; Chen, Qi, Wang, & Li, 2016). Naringin is a flavanone glycoside comprised of naringenin, an aglycone, and neohesperidose attached to the hydroxyl group at C-7. It has been reported to have a bitter taste because of the presence of glucose moiety (Bharti, Rani, Krishnamurthy, & Arya 2014). In addition, naringin, alternatively known as 5,7,4′-trihydroxy flavanone-7-O-rhamnoglucoside, is a well-investigated flavanone glycoside of grapefruits and citrus fruits (Zeng et al., 2019). Various reports have disclosed that naringin might modulate several signaling pathways; consequently, it presents extensive pharmacological indexes, such as anti-inflammatory, anti-cancer, prokinetic, pro-osteogenic, antiresorptive, and antiadipogenic responses. Moreover, naringin can positively affect cardiovascular diseases, metabolic syndrome, oxidative stress, genetic damage, and neurological ailments (Li, Wu, Wang, & Su 2020).

Herein, the investigation of the potential central antinociceptive mechanism of naringin and the evaluation via pharmacological approaches of the role of the opioidergic, serotonergic, adrenergic, and cholinergic pathways in its analgesic effects, took place. Therefore, the current study provides further insights into the mechanism of antinociception induced by Naringin.

MATERIALS AND METHODS

Chemicals and materials

Naringin, carboxymethylcellulose, yohimbine hydrochloride, diclofenac sodium, ketanserin tartrate, mecamylamine hydrochloride, naloxone hydrochloride, and atropine sulfate were obtained from Sigma, Germany. All the other used chemicals were of analytical grade, if not otherwise stated.

Animals

In the current study, Adult Swiss Albino male mice with a weight of almost 20-30 g were utilized. A temperature at 20-25°C, 55±15% relative humidity, 12 h light/dark cycles were adjusted for the experiments. The mice were acclimated for seven days prior to the dose initiation and they had free access to food and water. Moreover, the animals fasted overnight prior to the behavioral tests in order to reduce variability in investigatory parameters such as food-naringin interaction, while the analyses were performed between 9.00-13.00 a.m. The experimental protocols were carried out with the principles and guidelines adopted by the Guide for the Care and Use of Laboratory Animals (NIH Publication No.85-23, revised in 1985). The ethical committee of Ege University approved all the experiments while the experiments performed on mice were done with extra care and concern (Approval no:2020/079).

Drugs and treatments

The animals were grouped into seventeen and each group comprised of six mice. The control group received only solvent vehicles. Diclofenac (10 mg/kg) was used as a reference drug (Moniruzzaman & Imam, 2014; Afify, Alkreathy, Ali, Alfaifi, & Khan 2017). Naringin was administered intraperitonally (i.p.) at the doses of 20, 40, and 80 mg/kg. All drugs were injected intraperitoneally. For investigating the mechanisms of action, the mice were pre-treated with 5 mg/kg muscarinic receptor antagonist atropine 15 min before, 1 mg/kg nicotinic receptor antagonist mecamylamine 20 min before, 1mg/kg serotonin 5-HT2A/2C receptor antagonist ketanserin 30 min before, 1 mg/kg α2-adrenoceptor antagonist yohimbine 30 min before, and 5 mg/kg opioid antagonist naloxone 15 min before the administration of 80 mg/kg naringin. The measurements of pain threshold were performed using hot-plate and tail-immersion tests 30 min after Naringin administration. Doses and drug administration schedules were selected based on previous reports (Ben-Azu et al., 2018).

Analgesia test procedures

Hot-plate test

Eddy's hot plate method was performed to assess the antinociceptive effect of naringin. Mice showing quick responses like jumping and withdrawal within 15s to thermal stress were selected for the hot plate test analysis. Twenty-four hours before the experimental procedure the mice selection was performed. The pain reflexes concerning a thermal stimulus were estimated using a temperature-controlled plate (Hot/Cold Plate, Ugo Basile, Italy). The mice from each group were placed on the plate at 55.0±0.5 °C. The reaction times of the nociceptive responses (fore paw licking, withdrawal of paw, or jumping) were recorded. To minimize tissue damage, a cut-off time of 20s was selected. The behaviors of the mice were recorded before as well as after treatment (Bektaş & Arslan, 2016; Arslan, Aydin, Samur, & Bektas, 2018).

Tail- immersion test

A hot water bath was used to dip the mice tails tips in order to assess the painful reaction of the mice induced by thermal stimulus at 52±0.2°C. More precisely, a specific area of the mice tail was immersed in hot water. Sudden removal of the tail from the hot water was considered as a pain response. A cut of period of 15s was observed to avert tail tissue destruction of the mice (Bektaş & Arslan, 2016; Arslan et al., 2018).

The antinociceptive activity was expressed as the percent maximum possible effect (%MPE) calculated as:

%MPE = [{(Postdrug latency) – (Predrug latency)}{(Cut off time)– (Predrug latency)}] X 100.

Statistical analysis

Data were analyzed by using GraphPad Prism 5.0 software, Inc., San Diego, CA. The data have been represented as mean±SEM (standard error of the mean). Moreover, the data was interpreted by using one-way analysis of variance accompanied by post hoc analysis with Tukey's test. A p-value less than 0.05 was considered significant.

RESULTS

Analysis of the possible mechanism of action of naringin

The i.p. administration of naringin 20 mg/kg, naringin 40 mg/kg ($p<0.05$), and naringin 80 mg/kg ($p<0.01$) produced a dose-dependent prolongation of the latency for mice to respond to pain stimulation induced by heat. However, naringin 20 mg/kg did not demonstrate any significant change in the antinociceptive response of the mice when compared with vehicle control. The injection of the naringin vehicle did not induce hyperalgesia or any anti-hyperalgesic effect. Meanwhile, treatment with the reference drug, diclofenac (10 mg/kg, i.p.) exerted a significant increase ($p<0.001$) in response latency against thermal stimulus-induced nociception compared to the control. Figure 1 depicts the MPE% values which illustrate the antinociceptive effect of naringin according to hot-plate and tail-immersion analyses, respectively.

Mechanism of action studies

It can be said that atropine (5 mg/kg, i.p.), mecamylamine (1 mg/kg, i.p.), ketanserin (1 mg/kg, i.p.), yohimbine (1 mg/kg, i.p.) and naloxone (1 mg/kg, i.p.) did not exhibit any significant effect on the pain threshold, according to the results of hot-plate and tail-immersion tests in mice.

As it is shown in Figure 2, the antinociceptive action of naringin was prevented due to the administration of naloxone (opioid receptor antagonist) in the hot-plate ($p<0.01$) and the tail-immersion ($p<0.001$) analyses.

As shown in Figure 3, it was revealed that pre-treatment with mecamylamine reversed the pain relief effects of the naringin in hot-plate ($p<0.001$) and tail-immersion ($p<0.01$) tests.

On the contrary, Figure 4 shows that the pre-treatment with atropine did not significantly alter the pain thresholds in both the tail-immersion and hot-plate tests.

As shown in Figure 5, ketanserin alone did not significantly alter the pain thresholds. Naringin (80 mg/kg) plus ketanserin showed a significant effect on pain response as compared to the control group in the hot-plate ($p<0.01$) and tail-immersion ($p<0.001$) tests. The results indicate that ketanserin did not reverse the antinociceptive effect of naringin in a significant way.

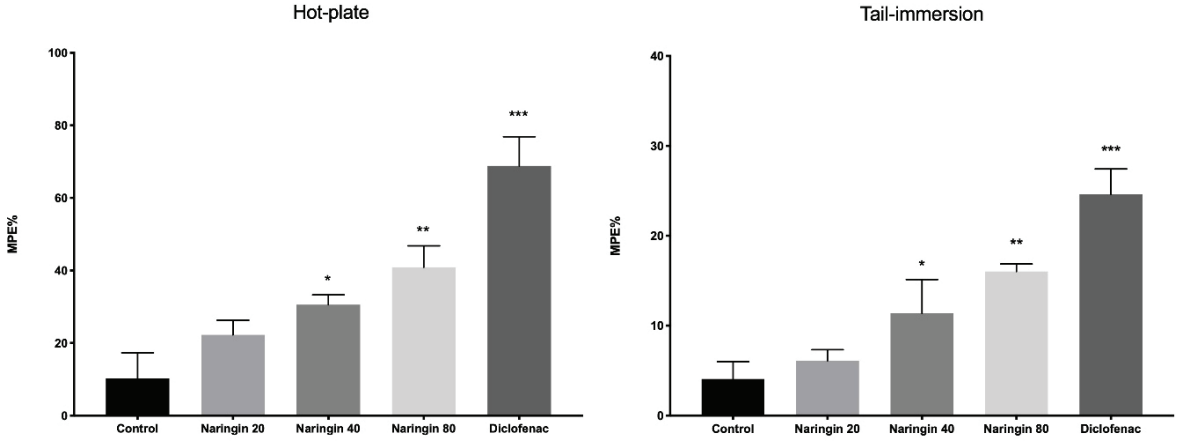

Figure 1. The induction of antinociception after naringin administration (20, 40, and 80 mg/kg) (ip) according to hot-plate and tail-immersion analyses. *p<0.05, **p<0.01, ***p<0.001 vs. Control.

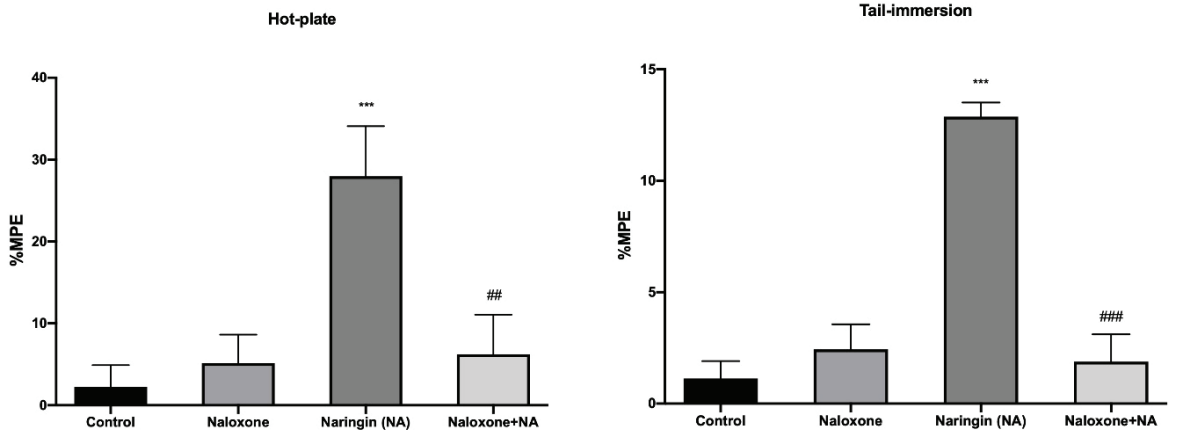

Figure 2. The reversal effect of 5 mg/kg naloxone (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. ***p<0.001 vs. Control; ##p<0.01, ###p<0.001 vs. naringin alone.

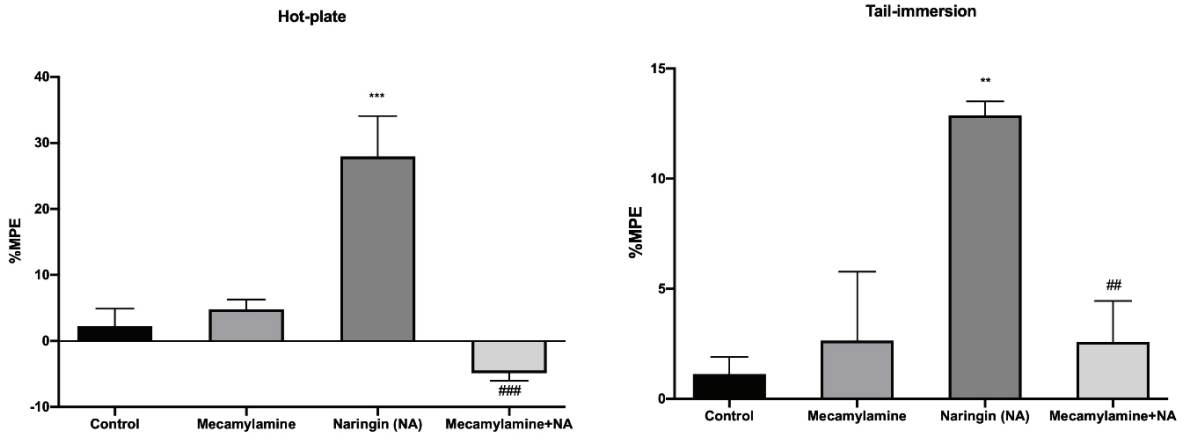

Figure 3. The reversal effect of 1mg/kg mecamylamine (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. **p<0.01, ***p<0.001 vs. Control; ##p<0.01, ###p<0.001 vs. naringin alone.

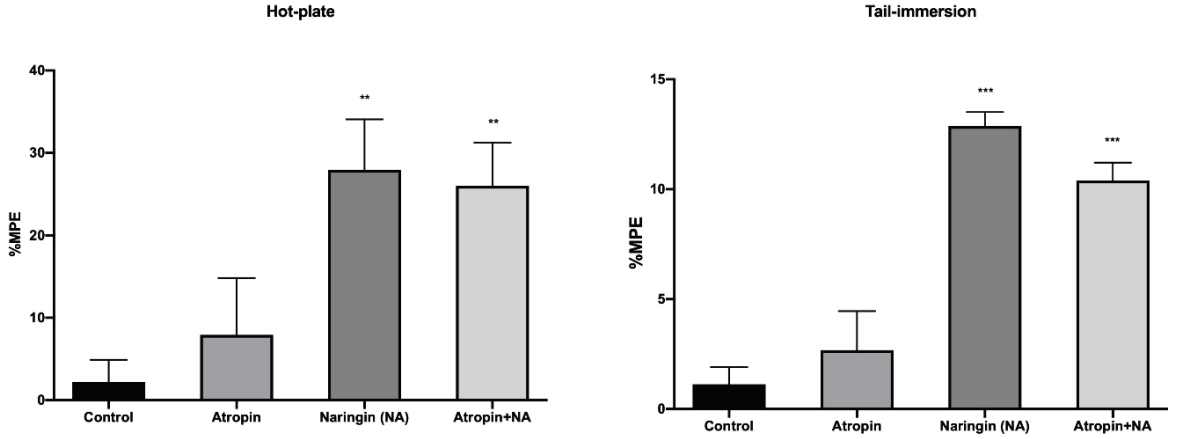

Figure 4. The reversal effect of 5 mg/kg atropine (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. **p<0.01, ***p<0.001 vs. Control.

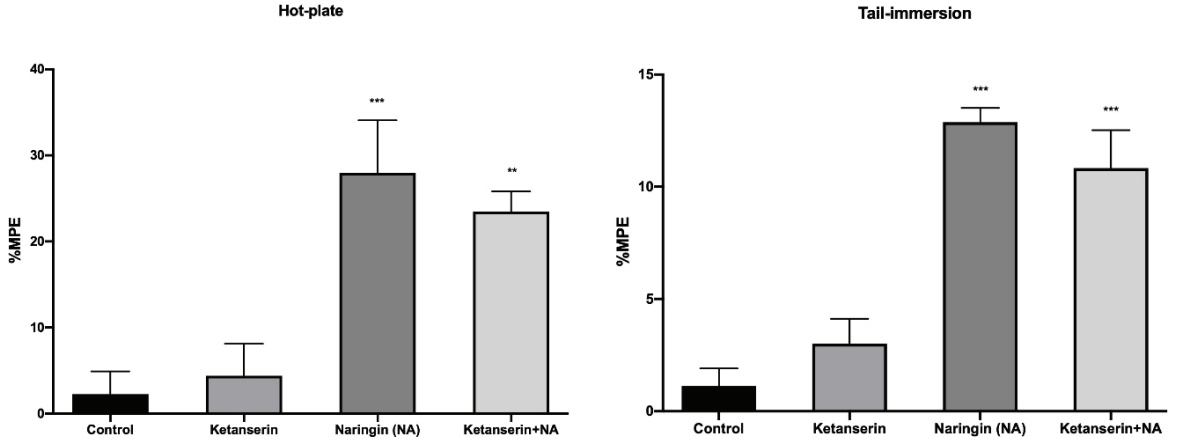

Figure 5. The reversal effect of 1 mg/kg ketanserin (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. **p<0.01, ***p<0.001 vs. Control.

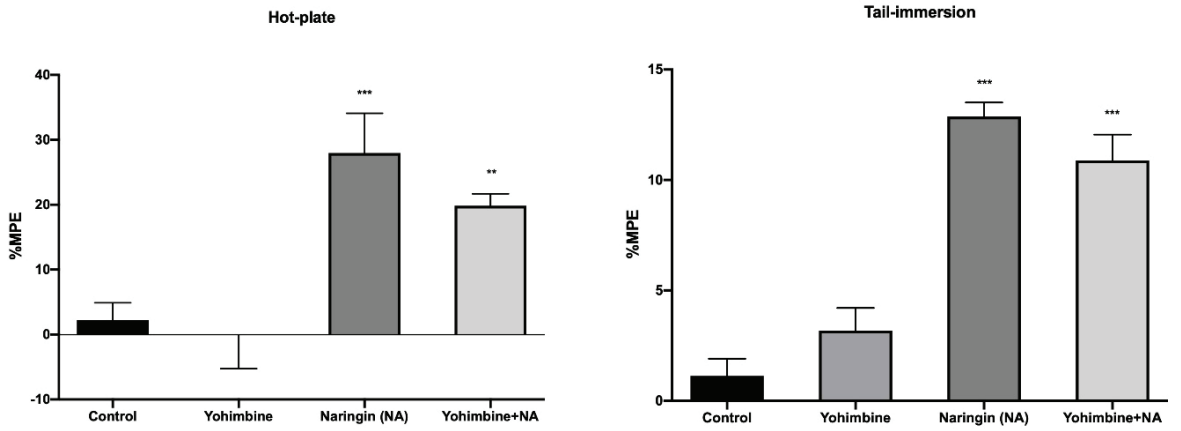

Figure 6. The reversal effect of 1 mg/kg yohimbine (ip) on 80 mg/kg (ip) naringin-induced antinociception in the hot-plate and tail-immersion tests. **p<0.01, ***p<0.001 vs. Control.

Figure 6 demonstrates that the pre-treatment with yohimbine did not show an antagonistic effect on antinociceptive activity of naringin (80 mg/kg) in the tail-immersion (p<0.01) and hot-plate (p<0.001) tests.

DISCUSSION

Herein, an investigation on the possible central antinociceptive effects of naringin through several pharmacological paths, such as hot-plate test and tail-immersion test, took place. The obtained results revealed that Naringin demonstrates statistically significant antinociceptive effects on the treated mice in comparison to control treated mice. It can be suggested that the antinociceptive effect as demonstrated by the tail-immersion and hot-plate tests, was mostly mediated by opioidergic and cholinergic systems.

In general, nociception is a reflex response of an organism due to external stimuli. Various nociceptive tests can be executed in order to examine the pharmacological effect of drugs to decrease pain. These tests can be implemented via the application of stimuli such as thermal, mechanical, or electrical. Herein, the antinociceptive potential of Naringin against the thermal stimulus provoked nociception was examined through hot plate and tail-immersion analyses; these tests are widely applied for the detection of possible analgesic properties of drugs (Xue, Wu, Wu, & Wang 2019). More specifically, the hot-plate test can be used in order to evaluate analgesics that centrally act and increase the pain threshold of animals regarding heat. It is thought to be responsive to opioids and measures the complex response to a non-inflammatory, acute nociceptive stimulus (Meshram, Kumar, Rizvi, Tripathi, & Khan, 2015). Moreover, the simple tail immerison as an endpoint of the test may be mediated as a spinal reflex. It is believed that the escape reaction is a complex occurrence liaised by the brain. Subsequently, the drug influence on the brain can be assessed by observing the escape reaction (Meshram et al., 2015). In addition, the hot-plate analysis is applied in order to assess the effects of centrally but not peripherally acting analgesics. Accordingly, the tail-flick method is useful so as to establish acute spinally mediated-nociception to thermal noxious stimuli (Chung, Li, Lin, & Tsai, 2019).

Naringin, after its oral administration, is mostly hydrolyzed to its aglycon, naringenin, by the enterobacterial enzymes, including b-rhamnosidase and a-glucosidase (Zeng et al., 2019; Li et al., 2013). Naringenin presents several pharmacological effects, such as antioxidant, anti-inflammatory, analgesic, etc. The antinociceptive properties of naringenin (66 mg/kg, i.p.) were shown with the participation of glutamatergic and opioid systems. Naringenin also reduced the nociceptive response of the formalin test (the inflammatory phase), bradykinin, and prostaglandin E2 (Dallazen et al., 2017). Only small amounts of naringin are available in plasma after oral intake and thus they might not adequately reach target tissues, such as the brain. Nonetheless, if it is given i.p., central activities of naringin have been revealed in mice (Yow et al., 2011; Nagi, Pineyro, Swayne, Tian, & Dascal 2014).

Naringin has been classified as a relatively harmless or non-toxic substance. The oral single dose of 16 g/kg of naringin has not provoked any acute toxicity when studied in the rat model (Li et al., 2013). Furthermore, according to recent research, the oral exposure of rats with a single dose of naringin (5 g/kg) did not show any mortality or unfavorable clinical effects. In addition, gross pathological signs, abnormal alteration in body weights, or toxicologically relevant modifications in serum biochemistry, hematology, urinalysis as well as other findings were not observed (Li et al., 2020).

According to the present results obtained from the tail-immersion and hot-plate tests, the pre-treatment with naloxone, an opioid receptor antagonist of μ-, κ- and δ-opioid receptors (MOR, DOR and KOR, respectively) (Araújo et al., 2017) has antagonized the antinociceptive effect of naringin. Flavonoids are potential opioid receptor ligands, and systemic administration of some flavonoids have been shown to elicit a dose-dependent inhibition of the nociceptive behavioral response (Alghamdi, 2020). The data indicate that naringin induced-central antinociceptive effects can be associated with the opioid receptor activation.

The potential role of serotonergic receptors in Naringin's analgesic activity has been investigated, but no relation was found with the antinociceptive effect of Naringin in hot-plate and

tail-immersion studies. It has been documented that activation of 5-HT receptors in the spinal cord causes antinociception (Sousa et al., 2017). This finding implies that naringin's antinociceptive action is not mediated by the serotonergic system.

The administration of α2-adrenoceptor agonists produce antinociception by inhibiting synaptic transmission in the dorsal horn of the spinal cord and there is evidence that stimulation of the descending noradrenergic system results in the activation of spinal α2-adrenoceptor and antinociception (Abubakar Nazifi, Odoma, Shehu, & Danjuma,, 2020). In this study, pretreatment of mice with yohimbine did not attenuate the antinociceptive activity of naringin. This implies that α2-adrenergic receptors do not play a role in its antinociceptive activity.

Modulation of nociception from acetylcholine involves the participation of multiple classes of receptors, including nicotinic and muscarinic receptors. The antinociceptive effects of acetylcholine in the dorsal horn of the spinal cord occur through mechanisms involving muscarinic receptors (M2, M3 and possibly M4) (Oliveira et al., 2018). The present results indicate that intraperitoneal administration of atropine did not impair the prolongation of reaction time induced by naringin.

Another point of this present investigation was to determine the connection of the cholinergic (nicotinic) modulation with naringin-induced antinociception. Analgesia can be achieved by influencing pathways other than the opioidergic pathways and one promising alternate avenue outside of opioid agents is to exploit the antinociceptive effects of the neuronal nicotinic acetylcholine receptors of this neurotransmitter system (Nissen et al., 2018). Mecamylamine is almost completely absorbed and readily crosses the blood–brain barrier where it acts as an acetylcholinergic nicotinic receptors antagonist (Shytle et al., 2002). In previous studies, mecamylamine administered prior to neuronal nicotinic receptor agonists prevented the antinociceptive effect in mice (Arihan, Boz, Iskit, & Ilhan,2009). In this study the antinociceptive effect of Naringin was also blocked by nicotinic receptor antagonist mecamylamine. The results indicate that there is an involvement of nicotinic receptors since mecamylamine inhibited the antinociceptive effect of naringin. Nicotinic acetylcholine receptors (major subtypes; α4β2 and α3β4) are expressed in the central nervous system, including many areas contributing to pain such as the midbrain, medulla, nucleus raphe magnus, thalamus, pedunculopontine tegmental nucleus, and spinal cord. α4β2 nicotinic full agonists were reported to display a wide-range profile of antinociceptive activity (AlSharari, Carroll, McIntosh, & Damaj, 2012).

Besides, the activation of supraspinal nicotinic acetylcholine receptors can be proved as an analgesic target for animal models of acute and chronic pain. The nicotinic acetylcholine receptors agonist ABT-594 displayed antinociceptive efficacy similar to μ-opioid receptor (MOR) agonists (Dziechciaż et al., 2013). Opioid receptors can significantly contribute to antinociception in the majority of organisms; it has been suggested that cholinergic receptor agonists effects in the analgesiometric tests are mediated via a pathway including opioid receptors. Consequently, it was believed that the co-administration with naloxone reversed the antinociceptive effects of the nicotinic receptor agonist, epibatidine, in the naked mole-rat models (Tdulu, Kanui, Towett, Maloiy, & Abelson, 2014). Similarly, the pain signaling of the dorsal horn of the spinal cord, which also expresses neuronal nicotinic receptors, emerged as a research field of interest. To conclude, the spinal neuronal nicotinic receptors have been also connected with nociceptive and antinociceptive roles (Nissen et al., 2018).

Previous studies demonstrated that daily intraperitoneal injection of naringin can promote neuroprotective roles in a rat model of Parkinson's disorder (Jung & Kim, 2014). Accordingly, the ip injection of naringin notably enhanced the glia-derived neurotrophic factor (GDNF) levels through the activation of (mTORC1) in nigral dopaminergic neurons (Leem et al., 2014; Jung, Leem, & Kim, 2014). It can be revealed that naringin increased dopamine levels in all the regions (Kola, Akula, NissankaraRao, & Danduga, 2017). On the other hand, it can be suggested that peripheral dopamine receptors might be involved in an antinociceptive action (Okumura et al., 2015).

KIR3 channels contribute to regulating postsynaptic potentials in the central and peripheral nervous systems. Besides, candidates which can regulate neuronal excitability via KIR3 channels can potently relieve the pain. It was revealed that Naringin activates KIR3 channels (Yow et al., 2011). The activation of KIR3 channels as a pervasive analgesic mechanism, in addition to opioids, mediates pain modulation. The evolution of direct KIR3 channel activators could be recommended as a potent plan to develop innovative analgesics. Thalamus and limbic cortex which express KIR3.1, 3.2, and 3.3 subunits as well as opioid receptors can be suggested as possible sites of supraspinal KIR3-mediated analgesia (Nagi et al., 2014). Thus, it can be suggested that the antinociceptive activity of naringin may be related to KIR-3 channel activity.

To summarize, various systems play a partial role in pain relief which is rational given that the central control of pain is coordinated through many neurotransmitters i.e. acetylcholine and endo-opioids. Therefore, adjunct studies evaluating the combination of several antagonists can be carried out to determine their possible involvement in pain control.

CONCLUSION

In summary, naringin presents a central antinociceptive property in mice which is possibly arranged by spinal/supraspinal-mediated opioidergic and nicotinergic modulation. It can be also assumed that the stimulation of KIR3 channels and the dopaminergic system might manage the antinociception of naringin. Consequently, naringin might act as a favorable candidate for pain relief. Additionally, the clarification of the effect and mechanisms of actions of the citrus flavonoid, naringin will contribute to new therapeutic approaches and provide guidance for new drug development studies. Therefore, this study opens up a new window for pain management using the naringin molecule. Future studies may involve clinical trials in order to clarify whether humans can be subjected to pain reduction via naringin i.p. administration.

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Author contributions: Conception/Design of Study- M.E.O.; Data Acquisition- M.E.O., Ç.K.K.; Data Analysis/Interpretation- M.E.O., Ç.K.K.; Drafting Manuscript- M.E.O.; Critical Revision of Manuscript- M.E.O., Ç.K.K.; Final Approval and Accountability- M.E.O., Ç.K.K.

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

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Original Article

Anti-fertility and antisteroidogenic activity of *Clerodendrum serratum* in mature male rats

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ABSTRACT

Background and Aims: The study was designed to evaluate the anti-fertility effect of methanolic extract of *Clerodendrum serratum* (MECS) on mature male rats.
Methods: The vehicles and MECS were administered orally to four groups of forty male albino rats on consecutive days for 30 days. At the end of the experimental period, five animals from both the control and experimental groups were given anesthesia and the animal reproductive organs were collected and anti-fertility effect evaluated by studying the following parameters (i) Body and reproductive organ weight (ii) Biochemical estimation (iii) Reproductive hormone analysis (iv) Abnormalities in reproductive organ histology. Another five rats in each group were subjected to fertility testing.
Results: This study shows that female rats which mated with MECS treated male rats delivered fewer pups than those which mated with the control rats. An antisteroidogenic effect was revealed through the increased concentration of testicular cholesterol and ascorbic acid in MECS treated rats. However, testicular Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD), glucose-6-phosphate dehydrogenase (G-6-PD); serum reproductive hormones testosterone, FSH and LH were significantly reduced.
Conclusion: The present investigation discovered the antisteroidogenic effect of MECS, which confirms the male antifertility effect of this plant. This encourages the traditional use of this plant in the Indian subcontinent as a male contraceptive.

Keywords: *Clerodendrum serratum*, Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD), glucose-6-phosphate dehydrogenase (G-6-PD), reproductive hormones

INTRODUCTION

The population of India is increasing at an alarming rate and has gone above 1.5 billion. Regulation of fertility has become a major distress factor for many people (Savadi & Alagawadi, 2009). A growing population is not only a worldwide issue but also a national public well-being concern. Birth control measures have become an essential part of our live. There are limited contraceptive agents available for men and these are only to be found in the market. However, women have access to various synthetic contraceptive agents, but these come with severe side effects (Joshi, Sharma & Chaturvedi, 2011). The WHO recommended the practice of utilization of traditional

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medicine for conception prevention instead of using synthetic drugs as this is more cost-effective (Umadevi Kumar, Bhowmik, & Duraivel 2013).

Clerodendrum serratum (L.) Moon (Verbenaceae) is a vital medicinal plant distributed throughout the tropical forest region in India and Sri Lanka and other warm temperature regions like South Asia, Malaysia and Africa. In the Indian system of medicine, *C. serratum* is an important medicinal plant and its leaves and roots are used for various ailments. *C. serratum* (English: Blue-floral glory, Tamil: Cherutekku) leaves can be used as a febrifuge and the roots are used as antiasthmatic, antihistaminic, antispasmodic, antitussive carminative and febrifuge (Khare, 2007). The 50% ethanolic extract of *C. serratum* demonstrated *in vitro* spermicidal activity in rat and human semen. A N-butanol fraction of 50% ethanolic *C. serratum* extracts also exhibited *in vitro* spermicidal activity in humans (Setty Kamboj, & Khanna 1977). As per our earlier research this plant shows changes in sperm concentration and motility with abnormalities in sperm morphology indicating that the plants have a potential spermatotoxic effect on male mature rats. *C. serratum* is a traditional tribal folk medicine which works as a male contraceptive (Pokharkar, Saraswat, & Kotkar, 2010).

The major groups of chemical constituents present in the *C. serratum* are carbohydrates, serratagenic acid, acteoside, indolizino and verbascoside, leucoanthocyanidins, flavanones, flavanonols, betulin, oleanolic acid, clerodermic acid β-sitosterol, γ-sitosterol and compesterol (Singh, Khare, Iyer, & Tripathi, 2012).

Nevertheless, scientific evidence about the potential effects of this plant on male antifertility effect is still lacking. In this study, we sought to extend the existing literature dealing with the investigation of the anti steroidogenic effect of *C. serratum* in male mature rats. The present work, therefore, attempts to report the preliminary results based on our *in vivo* studies to justify traditional and folklore beliefs.

MATERIALS AND METHODS

Animals

We utilized 40 Sprague-Dawley rats which weight ranged from 150 to 200 g. The rats were kept in certain conditions including a 12 h dark /12 h light cycle with free running water and pellets of rat feed. They were kept in relative humidity of 55±10% and constant temperature (21±2°C). All the animals were prepared for acclimatization for half a month. The experimental protocols were assessed and agreed by the Institutional Animal Ethics Committee (IAEC) of the institute (No. SRCP/IAEC/B.Pharm. Project /01 /2016-17).

Preparation of extract

The aerial parts of *C. serratum* were collected from the eastern part of the Seshachalam hill ranges and from the area lying within the geographical coordinates 14.3333°N, 78.2500°E in Thirumala, Andhra Pradesh, India. Taxonomical identification was made using the Botanical Survey of Medicinal Plants Unit at Sri Venkateshwara University, Thirupathi, Andhra Pradesh, India in August 2012. Figure 1 shows the aerial parts of the plant which were used for this study (Singh *et al.,* 2012).

The aerial parts of *C. serratum* were dried at 28°C, pulverized by a mechanical grinder and passed through 22 mesh sieves. The powder was subjected to defatting with petroleum ether (40-60°C), followed by Soxhlet extraction with 70% v/v ethanol at 68°C. The extracts were collected and filtered. The solvent was dried under reduced pressure using the Eyela Rotary evaporator at 40-45°C. Finally, the crude extract was stored in vacuum desiccators. Based on our survey of the literature, we chose methanol solvent to extract the following active components from this plant: terpenoids, saponins, tannins, xanthoxyllines, totarol, quassinoids, lactones, flavones, phenones, and polyphenols (Pandey & Tripathi, 2014).

Design of the experiments

The Sprague-Dawley rats were housed and acclimatized for one week before the experiment started. Forty healthy male albino rats were selected and divided into four groups containing 10 rats each and treated as follows:

Group 1 received distilled water (10 mL kg^{-1} b.wt. p.o.) as the normal control; Group 2 received MECS (100 mg kg^{-1} b.wt. p.o.); Groups 3 and 4 received MECS at doses of 300 and 500 mg kg^{-1} b.wt p.o.), respectively.

The vehicles and MECS were administered orally on 30 consecutive days. At the end of the experimental period, five animals from both the control and experimental groups were given anesthesia under sodium pentobarbital 24 h after the last dose

Botany
The taxonomical position is as follows: [6]

Kingdom:	Plantae
Sub-kingdom:	Viridaeplantae
Division:	Angiospermae
Class:	Magnoliopsida
Subclass:	Lamiidae
Order:	Lamiales
Family:	Lamiaceae/ Verbenaceae
Genus:	Clerodendrum
Species:	serratum

Figure 1. Aerial parts of the *C. serratum*.

and 18 h after fasting. The animal reproductive organs were utilized for histology studies, including measurement of reproductive organ weight, biochemical estimation, and reproductive hormone analysis. Another five rats in each group were subjected to fertility testing.

Gravimetric analysis of body weight and weights of reproductive organs

The body weights of the animals were recorded prior to and after treatment. Testis, epididymis, and seminal vesicles were weighed. Relative organ weight (ROW) for each organ collected was ascertained using the following expression:

Determination of reproductive performance

Every animal's fertility index was calculated as follows. After completion of teatment schdule, each male animal was paired with two fertile females, and their mating behavior was observed. Samples from vaginal smears were analyzed the day after the mating exposure. Day 0 post coitum was considered if spermatozoa were present. On the 15th post-coitum day, the mated females were laparotomized, the resorption site and/or several implantations were registered, and the term was allowed to run its course. The number of females who gave birth, as well as the number of litters born, was also reported (WHO Protocol MB-50, 1983).

Biochemical estimations

Estimation of cholesterol

An approximate weight of 3 mg of testicular tissues was carefully homogenized in Potter-Elvejem homogenizer using the combination of chloroform: ethanol mixture (2:1), non-polar parts were extracted out, and then the total cholesterol content was estimated. A standard curve was prepared by dissolving cholesterol (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/mL) in chloroform, mixed with glacial acetic acid and color reagent accordingly. The optical density was determined in the spectrophotometer at 620 nm against a blank (chloroform) and the total cholesterol content of the testis was determined from the standard curve (Speery & Webb, 1950).

Estimation of ascorbic acid

Testicular tissues weighing around 5 mg were homogenized in a Potter-Elvejem homogenizer with 45 µL of ice-cold 5% metaphosphoric acid and centrifuged at 3500 rpm. The optical density was estimated at 540 nm using 30 µL of supernatant and 15 µL of 2.6-dichlorophenol-indophenol sodium (0.1 mg/mL). The standard curve was drawn against the established ascorbic acid concentration. The standard curve was used to calculate the amount of ascorbic acid present in the testis (Omaye, Turnbull, & Souberlich, 1979).

Estimation of Glucose-6-Phosphate Dehydrogenase (G-6-PD) activity

Testes were homogenized and the mixture was centrifuged at 1000g for 5 minutes at 0°C, the supernatant was again centrifuged at 10,000 g for 10 minutes at 0ºC. 0.2 mL of Tris HCl buffer (pH 8.3, 0.5 M), 0.01 mL of 20 mM Nicotinamide adeninen dinucleotide phosphate (NADP), 0.025 mL of tissue extract and 0.025 mL of glass distilled water were added and mixed well. The absorbance of this mixture was read was kept in a spectrophotometer at 340 nm against a blank (distilled water) (Lohr & Waller, 1974)

Estimation of Δ^5-3β-Hydroxysteroid Dehydrogenase (Δ^5-3β- HSD) activity

The weighed testes were homogenized in 0.1 M phosphate buffer (pH 7.4) and centrifuged at 10000×g. for 10 minutes at 0°C. Then the supernatant was taken in a conical flask (50 mL) and 0.2 mL of Nicotinamide adenine dinucleotide (NAD), 0.1 mL of dehydroepiandrosterone were added and mixed well. This solution was kept in a shaking incubator at 37°C for 90 minutes, and acidified with 0.1 mL of 3 M acetate buffer (pH 5.0). This solution was then extracted with 10 mL of ethyl acetate and evaporated until it was dry. The residue was dissolved in 2 mL of ethanol and optical density was read by a spectrophotometer at 240 nm against ethanol as the blank. The protein content of the tissue was determined and specific activity was expressed per mg of protein (Rabin, Leipsner, & Deane,1961).

Estimation of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Testosterone

Serum FSH, LH, and testosterone levels were assessed using an enzyme-linked immunosorbent assay kit from UNITED BIOTEK, Canada. In 96 well plates, 50 µL rabbit anti-hormone reagent, 100 µL of working hormone-HRP conjugate reagent, and 25 µL standard and samples, were added to each well and incubated at 37°C for 1½ hours. The wells were rinsed five times with distilled water, followed by the addition of 100 µL TMB and incubated at room temperature. The solution 1N HCl was used to stop the reaction. Absorbance was measured at 450 nm (Auletta, Caldwell, & Hamilton, 1979).

Estimation of fructose in seminal vesicles and epididymal protein content

Fructose content in seminal vesicles was measured using the previously described procedure in keeping with the WHO laboratory manual (Padashetty & Mishra, 2007). Standard curve was prepared by dissolving fructose (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mM/mL) in water and mixing it with indole reagent. Finally, the absorbance was noted at 470 nm wavelength against blank. Protein was estimated with Folin's phenol reagent and the activities of the enzyme were expressed in unit per mg of protein as per the previously described method (Lowry, Rosenbrough, Farm, & Randall, 1951). Standard curve was prepared by dissolving BSA (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL) in distilled water. Optical density was recorded at 660 nm against the reagent blank

Histological studies of rat testis and epididymis

The histology of the tissue was studied adopting the routine paraffin method (Hamilton, 1975) and resin embedding method (Hayat, 1981). A section of tissue was mounted over the slide for the microscopic studies and the following steps were taken.

The reproductive organs were separated from the experimental and control rats, then washed altogether with normal saline, cut to pieces of the desired size and fixed in Bouin's

liquid fixative promptly after autopsy examination. Fixation continued at room temperature for one day, after which the tissues were transferred into 70% alcohol. The tissues were then dried out by passing them through ascending grades in alcohol, cleared in a solution of xylene, penetrated with liquid paraffin, and lastly implanted in paraffin wax (58°C MP). 5 µm thickness sections were acquired utilizing a rotary microtome (Leica, Germany). The section was stained in Harris' hematoxylin and eosin was, dried out utilizing alcohol, cleared in xylene and mounted using dihydroxyphthalate xylol (DPX). The stained slides images were captured using a research microscope.

Statistical analysis

The statistical analysis was performed by Graph Pad Prism 5.0 Version (Graph Pad Software, Inc., San Diego, California, USA). All data are presented as mean±SEM and comparisons done by one-way ANOVA followed by Tukey's test as a post hoc test. Values were considered significant at $p<0.05$ or less.

RESULTS

Body weights and reproductive organ weights

The final body weights of rats of all groups increased markedly when compared with their respective initial body weights and are shown in Table 1. A great decline in the weights of testis, epididymis and seminal vesicle was observed in all treatment groups when compared with Group I animals and this is shown in Table 1. Oral administration of the extract at the dose of 100 mg kg^{-1} b.wt, and 300 mg kg^{-1} b.wt showed significant ($p<0.05$) decrease in testis, epididymis and vas defenses compared with the control. The most significant reduction ($p<0.01$) of this reproductive organ weight was observed at the dose of 500 mg kg^{-1} b.wt compared as with control.

Reproductive performance

The female rats who mated with the MECS 100,300 and 500 mg kg^{-1} b.wt. p.o. treated male rats delivered fewer pups than those mated with the control. However, the female rats who mated with MECS 500 mg kg^{-1} body weight treated male rats, were not delivered of pups. That is, the MECS 500 mg kg^{-1} body weight showed 0% fertility, followed by MECS 300 mg kg^{-1} b.wt. p.o (16.7%) and MECS 100 mg kg^{-1} b.wt. p.o (33.3%), at a tested dose when compared to the control (100%). These results are shown in Table 2.

Antisteroidogenic property

In the current study, the decrease in protein concentration by MECS-treated rats can be attributed to a decrease in secretory activity. Figure 2A shows that the methanol extract of the aerial portion of *C. serratum* significantly increased cholesterol and

Table 1. Effect of MECS on body and reproductive organ weights of Male albino rats.

Groups	Body Weights (Grams)		Reproductive Organ Weights (%)		
	Initial	Final	Testis	Epididymis	Seminal Vesicles
Group I- Control DW (10ml kg^{-1}b.wt. p.o.)	171.3±2.716	211.5±3.89	0.745±0.039	1.65±0.047	3.27±0.045
Group II- MECS (100mg kg^{-1}b.wt. p.o.)	171.8±3.55	210.8±1.30	0.69±0.022	0.14±0.025*	2.67±0.25*
Group III- MECS (300mg kg^{-1}b.wt. p.o.)	169.8±3.21	207.7± 2.53	0.64±0.013*	0.12±0.054*	0.22±0.03*
Group IV- MECS (500mg kg^{-1}b.wt. p.o.)	170.7±3.52	210.2±1.74	0.59±0.020**	0.10±0.032**	0.17±0.12**

'n = 5' in each group; values are expressed as Mean±SEM. *$p<0.05$, ** $p<0.01$, compared with normal control.. Statistical test employed was one way ANOVA followed by Tukey multiple comparison test.

Table 2. Effect of MECS on Reproductive Performance by Fertility testing in Female rats after 30 days of treatment.

Design of treatment	Number of mated males/females	Number of females delivered	Litter size of the mated female	Percent Fertility
Group I- Control DW (10ml kg^{-1}b.wt. p.o.)	3/6	6	59	100
Group II- MECS (100mg kg^{-1}b.wt. p.o.)	3/6	2	8	33.3
Group III- MECS (300mg kg^{-1}b.wt. p.o.)	3/6	1	2	16.7
Group IV- MECS (500mg kg^{-1}b.wt. p.o.)	3/6	0	0	0

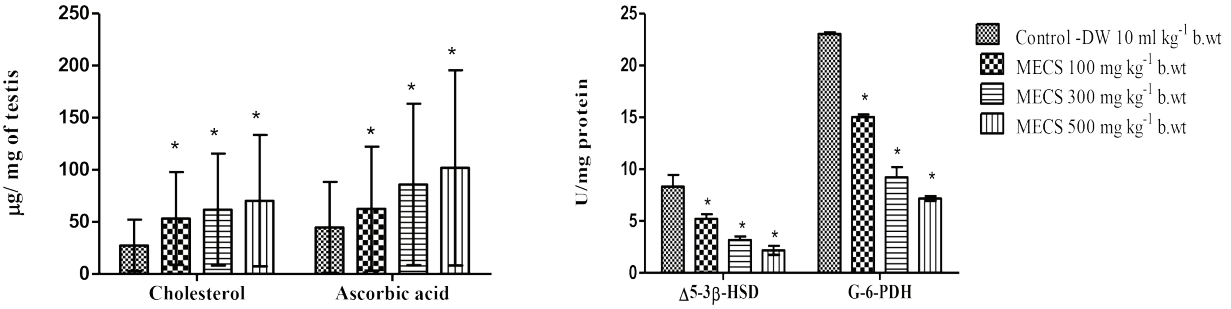

Figure 2. A. Effect of MECS on testicular cholesterol and ascorbic content in male rats after 30 days of treatment. **2. B.** Effect of MECS on testicular Δ^5-3β–HSD and G-6-PDH enzymes content in male rats after 30 days of treatment MECS- Methanolic extracts of *C. Serratum*, DW – Distill water. Data are presented as Mean±SEM (n=5); for each dose group. *Mean difference values compared to control group are significant at the 0.05 level.

ascorbic acid content in the testicular tissues of rats at all doses of 100, 300, and 500 mg kg^{-1} b.wt. When compared to control animals, the MECS 500 mg kg^{-1} b.wt displayed a significant increase in cholesterol and ascorbic acid content of 133.46 g/mg and 195.52 g/mg, respectively.

The MECS-treatements also suppressed the activities of two primary testicular steroidogenic enzymes, G-6-PD and 5-3β-HSD, significantly ($p<0.05$). In Figure 2B, the MECS-treated rats had the highest suppression of G-6-PD and 5-3β -HSD at 500 mg kg^{-1} b.wt, with 7.17 and 2.18 U/mg protein, respectively, as compared to control-treated animals. Among the therapies, this extract demonstrated that this drug has an antisteroidogenic effect in male rats.

Oral administration of the extract at the dose of 100 mg kg^{-1} b.wt, 300 mg kg^{-1} b.wt, and 500 mg kg^{-1} b.wt showed very significant ($p<0.005$) reduction in serum testosterone, FSH and LH levels comparable to that of the control has shown in Table 3. The results are summarized in Table 4. Among all groups, the MECS 500 mg kg^{-1} b.wt treated rats showed more of a reduction in the content of fructose in seminal vesicles, and epididymal protein content was 1.5% and 55.54%, respectively when compared to the control.

Table 3. Effect of MECS on fructose content in seminal vesicle and epididymal protein content of male rats.

Design of treatment	Fructose in seminal vesicle (mg/g)	Protein in epididymis (mg/g)
Group I- Control DW (10ml kg^{-1}b.wt. p.o.)	4.88±0.21	237.23±5.19
Group II- MECS (100mg kg^{-1}b.wt. p.o.)	3.33±0.11*	131.08±3.78*
Group III- MECS (300mg kg^{-1}b.wt. p.o.)	2.45±0.11*	77.42±2.30*
Group IV- MECS (500mg kg^{-1}b.wt. p.o.)	1.5±0.05*	55.54±1.47*

'n = 5' in each group; values are expressed as Mean±SEM. *$p<0.05$, compared with normal control. Statistical test employed was one way ANOVA followed by Tukey multiple comparison test.

Table 4. Effects of MECS on hormone profile of male albino rats.

Groups	Testosterone	LH	FSH
Group I- Control DW (10ml kg^{-1}b.wt. p.o.)	5.160±0.025	4.470±0.560	3.397±0.352
Group II- MECS (100mg kg^{-1}b.wt. p.o.)	3.367±0.465*	2.310±0.223*	1.990±0.054*
Group III- MECS (300mg kg^{-1}b.wt. p.o.)	2.087±0.094**	2.087±0.076**	1.950±0.043**
Group IV- MECS (500mg kg^{-1}b.wt. p.o.)	1.612±0.063***	1.680±0.329***	1.180±0.043***

'n = 5' in each group; values are expressed as Mean±SEM. *$p<0.05$, ** $p<0.01$, ***$p<0.001$ compared with normal control. Statistical test employed was one way ANOVA followed by Tukey multiple comparison test.

Effects of MECS on tissue histology

Testicular histology

As shown in Figure 3, the histological results indicated that the control animals had the usual histological structure of rat testis. Standard seminiferous tubules with multiple germ cells are visible in this segment. There is evidence of spermatogenesis, as shown by the orderly maturation of germ cells from the lumen's base to its middle. In MECS-treated testes (Sections B, C, and D), there were fewer secondary spermatocytes, spermatids, and Leydig cells, as well as histological damage to the testes with an increased diameter of seminiferous tubules, there are areas of degenerating cells and debris.

Epididymis histology

The findings revealed that the control animal had typical histological structure of the rat epididymis. Figure 4, Section A of the control epididymis reveals multiple tubules lined by ciliated epithelium extending into the lumen, as well as normal interstitial cells. There was an significant improvement in the histological composition of the epididymis in MECS treated rats relative to controls, so that (Figure 4, Section B, C, & D) revealed derangement, shrinkage of interstitial cells, and dramatic changes in cellular integrity. When these MECS-treated groups were compared to the control groups, there was a biologically significant difference in the histological parts.

DISCUSSION

Several medicinal plants have been stated to have antifertility properties in India as well as other parts of the world. Medical historians have identified a number of plants with abortifacient, contraceptive, and emmenagogue properties. With this in mind, the aim of this study was to test whether MECS had an antisteroidogenic impact in male rodents.

Our earlier research on the phytochemical screening of methanol extracts of *C. serratum* revealed the presence of carbohydrates, flavonoids, tannins, sterols and terpenoids. As per the OECD guideline 420 (acute oral toxicity-Acute Toxic Class

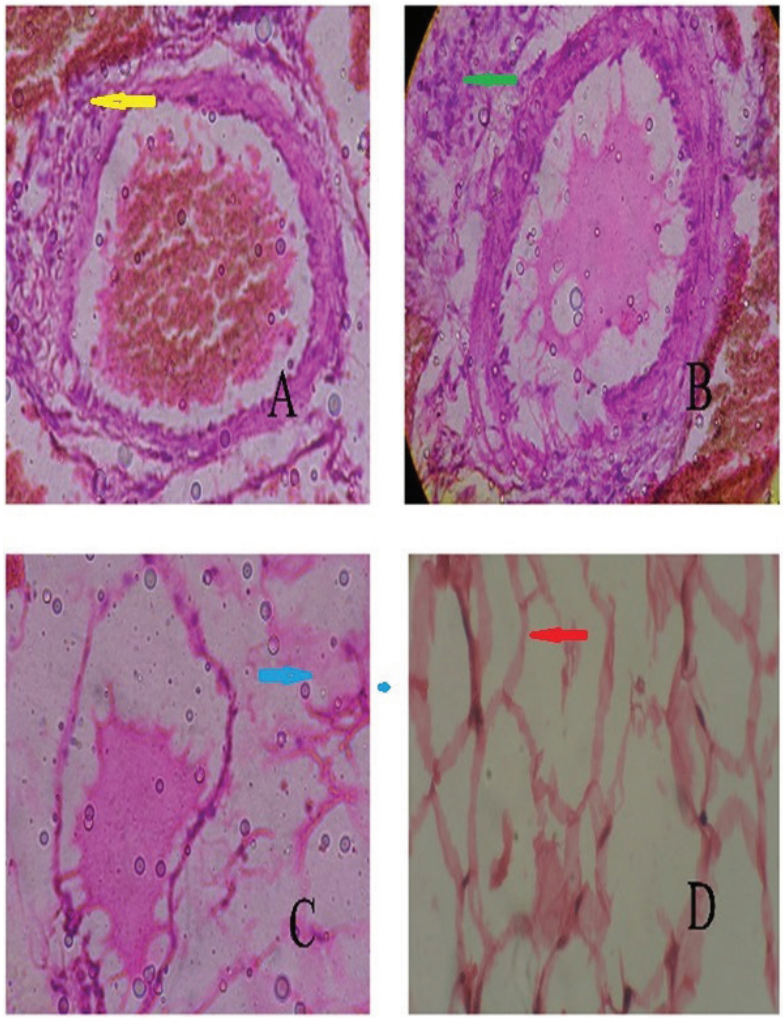

Figure 4. Effects of MECS on histological structure of rat epidydimis

Section A. Control, Distilled water (10 ml kg^{-1} b.wt. p.o.) showed the normal interstitial cells (Yellow arrow). **Section B.** MECS (100 mg kg^{-1} b.wt. p.o.) exhibited the derangement of interstitial cells (Green arrow). **Section C.** MECS (300 mg kg^{-1} b.wt. p.o.), the shrinkage of interstitial cells (Blue arrow) was present. **Section D.** MECS (500 mg kg^{-1} b.wt. p.o.) showed the drastic changes in the cellular integrity (Red arrow).

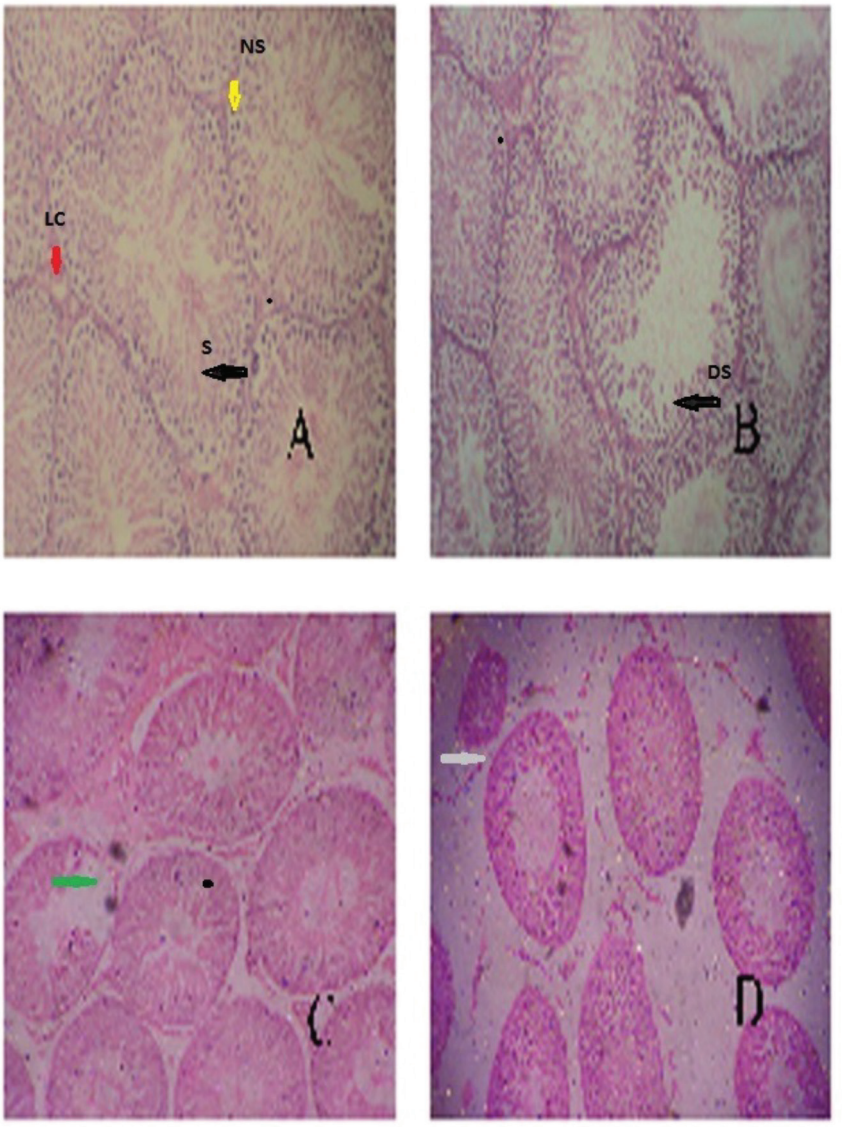

Figure 4. Effects of MECS on histological structure of rat testis.

Section A. Control, Distilled water (10 ml kg^{-1} b.wt. p.o.) showed the normal arrangement of sertoli cells (Black arrow), spermatogonia (yellow colour) and leydig cells (Red arrow). **Section B.** MECS (100 mg kg^{-1} b.wt. p.o.) showed the distorted seminiferous tubule (Black arrow). **Section C.** MECS (300 mg kg^{-1} b.wt. p.o.) exhibited the disorganized population of spermatogenic cells (Green arrow). **Section D.** MECS (500 mg kg^{-1} b.wt. p.o.) the Spermatocytes within the lumen are very few with evidence of reduction spermatogenesis, showing severe hypercellularity of leydig cells (Grey arrow).

Method) the acute toxicity of plant *of C. serratum* was reported this plant extract was not not cause lethal to the male rats even at the dose of 2000 mg kg^{-1} b.wt (Sarathchandiran, Kadalmani, & Navaneetha Krishnan, 2014 b).

The anti-fertility effect of MECS was confirmed by the following measures. A great decline in the weights of testis, epididymis and seminal vesicle were observed in all treatment groups when compared with the control. Reduction of reproductive organ weights, the disintegration of Leydig cells and regressive degenerative changes in testis, vas deferense, and epididymis indicated the anti-androgenic activity (Hiremath, Badami, Swamy, Londonkar, & Patil, 1997).

In the androgenic pathway, cholesterol and ascorbic acid are essential principle precursors for the formation of androgen in testis. This is involved in the process of steroidogenesis in rat testis (Rajnish *et al.,* 2011; Sharma & Jacob, 2001). *Feronia limonia* fruit pulp extract-treated rats show impairment of spermatogenesis due to an accumulation of cholesterol and ascorbic acid in rat testis (Dhanapal, Ratna, Sarathchandran, & Gupta, 2012). Mycotoxin MT81 and its benzoylated derivative induced an increased level of testicular cholesterol and ascorbic acid in male mature rats leading to impaired spermatogenesis (Choudhury, Gupta, & Majumder, 2011). The present study showed an elevated level of cholesterol and ascorbic acid in MECS treated rats testicular tissues, indicating the impaired spermatogenesis.

In our earlier studies, the MECS showed a reduction in sperm count and their motility. This indicates the inhibition of testosterone synthesis (Sarathchandiran, Kadalmani, & Navaneetha

Krishnan, 2014a). This theory was reinforced by changes in cholesterol and ascorbic acid, and it is further supported by the decreased importance of G-6-PD and Δ^5-3β-HSD activities in testicular tissues. G-6-PD and ~5-3ß-HSD have been successively identified to form two major androgen biosynthesis enzymes. The synthesis of androgen, based on the G-6-PD and Δ^5-3β-HSD enzyme on Knorr's well-documented knowledge. The suppressed steroid activity of the testicles could prevent cholesterol from being converted into testosterone by damaging the activities of these two basic androgen-based enzymes. (Anuja, Nithya, Rajamanickam, & Madambath, 2010; Pankajakshy & Madambath, 2009).

The decrease in Leydig cells observed in the histological structure of rat testis may be correlated with decreased testosterone production (Eik-Nes, 1970). Diminution of testosterone levels may indicate the delayed maturation of spermatozoa and the lower FSH and LH levels may affect the Sertoli cell function present in the seminiferous tubules resulting in the disturbing facilitatory function of these cells. The testosterone surge is required to stimulate the accessory glands fully. Decreased endogenous testosterone secretion from the testis deprive the development of mature sperm and also it suppresses testicular steroidogenesis and spermatogenesis (Kusemiju, Osinubi, Noronha, & Okanlawon, 2010). The fructose contents in seminal vesicles and epididymal protein were shown to be significantly ($p<0.05$) reduced in all doses of MECS treated rats. Fructose and citric acid play an essential role in sperm motility and concentration, especially with regards to energy metabolism (Videla, Blanco, Galli, & Fernández-Collazo, 1981). Fructose is a fundamental sugar in semen. Sperm motility and sperm count were diminished in aluminum chloride treated rats due to an absence of fructose content in seminal vesicles of mice (Chinoy & Ranga Geetha, 1984). The protein level is directly associated with the secretory activity of epididymis, which in turn depends on the androgen levels (Jones, 1977).

The antisteroidogenic property findings are additionally upheld by histopathological changes in the testicles. Leydig cells were lessened which demonstrates the inadequacy of these cells to synthesize testosterone. The quantity of Leydig cells has an immediate bearing on spermatogenesis (Gupta, Kumar, Dixit, & Dobhal, 2000). The reduction in the number of secondary spermatocytes and spermatids may be due to an insufficient amount of testosterone (De Kretser & Kerr, 1994). Distorted epithelial layer patterns and pyknosis were observed among the epithelial cells. The interstitial spaces were filled with loose connective tissue (Vengaiah, Govardhan, & Changamma, 2015). Ethanolic extract of *C. papaya* seeds on the epididymal histology revealed changes such as vacuolation in the tubules, loose contacts of the principal cells in the epithelium of epididymis and presence of degenerated late spermatids (Madan, 2013). Similar findings were observed in MECS treated animal testes such that a reduced number of secondary spermatocytes, spermatids and Leydig cells with changes of epididymal cellular integrity were eliciting adverse histological damage in the testes and epididymis. As per the previous reports, in rabbits, intravaginal administration of 0.5 mg β -sitosterylsulphate in KY jelly reduced pregnancy rates (Burck, Thakkar, & Zimmerman, 1982). Chronic administration of β -sitosterol at doses ranging from 50 to 500 pg triggered testicular lesions and prevented the process of spermatogenesis in males (Ghannudi, Shareha, Elsamannoudy, Ibrahim, & Elmougy, 1978; Malini, 1987). Based on the information presented above, we concluded that β-sitosterol plays an important role in male anti-fertility.

CONCLUSION

In conclusion, the testicular steroidogenesis and fertilities were lower in the MECS-treated rats, and anti-androgenic results were reported in hormonal study. Furthermore, histopathological findings confirmed the drug's male contraceptive function. More research is needed to determine the antifertility effect of MECS in various animal species, as well as to identify the bioactive principles responsible for this effect and to comprehen the cellular behavior's underlying mechanism.

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Original Article

Curcumin prevents tenofovir/lamivudine/efavirenz-induced nephrotoxicity in rats

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ABSTRACT

Background and Aims: Nephrotoxicity is an adverse effect, which may occur with the use of tenofovir/lamivudine/efavirenz (TLE) in the treatment of human immunodeficiency virus (HIV) infection. Curcumin (CUM), an isolate of *Curcuma longa* L. is used in folk medicine for the treatment of ailments. This study attempts to establish whether CUM supplementation can protect against a rat model of TLE-induced nephrotoxicity.
Materials and Methods: Adult male Wistar rats (n=40) were randomly grouped and supplemented orally with CUM (50, 100 and 200 mg/kg/day) prior to the oral administration of TLE (300/300/600 mg/kg/day) for 30 days. After the treatment, the rats were fasted overnight, weighed and anesthetized. Blood samples were collected, and sera were extracted for biochemical analyses. Kidney samples were excised, weighed and processed for oxidative stress markers and histology.
Results: Body weight was decreased ($p<0.01$) whereas kidney weight was increased ($p<0.01$) in TLE administered rats when compared to the control. Significant ($p<0.001$) increments in serum uric acid, creatinine, urea and kidney malondialdehyde levels were observed in TLE-administered rats. Significant ($p<0.001$) decreases in serum total protein, albumin, electrolytes, kidney superoxide dismutase, glutathione, catalase and glutathione peroxidase levels were observed in TLE administered rats when compared to the control. TLE produced tubular necrosis and hypercellular glomerulus with mesangial proliferation in the kidneys of treated rats. However, CUM (50, 100 and 200 mg/kg) supplementation abrogates TLE-induced nephrotoxicity in a dose-related manner at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, when compared to TLE group.
Conclusion: CUM seems effective against TLE-induced nephrotoxicity.

Keywords: Antiretroviral, curcumin, nephrotoxicity, rat

INTRODUCTION

Highly active antiretroviral therapy (HAART) consists of three drugs active against human immununodeficiency virus (HIV) infection. HAART has significantly decreased HIV progression and prevents HIV-related infections. The success of HAART reflects on the reductions of HIV related morbidity and mortality in the world (Palella et al., 1998; Montaner et al., 2010). However, the use of HAART has been associated with a myriad of toxicities, especially nephrotoxicity. Acute kidney injury, tubulopathies, chronic kidney disease, and end-stage renal disease requiring renal replacement therapy have been documented with the use of HAART (Kalyesubula & Perazella, 2016).

Tenofovir -lamivudine -efavirenz (TLE) is an integral part of the preferred first-line regimens for the treatment of HIV in adolescents and antiretroviral-naive adults especially in resource-limited settings (WHO, 2013). It has reduced the incidence of HIV related death, but the incidence of nephrotoxicity attributed to its tenofovir component, which can be aggravated by partner drugs is a worrisome challenge. An incidence of 17–22% of tubular dysfunction was observed in tenofovir containing regi-

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mens. Also, an incidence of 18.3% moderate renal impairment and 2.3% severe renal impairment were documented with the use of tenofovir containing regimens (Nartey et al., 2019). The primary clinical presentations of nephrotoxicity caused by tenofovir include proximal tubular dysfunction, electrolytes and acid-base disorder (Perazella, 2010). Kidney histological aberrations include chronic tubular-interstitial scarring, necrosis, tubular atrophy and interstitial fibrosis (Herlitz et al., 2010).

Curcumin (diferuloyl methane) is a low-molecular weight compound extracted from the roots of *Curcuma longa* L. (Zingiberaceae). It is traditionally used for centuries in Asia and other parts of the world for medicinal and culinary purposes. CUM has a diverse and wide range of targets at molecular and cellular levels (Noorafshan & Ashkani-Esfahani, 2013). Substantial number of *in-vitro* and *in-vivo* studies showed that it has essential pharmacological activities including anti-inflammatory, antispasmodic, antioxidant, anti-cancer, and antimicrobial effects (Akram et al., 2010). In folk medicine, it is used as treatments for sprains, liver disorders, anorexia, rheumatism, diabetes, cough, sinusitis and inflammation (Noorafshan & Ashkani-Esfahani, 2013). It has redox regulatory effects such as scavenging of free radicals and increased antioxidant activities (Hewlings & Kalman, 2007). Its anti-inflammatory activity has been characterized by reduction in pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and the inhibition of NF-kappa B pathway (Noorafshan & Ashkani-Esfahani, 2013). CUM has been shown to have potential protective effect on experimentally induced toxicities including hepatotoxicity (Farghaly & Hussein, 2010) cardiotoxicity (Mohantya et al., 2004) and nephrotoxicity (El-Zawahry & Abu El Kheir, 2007). This study examined its protective activity against TLE-induced nephrotoxicity in Wistar rats, which is a novel study.

MATERIALS AND METHODS

Animals, drugs, chemicals and treatment

Tenofovir disoproxil fumarate/lamivudine/ efavirenz (TLE), *Curcuma longa* (CUM), and piperine were used.

Adult male Wistar rats (n=40) were randomly grouped into 8 of n=5/group and used. The rats were acclimated for 2 weeks in cages in a standardized condition (12 h light/day cycles, 25°C±5°C) with *ad libitum* access to food and water. The rats were purchased from the animal research unit of the Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Rivers State, Nigeria. The guideline (2020/569/*EU)* on animal handling prepared by European Parliament and of the Council was used for this study. Ethical approval for this study (NDU/PHARM/PCO/AEC/064B) was provided by the Research Ethics Committee of the Department of Pharmacology/Toxicology, Faculty of Pharmacy, Niger Delta University on 4 August 2020.

Animal treatment

Group 1 (Control) received normal saline (0.2 mL/day/p.o). Groups 2-4 received CUM (50, 100 and 200 mg/kg/day/p.o) (Lee et al., 2016). Group 5 received TLE (300/300/600 mg/kg/day/p.o). Groups 6-8 received CUM (50, 100 and 200 mg/kg/day/p.o) before receiving TLE (300/300/600 mg/kg/day/p.o). Piperine (20 mg/kg/p.o) was added to CUM to improve bioavailability (Shoba et al., 1998). All the rats were treated for 30 days. After the treatment, the rats were allowed to fast overnight, weighed and anesthetized through inhalation in a chamber of diethyl ether. Blood samples were collected from the heart, centrifuged (1500 rpm for 20 min) and sera were extracted for biochemical assessments. Kidney samples were collected, rinsed in cold saline and homogenized in 0.1 M Tris-HCl solution buffered (pH 7.4). The homogenates were centrifuged (3000 rpm for 15 min), supernatants decanted and assayed for oxidative stress markers.

Assessment of serum biochemical markers

Serum total protein, creatinine, albumin, uric acid, urea, sodium, bicarbonate potassium, and chloride and concentrations were measured using laboratory test kits.

Assessment of kidney oxidative stress markers

Malondialdehyde (MDA) was assayed as reported by Buege & Aust, 1978. Reduced glutathione (GSH) was assayed using the method reported by Sedlak & Lindsay, 1968. Catalase (CAT) was assayed as described by Aebi, 1984. Glutathione peroxidase (GPx) was measured according to Rotruck et al., 1973. Superoxide dismutase (SOD) was assessed as reported by Sun & Zigman, 1978.

Histological analysis

Kidney samples were collected, blotted and fixed in 10% buffered formaldehyde. Kidney samples were processed and embedded in paraffin. Sections (3 µm in thickness) were produced on slides and stained with hematoxylin and eosin (H&E). Stained sections were assessed for histological changes using a light microscope.

Statistical analysis

Mean±standard error of mean (SEM) for the results for all groups (n=5) was determined. Data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *posthoc test*. Graph Pad Prism 5 Software (San Diego, CA USA) was used for data analysis. Significance was set at $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

Effects of curcumin on body and kidney weights of tenofovir/lamivudine/efavirenz -treated rats

The effects of CUM on body and kidney weights were not significant ($p>0.05$) when compared to control (Table 1). The administration of TLE produced a significant ($p<0.01$) decrease in body weight with a significant increase in kidney weight ($p<0.01$) when compared to control (Table 1). However, body and kidney weights were restored in CUM (50, 100, and 200 mg/kg) supplemented rats at $p<0.05$, $p<0.01$, and $p<0.01$, respectively when compared to TLE group (Table 1).

Effect of curcumin on serum kidney function markers of tenofovir/lamivudine/efavirenz -treated rats

The administration of CUM had no significant ($p>0.05$) effects on serum total protein, potassium, chloride, sodium, bicarbonate, albumin, uric acid, creatinine and urea levels when

compared to control (Table 2) (Figures 1-5). In TLE administered rats, serum total protein, potassium, chloride, sodium, bicarbonate and albumin levels were decreased significantly ($p<0.001$) whereas uric acid, creatinine and urea levels were increased significantly ($p<0.001$) when compared to control (Table 2) (Figures 1-5). However, CUM (50, 100, and 200 mg/kg)

Table 1. Effects of curcumin on body and kidney weights of tenofovir/lamivudine/efavirenz -treated rats.

Dose (mg/kg)	FBW (g)	AKW(g)	RKW (%)
Control	255.8±17.7	0.65±0.06	0.25±0.06
CUM 50	250.1±15.1	0.67±0.01	0.27±0.03
CUM 100	252.7±13.0	0.63±0.09	0.25±0.01
CUM 200	250.2±15.9	0.66±0.04	0.26±0.06
TLE	161.5±17.6#	1.99±0.03#	1.23±0.09#
CUM 50 + TLE	180.3±16.6	1.70±0.02	0.94±0.07*
CUM 100 + TLE	200.7±16.1*	1.16±0.06*	0.58±0.05**
CUM 200 + TLE	250.8±18.6**	0.70±0.08**	0.28±0.01***

CUM: Curcumin, TLE: Tenofovir/lamivudine/efavirenz, FBW: Final body weight, AKW: Absolute kidney weight, RKW: Relative kidney weight, Data as mean±SEM, (Standard error of mean), n=5, #p<0.01 Significant differnce when compared to control, *p<0.05, **p<0.01, ***p<0.001 Significant difference when compared to TLE.

Table 2. Effect of curcumin on serum electrolytes of tenofovir/lamivudine/efavirenz-treated rats.

Dose (mg/kg)	Potassium (mmo/L)	Chloride (mmo/L)	Sodium (mmo/L)	Bicarbonate (mmo/L)
Control	3.70±0.19	111.02±12.0	125.63±11.3	12.86±1.45
CUM 50	3.71±0.04	112.11±13.9	126.16±12.0	12.67±2.17
CUM 100	3.73±0.30	114.12±10.7	128.87±13.2	12.52±3.63
CUM 200	3.78±0.06	116.76±12.8	129.74±14.8	12.35±2.33
TLE	1.57±0.17$^{\pi}$	48.10±5.57$^{\pi}$	48.03±4.66$^{\pi}$	5.00±0.76$^{\pi}$
CUM 50+TLE	2.18±0.06^{a}	65.02 ±6.86^{a}	67.16±6.92^{a}	7.02±0.09^{a}
CUM 100+TLE	2.89±0.43^{b}	85.07 ±8.66^{b}	88.34 ±8.55^{b}	9.00±1.22^{b}
CUM 200+TLE	3.60±0.36^{c}	109.11±10.7^{c}	118.78±11.1^{c}	12.48±1.60^{c}

CUM: Curcumin, TLE: Tenofovir/lamivudine/efavirenz, n=5, Data as mean ± SEM (Standard error of mean), $^{\pi}$p<0.001 Significant differnce when compared to control, ^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001 Significant differnce when compared to TLE.

Table 3. Effect of curcumin on kidney oxidative stress markers of tenofovir/lamivudine/efavirenz-treated rats.

Dose (mg/kg)	MDA (mmol/mg protein)	GSH (µmole/ mgprotein)	CAT (U/mgprotein)	SOD (U/ mgprotein)	GPx (U/ mgprotein)
Control	0.17±0.06	20.03±3.89	29.55±3.24	23.05±3.77	30.34±4.87
CUM 50	0.16±0.09	20.27±2.45	29.73±2.56	23.37±2.98	30.51±3.96
CUM 100	0.15±0.05	20.61±2.40	30.04±3.01	23.54±4.70	30.70±4.33
CUM 200	0.13±0.07	20.90±3.73	30.53±4.66	24.06±3.66	31.05±4.16
TLE	2.51±0.73$^{\pi}$	4.66±0.73$^{\pi}$	7.14±0.67$^{\pi}$	7.36±0.72$^{\pi}$	8.05±0.56$^{\pi}$
CUM 50+TLE	1.80±0.81^{a}	7.54±0.84^{a}	10.27±1.09^{a}	11.47±1.89^{a}	11.67±1.22^{a}
CUM 100+TLE	0.94±0.06^{b}	11.64±1.02^{b}	16.65±1.67^{b}	16.50±1.89^{b}	17.83±1.01^{b}
CUM 200+TLE	0.20±0.08^{c}	18.92±2.16^{c}	26.92±4.27^{c}	21.73±2.02^{c}	27.42±3.50^{c}

SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, MDA: Malondialdehyde, GPx: Glutathione peroxidase, CUM: Curcumin, TLE: Tenofovir/lamivudine/efavirenz, n=5, Data as mean±SEM (Standard error of mean), $^{\pi}$p<0.001 Significant differnce when compared to control, ^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001 Significant differnce when compared to TLE.

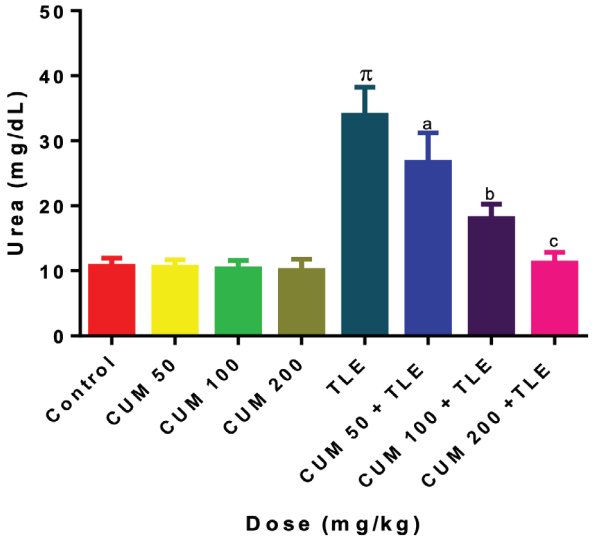


Figure 1. Effect of curcumin on serum urea levels of tenofovir/lamivudine/efavirenz -treated rats.

CUM: Curcumin, TLE: Tenofovir/lamivudine/efavirenz, Data as mean±SEM (Standard error of mean), n=5, $^{\pi}p<0.001$ significance when compared to control, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ significance when compared to TLE.

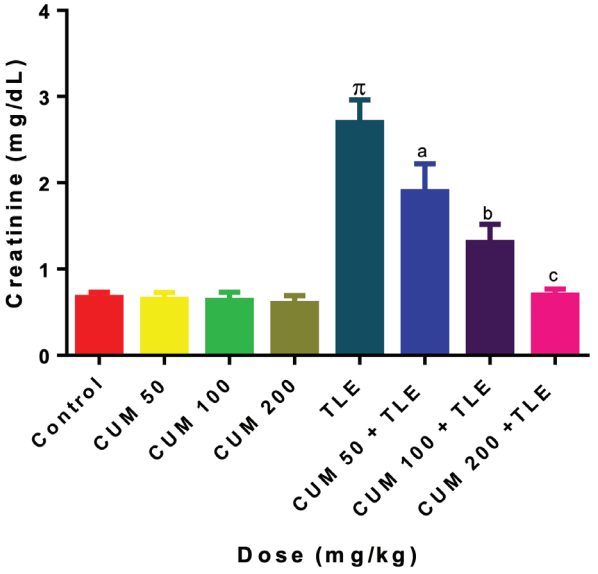


Figure 2. Effect of curcumin on serum creatinine levels of tenofovir/lamivudine/efavirenz -treated rats.

CUM: Curcumin, TLE: Tenofovir/lamivudine/efavirenz, Data as mean±SEM (Standard error of mean), n=5, $^{\pi}p<0.001$ significant difference when compared to control, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ significant difference when compared to TLE.

supplementation increased serum total protein, potassium, chloride, sodium, bicarbonate and albumin levels significantly at p<0.05, p<0.01, and p<0.01, respectively when compared to TLE. CUM (50, 100, and 200 mg/kg) supplementation decreased serum uric acid, creatinine and urea levels in a dose-related manner at p<0.05, p<0.01, and p<0.001, respectively when compared to TLE (Table 2) (Figures 1-5).

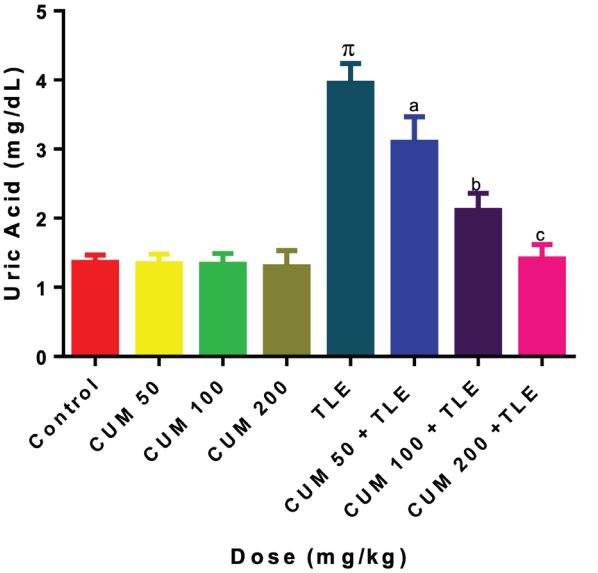


Figure 3. Effect of curcumin on serum uric acid levels of tenofovir/lamivudine/efavirenz -treated rats.

CUM: Curcumin, TLE: Tenofovir/lamivudine/efavirenz, Data as mean±SEM (Standard error of mean), n=5, $^{\pi}p<0.001$ significant difference when compared to control, $^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p<0.001$ significant difference when compared to TLE.

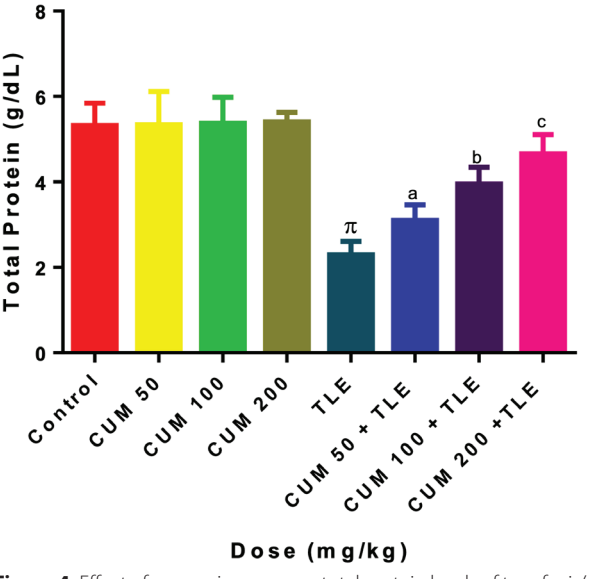


Figure 4. Effect of curcumin on serum total protein levels of tenofovir/lamivudine/efavirenz -treated rats.

CUM: Curcumin, TLE: Tenofovir/lamivudine/efavirenz, Data as mean±SEM (Standard error of mean), n=5, $^{\pi}p<0.001$ significant difference when compared to control, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ significant difference when compared to TLE.

Effects of curcumin on serum kidney oxidative markers and histology of tenofovir/lamivudine/efavirenz -treated rats

Kidney antioxidants (SOD, CAT, GSH and GPx) and MDA levels were normal (p>0.05) in CUM-administered rats when compared to control. TLE administration significantly (p<0.001)

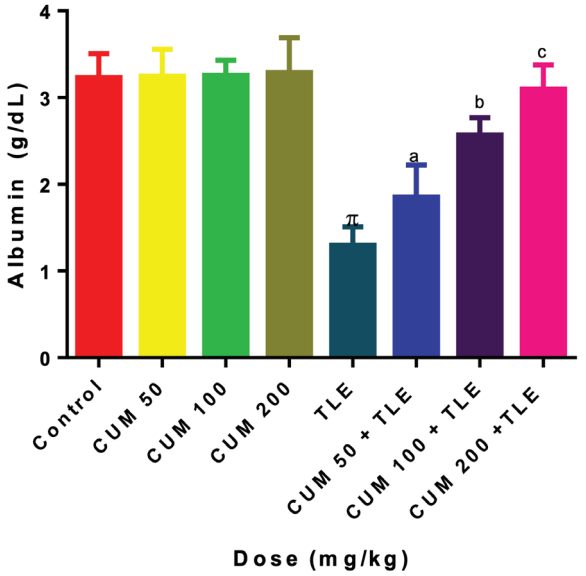


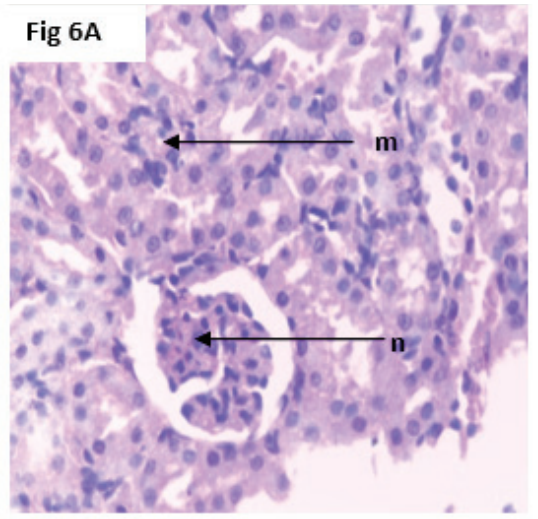
Figure 5. Effect of curcuminon serum albumin of tenofovir/lamivudine/efavirenz-treated rats.

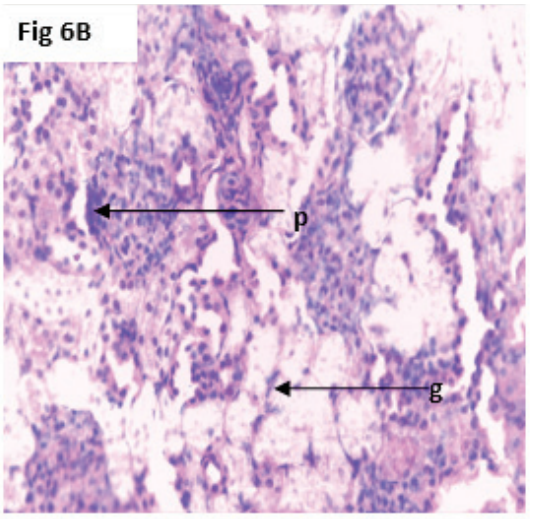
CUM: Curcumin, TLE: Tenofovir/lamivudine/efavirenz, Data as mean±SEM (Standard error of mean), n=5, $^{π}p<0.001$ significant difference when compared to control, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ significant difference when compared to TLE.

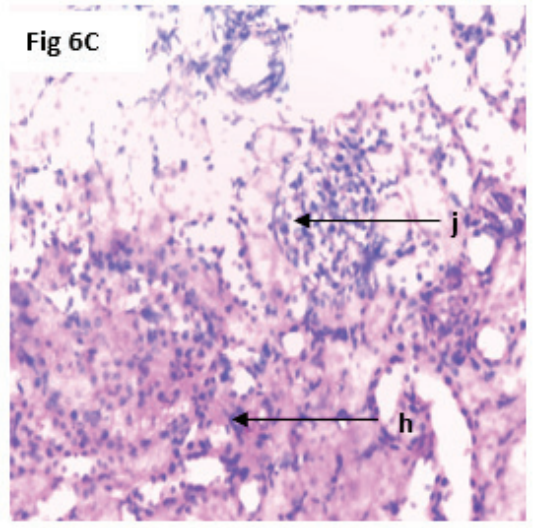
decreased kidney antioxidants, but significantly ($p<0.001$) increased kidney MDA levels when compared to control (Table 3). However, CUM (50, 100, and 200 mg/kg) supplementation significantly increased kidney antioxidants, and significantly decreased kidney MDA levels in a dose-related manner at $p<0.05$, $p<0.01$, and $p<0.001$, respectively when compared to TLE treated rats (Table 3). The kidney of the control rats showed normal renal tubule and glomerulus (Fig 6A), but the kidney of TLE-treated rat showed tubular necrosis and hypercellular glomerulus with mesangial proliferation (Figure 6B). The kidney of CUM (25 mg/kg) and CUM (50 mg/kg) supplemented rats showed normal renal tubules and hypercellular glomeruli with mesangial proliferations as shown in (Figure 6C) and (Figure 6D), respectively. However, the kidney of CUM (100 mg/kg) supplemented rat showed normal renal tubules and glomerulus (Figure 6E).

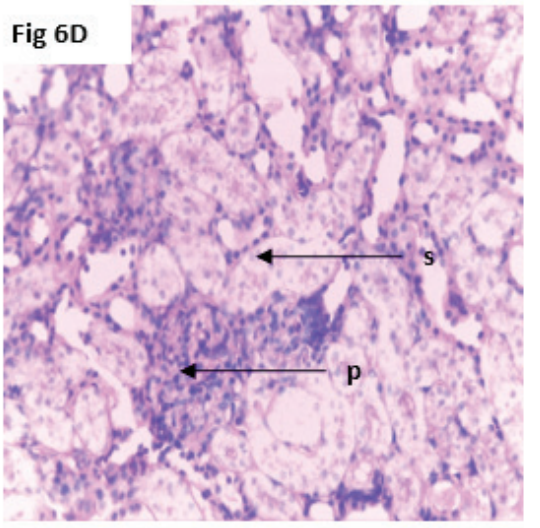
DISCUSSION

TLE-related nephrotoxicity can add to HIV infection associated socio-economic burden (Perazella, 2010). CUM is an isolate of turmeric with a wide spectrum of biological activity that is used in folk medicine (Noorafshan & Ashkani-Esfahani, 2013). This study attempts to establish whether CUM supplementation can prevent TLE-induced nephrotoxicity in a rat model. In the current study, the administration of CUM had no effects on all evaluated parameters at the serum and tissue levels. On the other hand, the administration of TLE increased kidney weight, but decreased body weight, which supports earlier reports (Jang et al., 2010). The observed decrease in body weight may be ascribed to decreased appetite whereas increase in kidney weight may be predicated on the induction of inflammation by TLE. In this study, the conspicuous incapacitation of kidney function by TLE was marked by elevated serum uric acid, urea, and creatinine levels with decreased serum total protein, albumin and serum electrolytes. This observation supports previous reports (Fenandez-Fernandez et al., 2011). The health status of the kidney is ascribed to its capacity to functionally regulate the serum concentrations of the aforementioned indices (Gowda et al., 2010). In the midst of perturbations caused by chemical assaults or diseases the functional capacity of the kidney is impaired causing aberrations in serum uric acid, urea, creatinine, total protein, albumin and serum electrolytes (Gowda et al., 2010). In the present study, TLE caused dysfunction in kidney reduction/oxidation status of treated rats characterized by decreased antioxidants and increased MDA levels. This observation supports earlier findings (Adikwu & Apiakise, 2016). Antioxidants form defensive network that prevents oxidative damage by scavenging and neutralizing free radicals, but could be consumed and depleted as a consequence of increased free radicals production beyond antioxidants regulation causing oxidative stress (Adikwu & Apiakise, 2016). Hence, depleted kidney antioxidants observed in TLE-treated rats established oxidative stress. MDA is used experimentally to mirror the occurrence of lipid peroxidation (LPO) in a pathologic process or condition (Adikwu & Apiakise, 2016). Therefore, TLE-induced elevation in MDA level established the occurrence of LPO. In the current study, TLE-induced nephrotoxicity was characterized by kidney tubular necrosis and hypercellular glomerulus with mesangial proliferation. This observation is in agreement with earlier findings (Herlitz et al., 2010). In the current study, CUM supplementation abrogates TLE-induced nephrotoxicity in a dose-related manner. This was characterized by restored body and kidney weights and up-regulation of serum total protein, albumin, electrolytes and kidney antioxidants. CUM supplementation caused down-regulation of serum uric acid, creatinine, urea and kidney MDA levels. Also, tubular necrosis and hypercellular glomerulus with mesangial proliferation were absent in the kidneys of rats supplemented with the highest dose of CUM. This finding correlates with the reported protective activity of CUM against gentamicin-induced nephrotoxicity in rats (El-Zawahry & Abu El Kheir, 2007). In the present study, the protective impact of CUM on TLE-induced nephrotoxicity may be ascribed to its antioxidant and anti-inflammatory activities. Studies have associated most therapeutic effects of CUM to its antioxidant and anti-inflammatory activities (Hewlings & Kalman, 2007). CUM, as an antioxidant, inhibits oxidative stress and LPO by scavenging and neutralizing ROS (Menon &Sudheer, 2007). It can up regulate the activities of endogenous antioxidants (SOD, CAT, and GSH) and increase antioxidants gene expression. CUM can inhibit enzymes including xanthine hydrogenase/oxidase and lipoxygenase/cyclooxygenase, which are facilitators of free radicals production and can decrease the gene expression of such enzymes (Lin et al., 2007). The anti-inflammatory action of CUM includes inhibitory effects on pro-inflammatory mediators such as cytokines (TNF-α and IL-6). It can also inhibits NF-kappaB activation pathway, which is essential for inflammation (Noorafshan & Ashkani-Esfahani, 2013; Lin et al., 2007).









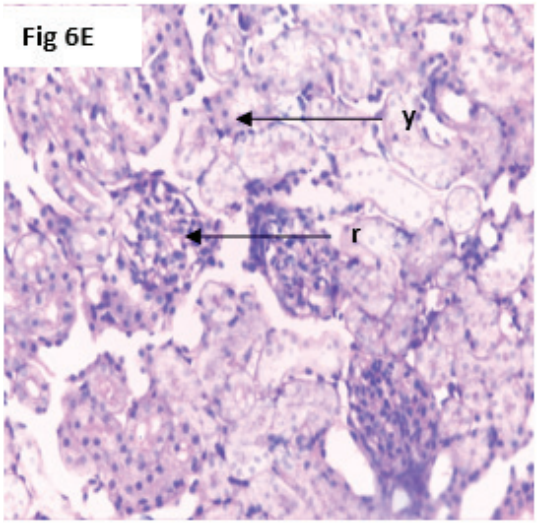


Figure 6. A: Kidney of control rat showed normal renal tubule (n) and glomerulus (m); B: Kidney of TLE-treated rat showed tubular necrosis (h) and hypercellular glomerulus with mesangial proliferation (j); C: Kidney of CUM (25mg/kg) supplemented rat showed normal renal tubule (h) and hypercellular glomerulus with mesangial proliferation (j); D: Kidney of CUM (50mg/kg) supplemented rat showed normal renal tubule (s) and hypercellular glomerulus with mesangial proliferation (p); E: Kidney of CUM (100mg/kg) supplemented rat showed normal renal tubule (y) and glomerulus (r).

CONCLUSION

Based on the observation in the current study, CUM may clinically protect against TLE-related nephrotoxicity.

Peer-review: Externally peer-reviewed.

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

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Original Article

Phytochemical profiling of the bioactive principles of *Alysicarpus glumaceus* (Vahl) DC. aerial parts

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ABSTRACT

Background and Aims: *Alysicarpus glumaceus* is a plant used in Africa, Asia and some parts of the Arabian Peninsula with folkloric claims of having anti-tussive, anti-asthmatic, anti-diarrheal, abortifacient, anti-psychotic, anti-inflammatory, diuretic and stimulatory activities. The aim of this study was to determine the phytoconstituents present in the methanol extract (ME) of *Alysicarpus glumaceus* and its fractions.

Methods: Standard qualitative phytochemical screening methods such as thin layer chromatography (TLC), gas chromatography mass spectrometry (GC-MS) and fourier-transform infrared (FT-IR) spectroscopy were employed for the profiling of the plant and identification of the phytoconstituents.

Results: The phytochemical screening revealed the presence of alkaloids, cardiac glycosides, flavonoids, saponins, steroids, tannins and terpenes. GC-MS chromatogram showed a total of 57 peaks with 38 different compounds identified, out of which 15 of the compounds were fatty acids mainly 3-Quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy-, ethyl ester; cis-Vaccenic acid; 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15- hexadecamethyl-;1-methyl-4-phenyl-5-thioxo-1,2,4-triazolidin-3-one; 2-Pentanone, 4-hydroxy-4-methyl-; Hexadecanoic acid; Palmitoleic acid; 9,12-Octadecadienoic acid and 9,17-Octadecadienal. While FT-IR spectras indicated the presence of carbonyl, alcohol, carboxylic acid, and aliphatic functional groups. Additionally, ethyl acetate fraction showed the peak characteristics of the aromatic (=C-H)/olefinic (=C-H) functional group.

Conclusion: The study showed that fatty acids were the major constituents of *Alysicarpus glumaceus*.

Keywords: *Alysicarpus glumaceus*, phytoconstituents, gas chromatography-mass spectrometry (GC-MS)

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INTRODUCTION

Medicinal plants have existed since the creation of man (Saric-Kundalic Dobe, Klatte-Asselmeyer & Saukel 2010). An estimated 14-28% of higher plant species are used for their therapeutic purposes and 74% of therapeutic agents in use were discovered from folkloric use of some of these plants (Ncube, Afolayan & Okoh , 2008). The search for chemical compounds in medicinal plants is of great importance because it gives information about the quality of the plant. The fundamental reason for quality control of herbal medicines is based on the concept of phytoequivalence of herbs, and then to use this concept to identify the real herbal medicine from the false one and further to doquality control (Ingole, 2016). Traditionally, medicinal plants possess different secondary metabolites with an array of pharmacological or biological activities such as antimicrobial, antioxidant, antifungal, antibacterial and antiviral agents.

Phytochemicals are natural bioactive compounds found in plants to protect against diseases and parasites along with the defense system of the body (Krishnaiah, Devi, Bono & Sarbatly, 2009). It is therefore important to know the phytochemical constituents of plants as that can lead to a search for the development of new and improved therapeutic agents (Phani, Anilakumar, & Naveen., 2015). Identification of chemical compounds in plants adds more knowledge to the medicinal value of plants as reported by Wintola & Afolayan (2011).

Alysicarpus glumaceus (*A. glumaceus*) is commonly known as alyce clover, alysicarpus, buffalo clover. It belongs to the family of Leguminonae. *It* is very similar to its other species *Alysicarpus rugosus* and *Alysicarpus ovalifolius* during the vegetative stages, thus difficult to identify (CIRAD, 2010). It is a shrubby, loosely branched, creeping and ascending non-climbing annual to 1m high in the grassy savanna mostly found in Northern Nigeria, where it is locally known as "*gadagi*" in hausa and "*bundiya*" in Fulani (Burkill, 1985).

The leaf is generally used for healing by application on old wounds, burns and leprosy; respiratory diseases including nasopharyngeal infections, cough and asthma; stomach aches and protection against the evil eye. The leaf and root are used as anti-motility agents. The root is used for its anti-inflammatory (gout and edema), aphrodisiac, abortifacient and is taken orally for snake bites (Haerdi, 1964; Burkill, 1985; Bekalo, Woodmatas & Woldemariam, 2009; Pandya, 2009; Umberto, 2016). The aerial parts are used for neuropsychiatric diseases especially depression and a poly herbal preparation having *Alysicarpus ovalifolius* as the main ingredient along with other substances of abuse is commonly available as "gadagi tea" in Kano State-Nigeria for over fifty years, believed to be medicinal. It is taken for extra energy and also increases alertness (Aminu, 2017), thus a stimulant.

The aim of this work was to identify the active principles in both the methanol extract of *A. glumaceus* and its fractions using phytochemical screening methods, thin layer chromatography (TLC), fourier transform infrared (FT-IR) spectroscopy and gas chromatography and mass spectrometry (GC-MS).

MATERIALS AND METHODS

Collection and identification of the plant

The whole plant of *A. glumaceus* was collected from Turunku of Igabi Local Government Area in Kaduna State, Nigeria in the month of September 2017 and was authenticated at the Department of Biological Sciences, Herbarium Section, Ahmadu Bello University Zaria, Nigeria by comparison with an existing specimen number, 446.

Plant extraction and fractionation

Two kg of shade dried coarse aerial parts of *A. glumaceus* were obtained after being pulverized in the pestle and mortar was macerated in methanol 70% for 10 days with occasional shaking. The menstrum was filtered after collection and left in an evaporating dish at room temperature for the filtrate to concentrate to a consistent weight. The concentrated filtrate was referred to as methanol extract (ME). The ME was successively partitioned using *n*-hexane, ethyl acetate, chloroform and *n*-butanol. These were subsequently concentrated as *n*-hexane (HEX), ethyl acetate (EAF), chloroform (CCF), *n*-butanol (BUT) and residual aqueous (RA) fractions respectively. The ME and its fractions were stored in a desiccator until use.

Qualitative phytochemical screening of the methanol extract of *Alysicarpus glumaceus* and its fractions

Phytochemical screening of the methanol extract of *A. glumaceus* and its fractions was carried out in accordance with standard protocols as described by Sofowora (1993) and Trease & Evans (2004).

Thin layer chromatography (TLC)

TLC ascending technique was employed and the stationary phase was pre-coated silica gel 60 PF_{254} (0.2 mm thick) TLC plate. Spots were applied manually using capillary tubes and the plates developed at room temperature in an air tight chromatographic tank containing various developing solvent systems. The developed chromatograms were air dried and visualized under ultraviolet light (254 and 366 nm), sprayed in the fume cupboard with 10% concentrated sulphuric acid followed by heating as a detecting agent. The positions of the various phytoconstituents were marked and their retention factor (R_f) was calculated (Harbone, 1973).

Identification of phytochemicals using gas chromatography and mass spectrometer (GC-MS)

The Phytochemical constituents from ME and fractions (HEX, EAF, CFF, N-But and RAF) of *A. glumaceus* were identified using gas chromatography-GC (model: Agilent, 7890A) interfaced with mass spectrometer-MS (Model: Agilent, 5977A) which was equipped with Agilent J & W GC capillary column with HP-5MS Ultra Inert (30 m x 250 µm x 0.25 µm) and composed of (5%-phenyl)-methylpolysiloxane. An electron ionization system with ionization energy of 70 eV with Emission current 0–315 µA was used. GC-grade helium gas (99.999%) was used as the carrier gas at a flow rate of 3.6839 mL/min and an injection volume of 3 µL (split ratio of 5:1). With the injector temperature at 250°C and ion-source temperature at 280°C, the oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10°C/min, to 200°C,

then 5°C/min to 280°C (isothermal for 9 min). Mass spectra were taken at 70 eV (a scan interval of 0.5 seconds and fragments from 40 to 550 Da). The total GC running time was 650 mins. The eluted constituents were detected by a flame ionization detector and the Gas chromatogram of each plant extract was recorded.

Phytochemicals present in the 6 samples were determined based on molecular weight and molecular structure. The relative percentage of each component was calculated by comparing its average peak area to the total area. The software used to handle the mass spectra and chromatograms was MassHunter and classic MSD ChemStation. The mass spectra of the compounds present in each of the samples were matched with National Institute of Standards and Technology (NIST) library 2014.

Identification of the functional groups using Fourier-transform infrared (FT-IR) Spectroscopy

FT-IR spectrum of methanol extract of *A. glumaceus* and its fractions was recorded using an Agilent Cary 630 FT-IR spectrometer with a resolution of 8.0 cm^{-1} in the transmission mode. The prepared sample was compressed into a self-supporting pellet and introduced as a spot on a thin film slide into an IR cell in the region 4000–650 cm^{-1}. The exposure time was about 15s and 16 scans were taken, IR spectrums were automatically generated after the analysis. The functional groups present in the 6 extracts were identified based on their wavelengths.

RESULTS

Yield of the methanol extract of *Alysicarpus glumaceus* and its fractions

2 kg of the powdered aerial parts of *A. glumaceus* gave a yield of 15.78% of the methanol extract. Partitioning of 250 g of the methanol extract with solvents of varying polarity resulted into *n*-hexane (45.53 g -Dark green), chloroform (10.06 g - light green), ethyl acetate (5.29 - light brown), *n*-butanol (57.17 g - Brownish red) and residual aqueous (126.59 g - coffee brown) fractions as shown in Table 1.

Table 1. Yields of the methanol extract of *Alysicarpus glumaceus* and its fractions.

Extract	Yield (g)	Yield (%)
ME	315.63	15.78
Fractions		
HEF	45.53	18.21
EAF	10.06	4.02
CCF	5.29	2.12
NBF	57.17	22.87
RAF	126.59	50.64

ME- Methanol Extract, HEF- Hexane Fraction, EAF- Ethyl Acetate Fraction, CCF-Chloroform Fraction, NBF- *n*-butanol Fraction, RAF- Residual Aqueous Fraction

Qualitative phytochemical screening of the methanol extract of *Alysicarpus glumaceus* and its fractions

The result of the phytochemical analysis revealed the presence of alkaloids, carbohydrates, cardiac glycosides, flavonoids, saponins, steroids, tannins and terpenes but antraquinones were absent in the methanol extract of *A. glumaceus* and all its fractions. The hexane fraction was devoid of carbohydrates, flavonoids, saponins and tannins while the ethyl acetate and *n*-butanol fraction showed an absence of steroids and terpenes. Besides, he residual aqueous fraction lacked saponins and steroids (Table 2).

Thin layer chromatography

The analysis from thin layer chromatography profile of the methanol extract, *n*-hexane fraction, chloroform fraction, ethyl acetate, *n*-butanol and residual aqueous fractions revealed the presence of various phytochemicals when developed in different solvent systems of varying polarity. The R_f values of the various spots and the number of spots obtained after the development in various solvent systems are presented in Table 3.

Compounds identified in the methanol extract of *Alysicarpus glumaceus* and its fractions following GC-MS analysis

A total of 57 peaks were identified through the NIST (2014) library search of GC-MS, in methanol extract and the fractions of the plant, however some were overlapped. Thirty-eight compounds in all the extracts were identified and are listed in Tables 4-9.

Pharmacological activities of the identified compounds by GC-MS

Some of the identified compounds by the GC-MS that gave the large peak area and their pharmacological activity or activities that have been reported in the literature are all summarized in Table 10.

Functional chemical groups identified by Fourier-transform-infrared (FT-IR) spectroscopy analysis in the methanol extract of *Alysicarpus glumaceus* and its fractions

The study revealed most of the functional groups identified were in the frequency range of 3306 – 1025 cm^{-1}. These groups were almost similar for the methanol extract and its fractions. They included the saturated carbons, hydroxyl and carboxylic acid as presented in Table 11. However, the ethyl acetate fraction also had unsaturated carbons and esters identified in addition to the others.

DISCUSSION

The aerial parts of *A. glumaceus* were extracted with 70% methanol (70% methanol:30% water) yielded 15.78%, a similar yield (13.86%) of the methanol extract of *A. glumaceus* was reported by Bawa (2012). The high yield obtained in the *n*-butanol fraction and residual aqueous fraction suggests that the phytoconstituents present in the aerial parts may be more polar thus more soluble in polar solvents.

The results obtained from the qualitative phytochemical screening indicated a similarity in the profile of phytochemicals obtained from the methanol extract of *A. glumaceus* and

Table 2. Phytochemical constituents of the methanol extract of *Alysicarpus glumaceus* and its fractions.

Chemical constituents	Test	ME	HEF	EAF	CCF	NBF	RAF
Carbohydrates	Benedicts	+	-	+	+	+	+
	Fehlings	+	-	+	+	+	+
	Molisch	+	-	+	+	+	+
Alkaloids	Dragendroffs'	+	+	+	+	+	+
	Mayers	+	+	+	+	+	+
Flavonoids	Sodium hydroxide	+	-	+	+	+	+
Anthraquinones	Free anthraquinone	-	-	-	-	-	-
	Combined anthraquinone	-	-	-	-	-	-
Saponins	Frothing	+	-	+	+	+	-
Tannins	Ferric chloride	+	-	+	+	+	+
Steroids		+	+	-	+	-	-
Terpenes		+	+	-	+	-	+
Cardiac glycosides		+	+	+	+	+	+

ME- Methanol Extract, HEF- Hexane Fraction, EAF- Ethyl Acetate Fraction, CCF-Chloroform Fraction, NBF- *n*-butanol Fraction, RAF- Residual Aqueous Fraction. += Presence of constituents, - = absence of constituents

its fractions. They included cardiac glycosides, steroids, triterpenes, saponins, flavonoids, alkaloids, tannins and devoid of both free and combined anthraquinones. Although, the hexane fraction was devoid of flavonoids, saponins and tannins, the result of this study correlates to a study done by Bawa (2012) on the same species. Similar classes of phytochemicals have also been reported to be present in another member of the genus, *Alysicarpus ovalifolius* and other members of the genus (Bashir, Uzair, & Bashir., 2018). Furthermore, alkaloids, saponins and tripenes have been previously isolated from the genus, Alysicarpus (Allen & Allen, 1981).

Phytochemicals have been reported to have many nutritive, biological and therapeutic properties (Benedec *et al*., 2013). They serve as useful taxonomic markers in identifying particular species as well as to distinguish it from related species, hence are helpful in the delimitation of taxa (Jonathan & Tom, 2008; Du *et al*., 2014). Phytochemicals are sources of energy required for a number of other physiological processes in humans (Hoffman, Friedmann, Saltman & Polich., 1999; Dingman, 2002). Flavonoids, saponins and tannins have been found to possess appreciable anti-inflammatory and central nervous system (CNS) action (Jäger & Saaby, 2011; Kauri & Arora, 2015). Flavonoids and neuroactive steroids were found to be ligands for gamma-aminobutyric acid (GABA) receptors in the CNS (SajidBijan, Zamiul, Mominul & Ekramul, ., 2013). Tannins have been used to tan animal hides and some isolated from certain plants have been shown to possess antidepressant and antihemorrhagic activities (Pemminati *et al*., 2010). Flavonoids, terpeniods and tannins possess antioxidant activity (Dutta, 2013; Phani *et al*., 2015). Triterpeniods have been reported to possess a wide range of neuropharmacological activities like anxiolytic, sedative, hypnotic, antidepressant and antinoceptive (Scott, Wright & Angus 2004; Morris,Dawson, Reynolds, Atack & Stephens ., 2006; Parmar *et al*., 2013). Alkaloids, flavonoids, terpenoids, tannins and saponins also give neuroprotection (Phani *et al*., 2015). Alkaloids and flavonoids have immunomodulatory activity (Middleton, Kandaswami & Theoharides 2000; Horrigan,Kelly & Connor 2006; Lantz, Chen, Sarihan, Sólyom, Jolad & Timmermann., 2007; Kure,Timmer & Stough ., 2017).

TLC profiling of the methanol extract of *A. glumaceus* and its fractions showed that there were a number of spots implying the presence of secondary metabolites. Various R_f values of the compounds gives an idea about their polarity that may also help in selecting a particular solvent system for further isolation of any compound from the plant extracts using chromatographic and spectroscopic techniques (Biradar & Rachetti, 2013). Compounds with high R_f value in less polar solvent system have low polarity while those with a low R_f value have high polarity (Talukdar, Choudhury, Chakraborty & Dutta (2010). In relation to other chromatographic methods, TLC offers the simplest and cheapest means of detecting natural product constituents.

GC-MS technique is used for the identification and quantification of compounds (Aneesh, Thomas, Thomas & Anandan., 2013; Senthil, Rameashkannan & Mani ., 2016). The 3, 7, 28, 6, 6 and 7 peaks were seen in the GC-MS chromatograms of methanol, hexane fraction, ethyl acetate fraction, chloroform fraction, *n*-butanol fraction and residual aqueous fraction, respectively. Fifteen out of the 38 different compounds that were identified in all the 6 extracts of *A. glumaceus* were fatty acids, thus indicating that they are the major constituents determined from the GC-MS analysis. Hexane and chloroform fractions had most fatty acids and occupied (match >90%) most of the total peak area while other extracts had less than 50%. These included both unsaturated (essential fatty acid 9, 12-octadecadienoic acid, methyl ester; [1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylic acid, 4,7-dihydro-7-imino-, ethyl ester; 9, 12, 15-octadecadienoic acid, methyl ester (Z,Z,Z)-; etc) and saturated fatty acids (hexadecanoic acid; pentanoic acid, heptyl ester; methyl stearate; pentadecanoic acid, 2,6,10,14-tetramethyl- methyl ester).

In addition, other classes of compounds like lactones, ethyl ester alkaloids, amide, ketones and unsaturated alcohols were also identified by the GC-MS.

Fatty acids are known to be responsible for many important physiological processes. They provide energy to the cell and act as substrates in the synthesis of fats, lipoproteins, liposac-

Table 3. Thin layer chromatography of the methanol extract of *Alysicarpus glumaceus* and its fractions.

Sample	Solvent system	No of Spots	R_f
Plate I & III			
ME	CF: EA		
	1:2	0	–
	2:1	3	0.89, 0.684, 0.937
CCF	CF: EA		
	1:2	7	0.073, 0.293, 0.489, 0.013, 0.756, 0.793, 0.866.
	2:1	7	0.165, 0.316, 0.379, 0.506, 0.684, 0.873, 0.949
EAF	CF: EA	2	0.013, 0.073
	1:2	2	0.063, 0.189
Plate II			
EAF			
	2:1	6	0.069, 0.139, 0.205, 0.625, 0.819, 0.889
	EA: CF: M: W		
	15:8:4:1		
RAF	EA: CF: M: W		
	15:8:4:1	0	–
Plate IV			
ME	Hexane : EA		
	3:2	3	0.571, 0.596, 0.974
HEX	Hexane : EA	6	0.532, 0.714, 0.819, 0.870, 0.909,
	3:2		0.974
CCF	Hexane : EA		
	3:2	7	0.104, 0.338, 0.530, 0.714, 0.819, 0.780, 0.909
Plate V	CF: Toluene		
HEX	90:10	5	0.063, 0.203, 0.281,0.438, 0.75
CCF	90:10	3	0.063, 0.438, 0.75
EAF	90:10	4	0.063, 0.203, 0.75
NBF	90:10	3	0.063, 0.203, 0.75
RAF	90:10	1	0.0469
Plate VI	CF: AA: M: W		
sNBF	64:32:12:8	4	0.246, 0.344, 0.557, 0.623
RAF	64:32:12:8	6	0.0492, 0.082, 0.328, 0.459, 0.557, 0.623

AA-Acetic Acid, CCF-Chloroform Fraction, CF-Chloroform, EA- Ethyl Acetate, EAF- Ethyl Acetate Fraction, HEF- Hexane Fraction, M-Methanol, ME- Methanol Extract, NBF- *n*-butanol Fraction, RAF- Residual Aqueous Fraction, R_f- Retention Factor, W-Water.

Table 4. Compounds identified in the methanol extract of *Alysicarpus glumaceus* following GC-MS analysis.

PK	RT	Peak Area	Library/ID/Class of the compound
1	5.3809	1.0341	2-Pentanone, 4-hydroxy-4-methyl- (KETONE)
2	55.9738	0.102	11,14,17-Eicosatrienoic acid, methyl ester (ESTER)
3	95.4662	98.8639	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15 hexadecamethyl- (LACTONE)

PK-Peak, RT-Retention Time, ID-Identity

Table 5. Compounds identified in the hexane fraction of *Alysicarpus glumaceus* following GC-MS analysis.

PK	RT	Peak Area	Library/ID/Class of the compound
1	6.7732	0.0977	Propanedinitrile, methylene- (NITRILE)
2	47.2548	0.1058	1,4-Cyclohexanediol, trans- (ALICYCLIC ALCOHOL)
3	50.149	1.3733	Hexadecanoic acid, methyl ester (FATTY ACID ESTERS)
4	55.7908	0.958	9,12-Octadecadienoic acid, methyl ester (FATTY ACID ESTERS)
5	56.0106	2.7726	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (FATTY ACID ESTERS)
6	56.8898	0.5085	Methyl stearate (FATTY ACID ESTERS)
7	98.5071	94.1841	[1,2,4]Triazolo[1,5-a]pyrimidine-6-carboxylic acid, 4,7-dihydro-7-imino-, ethyl ester (FATTY ACID ESTERS)

PK-Peak, RT-Retention Time, ID-Identity

Table 6. Compounds identified in the ethyl acetate fraction of *Alysicarpus glumaceus* following GC-MS analysis.

PK	RT	Peak Area	Library/ID/Class of the compound
1	5.3809	5.0383	2-Pentanone, 4-hydroxy-4-methyl- (KETONE)
2	10.5098	0.451	Methanamine, N-methoxy- (AMINE)
3	15.712	0.4783	Cyclopropane, 1,1-dimethyl- (ALICYCLIC HC)
4	35.0552	0.0632	2-(1,2,3,4-Tetrazol-1-yl)acetonitrile (NITRILE)
5	37.3998	0.0896	3-Methyl-4-amino-5-methylamino-1,2,4-triazole(AROMATIC HETEROCYCLE)
6	41.1732	0.3714	(SR)- or (RS)-4-methyl-2,3-pentanediol (ALCOHOL)
7	42.8218	0.1124	Propanoic acid (ORGANIC ACID)
8	44.8734	0.2417	4-Methyl-1,4-heptadiene (ALKENE)
9	45.8991	0.1319	1,2:4,5:9,10-Triepoxydecane (EPOXIDE)
10	46.5586	0.1532	1-Propanol, 2-methyl- (ALCOHOL)
11	47.0348	0.609	1-Methoxy-3-(2-hydroxyethyl) nonane (ALKANE)
12	47.2546	0.8256	Oxirane, [(dodecyloxy)methyl]- (OXIRANE)
13	47.9141	0.0973	2-Nonen-1-ol (UNSATURATED ALCOHOL)
14	48.0973	0.2592	Cyclopentyl-methyl-phosphinic acid, 2-isopropyl-5-methyl-cyclohexyl ester (ESTER)
15	48.5369	0.2575	1-Octadecyne (ALKYNE)
16	48.83	0.1028	Propanoic acid (ORGANIC ACID)
17	49.3428	0.2902	1,6-Octadiene, 5,7-dimethyl-, (R)- (ALKENE)
18	49.7458	0.1352	2-Octylcyclopropene-1-heptanol (ALCOHOL)
19	50.1488	8.2493	Hexadecanoic acid, methyl ester (FATTY ACID ESTER)
20	50.9548	0.0757	2-Pentyn-1-ol (UNSATURATED ALCOHOL)
21	51.431	3.4969	Dibutyl phthalate (ESTER)
22	52.4202	0.5035	2-Dodecanol (ALCOHOL)
23	53.5559	0.3231	13-Tetradecynoic acid, methyl ester (FATTY ACID ESTER)
24	55.7906	5.4353	9,12-Octadecadienoic acid, methyl ester (FATTY ACID ESTER)
25	56.047	15.079	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (FATTY ACID ESTER)
26	56.4867	0.4996	Undec-10-ynoic acid, undecyl ester (FATTY ACID ESTER)
27	58.9412	53.9042	cis-Vaccenic acid (ORGANIC ACID)
28	67.4039	2.7255	9,17-Octadecadienal, (Z)- (ALDEHYDE)

PK-Peak, RT-Retention Time, ID-Identity

Table 7. Compounds identified in the chloroform fraction of *Alysicarpus glumaceus* following GC-MS analysis.

PK	RT	Peak Area	Library/ID /Class of the compound
1	6.7731	0.1402	Pentanoic acid, heptyl ester (FATTY ACID ESTERS)
2	50.1488	1.0016	Hexadecanoic acid, methyl ester (FATTY ACID ESTERS)
3	55.7906	0.453	9,12-Octadecadienoic acid, methyl ester (FATTY ACID ESTERS)
4	56.0104	1.4074	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (FATTY ACID ESTERS)
5	56.853	0.3809	Methyl stearate (FATTY ACID ESTERS)
6	97.0415	96.6169	3-Quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy-, ethyl ester (FATTY ACID ESTERS)

PK-Peak, RT-Retention Time, ID-Identity

Table 8. Compounds identified in the *n*-butanol fraction of *Alysicarpus glumaceus* following GC-MS analysis.

PK	RT	Peak Area	Library/ID/Class of the compound
1	5.3811	1.3888	2-Pentanone, 4-hydroxy-4-methyl- (KETONE)
2	50.149	0.134	Hexadecanoic acid, methyl ester (FATTY ACID ESTER)
3	56.0106	0.3103	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (FATTY ACID ESTER)
4	98.8368	98.1669	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (ETHER)

PK-Peak, RT-Retention Time, ID-Identity

Table 9. Compounds identified in the residual aqueous fraction of *Alysicarpus glumaceus* following GC-MS analysis.

PK	RT	Area Pct	Library/ID/ Class of the compound
1	11.2425	0.1503	Methanamine, N-methoxy- (AMINE)
2	42.4921	0.2933	Propanamide, 2-methyl- (AMIDE)
3	52.4935	0.1174	Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester (ESTER)
4	60.773	4.409	Palmitoleic acid (FATTY ACID)
5	87.9194	95.03	1-methyl-4-phenyl-5-thioxo-1,2,4-triazolidin-3-one (KETONE)

PK-Peak, RT-Retention Time, ID-Identity

Table 10. Some of the chemical compounds identified from the methanol extract of *Alysicarpus glumaceus* and its fractions by GC-MS and their reported pharmacological activity.

S/No	Compound	Derivatives	Pharmacological activity
1.	[1,2,4]Triazolo[1,5-a]pyrimidine-6-carboxylic acid, 4,7-dihydro-7-imino-, ethyl ester	Fatty acid esters	Anti-inflammatory, analgesic, precursors for other drug molecules with activities like anticancer, anxiolytic, antioxidant, anti-Parkisons', antibacterial agents and antidepressants. (Rajeev, Prabodh & Mohammed. 2011; Aly, Hassan, Makhlouf, & Bräse, 2020),
2.	Hexadecanoic acid	methyl ester Fatty acid	Anti-inflammatory, antioxidant, inhibit production of uric acid activity, urine acidifier, mainly used to produce soaps, cosmetics, release agents, antifibrinolytic, heamolytic, lubricant, nematicide, antialopecic and antidepressant. (Rahman, Ahmad, Mohamed, & Ab Rahman, 2014)
3.	9,12-Octadecadienoic acid	methyl ester Fatty acid	Hepatoprotective,antihistaminic, hypocholesterolemic, antieczemic, antioxidant and anticancer properties (Yu., *et al,* 2005; Akpuaka, Ekwenchi, Dashak, & Dildar, 2013)
4.	3-Quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy	ethyl ester Alkaloid	Anti-inflammatory, inhibit production of uric acid activity, urine acidifier, increae aromatic amino acid decarboxylase acitivity, 17-beta-hydroxysteriod dehydrogenase inhibitor, testosterone-hydoxylase inducer,
5.	Cis-Vaccenic Acid	Fatty Acid	Anticancer and antimicrobial properties (Lim, *et al.,* 2013; Hamazakic *et al.,* 2016)
6.	Dibutyl phthalate	Fatty acid	Antimicrobial, antifouling, solvent for perfumes, nitrocellulose and cellulose acetate, alcohol denaturant (toilet preparations), nail polish, as a fixative in perfumes and in fingernail elongators as a plasticizer, ectoparasitic agent. (Senthil, Rameashkannan & Mani., 2016; Ingole, 2016)

Table 10. Continue.

S/No	Compound	Derivatives	Pharmacological activity
7.	Palmitoleic acid	Fatty acid	anti-thrombotic effects, lowers LDL cholesterol and higher high-density lipoprotein cholesterol, has beneficial effects on insulin sensitivity, cholesterol metabolism, and hemostasis. (Morgan & Dhayal, 2010)
8.	Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester	Fatty acid ester	saturated fatty acid, flavoring agent (Smedman, Gustafsson, Berglund, & Vessby 1999; Jost, 2002; Aneesh, Thomas, Thomas, & Anandan, 2013)
9.	Propranoic Acid	Organic Acid	Anti-inflammator(Prostaglandin Inhibitor (present in NSAIDS-Ibuprofen), anti-asthmatic drugs, preservative and as an anti-microbial agent in foods produced for human and livestock consumption. (Dracheva *et al.,* 2009; Flavouring agent, antifungal agent (Lim, Wong, Rosli, Ng, Seow, & Chong, 2009)
10.	2-Dodecanol	Alcohol	Antimicrobial (Falowo, Muchenje, Hugo, Aiyegoro. & Fayemi., 2017)
11.	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15 hexadecamethyl-	Lactone	

Table 11. Functional groups identified by Fourier-Transform-Infrared FT-IR spectroscopy analysis of the methanol extract of *Alysicarpus glumaceus* and its fractions.

Sample/Fraction	I.R Frequency(cm^{-1})	Assignment	Inference
Methanol	3306	O – H Str	- Saturated carbons
	2922	-C – H Str (asym)	- Alcohol
	2855	-C – H Str (sym)	- Carboxylic acids
	1718	-C = O	
	1036	-C – O	
n-Hexane	3354	O – H Str	- Saturated carbons
	2922	-C – H Str (asym)	- Alcohol
	2855	-C – H Str (sym)	- Carboxylic acids
	1710	-C = O	
	1039	-C – O	
Chloroform	3339	O – H Str	- Saturated carbons
	2922	-C – H Str (asym)	- Alcohol
	2855	-C – H Str (sym)	- Carboxylic acids
	1718	-C = O Str	
	1036	-C – O Str	
Ethyl acetate	3343	O – H Str	-Saturated and Unsaturated carbons
	3011	=C – H Str	
	2922	-C – H Str (asym)	-Carboxylic acid
	2855	-C – H Str (sym)	-Alcohols
	1710	-C = O Str	-Esters
	1073 – 1170	O - C – O Str	
Residual aqueous	3261	O – H Str	- Saturated carbons
	2926	-C – H Str	- Alcohol
	1580	-C = O Str	- Carboxylic acids
	1036	-C – O Str	
Butanol	3272	O – H Str	- Saturated carbons
	2929	-C – H Str	- Alcohol
	1707	-C = O Str	- Carboxylic acids
	1025	-C – O Str	

IR- Infar Red, Str-Stretching, sym-Symertic, asym- Asymertic

charides and eicosanoids (Ansorena, Gimeno, Astiasarán, & Bello., 2001). They aid in the of modulation of enzymes activities (Zamaria, 2004), receptor site (Facchini, 2001), as well as neurotransmission, cell survival and signaling pathways, which ultimately affects mood and cognition (Parekh, Smeeth, Milner & Thure2017; Fernandes,Mutch, & Leri ., 2017). Other fatty acids found in *A. glumaceus* with their reported pharmacological activities includes the following: essential fatty acid 9, 12-Octadecadienoic acid, methyl ester (Saradha & Paulsamy, 2013; Aneesh,Thomas, Thomas, & Anandan, 2013; Venkatesh, Vidya,& Kalaivani ,2014); dibutyl phthalate, hexadecanoic acid (Easwaran & Ramani, 2014; Senthil, Rameashkannan & Mani., 2016; Abbasi-Maleki &Mousavi, 2017) Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-; (Kumaradevan, Damodaran, Mani, Dineshkumar & Jayaseelan.,2015), Pentadecanoic acid (Smedman, Gustafsson, Berglund, & Vessby ., 1999; Jost, 2002, Aneesh, Thomas, Thomas, & Anandan, 2013)), Palmitoleic acid (Abraham, Riemersma, Wood, Elton, & Oliver, , 1989; Morgan & Dhayal, 2010; Mozaffarian *et al*., 2010) and [1,2,4] Triazolo[1,5-a] pyrimidine-6-carboxylic acid, 4,7-dihydro-7-imino-, ethyl ester (Rajeev, Prabodh & Mohammed., 2011).

The FT-IR data obtained for all the extracts indicated the presence of the carbonyl (C=O), alcohol and carboxylic acid (O-H), and aliphatic (-C-H) functional groups, as evident by the appearance of prominent bands observed at 1707-1718 cm^{-1} (C=O stretching vibrations), 1025-1039 cm^{-1} (C-O stretching vibrations), 3261–3354 cm^{-1} (O – H stretching vibrations) and 2990 cm^{-1} (Aliphatic Csp3-H stretching vibrations) for both the methanol and its fractions. Additionally, the ethyl acetate fractions showed peak characteristics of aromatic (=C-H)/olefinic (=C-H) functional group at 3011 cm^{-1} (aromatic Csp2-H stretching vibrations), although the presence of aromatic function in the other fractions could not be ruled out due to the broad O-H stretching peak which might be overlapping with it and also the presence of a peak around 1500 cm^{-1} (C=C stretching vibrations) (Crews, Rodriquez, Jaspars & Crews 1998; Pavia, Lampman, Kriz & Engel ., 2011) for all the extracts. These functional groups or pharmacophores are usually the ones responsible for the therapeutic action and similar pharmacophores found in the extracts have been reported in tricyclic antidepressants (Sagdinc, Azkeskin, & Eşme., 2018). The fact that there was no absorbance observed in the region 2220-2260 cm^{-1} indicated that there was no cyanide group which is a toxicophore and suggested that the plant can be considered as safe (Ragavendran, Sophia, Raj & Gopalakrishnan., 2011; Abdoun, Hassan, Gaber, & El- Sharekh., 2019).

These spectral data obtained from the extracts conformed with the qualitative phytochemical screening revealed the presence of flavonoids, saponins, tannins, steroids and cardiac glycosides in all the fractions except for hexane fraction which was devoid of flavonoids and saponins. The functional groups identified (alcohols, acids and ketones) are characteristics of these phytochemicals (Pavia, Lampman, Kriz & Engel., 2011). These identified functional groups further confirm the presence of the secondary metabolites in the methanol extract of *A. glumaceus* and its fractions.

In addition, the result from FT-IR spectrum correlate to the chromatograms obtained from GC-MS analysis of the methanol extract of *A. glumaceus* and its fractions where the most significant fragments (compounds) identified were found to be fatty acids, alcohols, ketones, and esters.

Further study is needed to isolate and characterize the specific active principle(s) responsible for the biological activity of the plant. Additional investigations are ongoing to evaluate its pharmacological activities and to elucidate the probable mechanism(s) of some of the activities including antidepressant, anxiolytic, analgesic and anti-inflammatory.

CONCLUSION

A. glumaceus contains several classes of phytoconstituents such as alkaloids, flavonoids, tannins, saponins, terpenes, steroids and cardiac glycosides. It also contains many compounds whose biological activities have been previously reported and this may provide a scientific rationale for various folkloric claims of the plant.

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Original Article

Quantitative determinations on commercial samples of Melissae folium and their antioxidant activity

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ABSTRACT

Background and Aims: *Melissa officinalis* L. (lemon balm) is a perennial herb. Melissae folium and their preparations have been used for their sedative, spasmolytic and antibacterial actions. The study was aimed to investigate the qualities and also to compare the antioxidant activity potentials of the drug samples available in herbal markets and pharmacies in Turkey
Methods: The percentages of the loss on drying and total ash were determined by gravimetric method and the percentage of total hydroxycinnamic derivatives was calculated by a spectrophotometric method according to European Pharmacopoeia. Drug samples were investigated for their potentials to scavenge the DPPH radical by using an *in vitro* method.
Results: The percentages of the loss on drying were found to be between 8.51-16.53 %; whereas total ash amounts were determined between 9.41-11.33%. The percentage of total hydroxycinnamic derivatives was found in the range of 4.45-12.97 %. The extracts of the samples were found to have DPPH radical scavenging activity with EC_{50} values ranging from 10.60 to 19.10 µg/ml.
Conclusion: In the assays for total ash and quantification of total hydroxy cinnamic derivatives all of the examined commercial samples were found to be compatible with standards in European Pharmacopoeia. Among the tested samples; a sample sold in pharmacy seems to have the best quality when its compared with the standards in European Pharmacopoeia.

Keywords: Melissae folium, European Pharmacopoeia, quality control analysis

INTRODUCTION

Melissa officinalis L., commonly known as lemon balm, is a perennial herb belonging to the Lamiaceae family. Preparations, which are introduced in folk medicine as infusion from dried *M. officinalis* leaves, are recommended against colds and are used in functional disorders of the circulation. Preparations of lemon balm have been used for their sedative, spasmolytic and antibacterial actions. They are, therefore, employed for gastrointestinal disorders of nervous origin, in psychosomatic cardiac disorders and against migraine (Wichtl & Bisset, 1994). The Lamiaceae are a promising source of natural antioxidants due to the large amount of phenolic acids found in many species of this family (Weitzel & Petersen, 2011; Barros et al., 2013). Rosmarinic acid (a hydroxycinnamic derivative), which is one of the main secondary metabolites (phenolic acids) in the leaves, is potent antioxidant. The rosmarinic acid is considered an analytical marker for *M. officinalis* (Petersen & Simmonds, 2003). In the monograph of European Pharmacopoeia 6th edition, the percentage of total hydroxycinnamic derivatives of the herbal drug is expressed as rosmarinic acid.

The purpose of this study is to compare some quality control parameters of the commercial samples sold in the pharmacy and herbal market in Turkey with respect to the methods available in European Pharmacopoeia. In this context, assays for loss on drying and total ash were carried out by the gravimetric method. The content of total hydoxycinnamic derivatives were determined (expressed as rosmarinic acid)

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by using a spectrophotometric method (European Pharmacopoeia 6th edition; Arnow, 1937; Vladimir-Knežević et al. 2011). Moreover, the antioxidant activity of the samples were examined by using the DPPH method, which is a widely used and simple method employed for the determination of antioxidant activity (Brand-Williams, Cuvelier & Berset 1995; Choi et al., 2002).

MATERIALS AND METHODS

Materials

DPPH (2,2-diphenyl-2-picrylhydrazyl) reagent and methanol were purchased from Sigma- Aldrich (Germany). All other reagents and solvents used were of analytical grade.

Sample Preparation

Dried commercial samples were purchased from herbal market and pharmacies in Turkey. A and D samples of Melissae folium were obtained from two different herbal markets; B and C samples were obtained from two different pharmacies. The drug specimens were finely powdered. Methanolic and ethanolic extracts of Melissae folium were used in the studies. For methanolic extract preparation; the pulverized sample was weighed (2 g) and extracted with methanol (20 mL) in an ultrasonic bath for 30 min, three times. The extraction was followed by filtration and the filtrate was evaporated by using a rotary evaporator (Choi et al 2002).

An ethanolic extract was prepared according to European Pharmacopoeia 6th edition. The powdered plant material (0.20 g) was extracted with 50% ethanol (190 mL) under a reflux condenser in a boiling water bath for 30 min. The cooled extract was filtered, the filter rinsed with ethanol, and then the filtrate and rinsing solution was combined and diluted to 200.0 mL with 50% ethanol.

Procedures recorded in the European Pharmacopoeia 6th edition were used to determine the amounts of total hydroxycinnamic derivatives, found in samples. The assays for loss on drying and total ash were performed according to European Pharmacopoeia 8th edition. All the experiments were performed in triplicate.

Quantitative determination of total hydroxycinnamic derivatives, expressed as rosmarinic acid

Determination of hydroxycinnamic acid derivatives was performed according to the procedure described in European Pharmacopoeia 6th edition. Briefly, an aliquot of the ethanolic extract (1.0 mL) was mixed with 0.5 M hydrochloric acid (2 mL), Arnow reagent (10% aqueous solution of sodium nitrite and sodium molybdate, 2 mL) and 8.5% sodium hydroxide (2 mL) and diluted to 10.0 mL with water. The absorbance of the test solution was measured immediately at 505nm against blank. The content of total hydroxycinnamic derivatives was calculated and expressed as rosmarinic acid, according to the following expression: (%)=A×5/m, where A is the absorbance of the test solution at 505 nm and m is the mass of the sample, in grams. (European Pharmacopoeia 6th edition; Vladimir-Knežević et al., 2011)

DPPH radical scavenging activity

The samples were extracted with methanol and analyzed for antioxidant activity by DPPH (2,2-diphenyl-2-picrylhydrazyl) method (Brand-Williams et al., 1995; Choi et al., 2002).

The free radical scavenging activities of the samples were measured using the stable DPPH radical, according to the method of Brand Williams. Briefly, 0.3 mM solution of DPPH in methanol was prepared and this solution (1 mL) was added to sample solution in methanol at different concentrations (2.5–100 µg/mL). The mixture was allowed to stand for 30 min in the dark, and the absorbance was then measured at 517 nm.

The capability to scavenge the DPPH radical (EC%) was calculated using the following equation: $EC\%=100-[Abs_{sample}- Abs_{blank}) x100/Abs_{cont}$), where Abs_{sample} is the absorbance obtained in the presence of the different extract concentrations and Abs_{blank} is that obtained in the absence of extracts. A methanol plus plant extract mixture was used as a blank. All the determinations were done in triplicate. Ascorbic acid was used as a positive control. The results are presented as mean±SD. EC_{50} correlation analysis was carried out with GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA.

RESULTS AND DISCUSSION

In the context of quality control experiments, the loss on drying and total ash contents of the specimens were determined. The amounts of loss on drying were found to be between 8.51-16.53% and the total ash contents were found to be between 9.41-11.33% (Table 1). In the European Pharmacopoeia 8th the limit for loss on drying was 10% and for total ash was 12%.

The total ash contents of all the samples were compatible with the standard values in the monograph, however the content of loss on drying was higher than the limit value in three samples. This might be due to the storage conditions of the samples.

The content of total hydroxycinnamic derivatives was determined by a spectrophotometric method using the Arnow reagent (Vladimir-Knežević et al., 2011). The results are expressed as rosmarinic acid. In European Pharmacopoeia 6th edition, it is indicated that pharmacopeial grade Melissae folium contains at least 4% total hydroxycinnamic acid derivatives expressed as rosmarinic acid. In our study, the range of total hydroxycinnamic acid derivatives was found between 4.45-12.97% (Table 1) and the results were compared with the results of the previous published data.

Carnat, Carnat, Fraisse & Lamaison, (1998), reported that, the total hydroxycinnamic acid content was determined by a spectrophotometric method with the Arnow reagent and as a result total hydroxycinnamic acids based on the dry weight of the leaf were found as 11.29%.

In a study by Aprotosoaie, Raileanu, Trifan, & Cioanca, (2013) total hydroxycinnamic acids expressed as g rosmarinic acid / 100 g dry weight in *Melissa officinalis* sample were found to be 4.15% using a spectrophotometric method.

Table 1. Quantitative determinations on lemon balm and its radical scavenging activity.

Sample code	Loss on drying % (±SD)	Total ash % (±SD)	Total hydroxycinnamic derivatives % (±SD)	Radical scavenging activity (EC_{50} µg/ml)* (±SD)
A	**12.08 (0.16)**	9.41 (0.10)	4.45 (0.09)	18.75 (3.46)
B	8.51 (0.09)	11.31(0.03)	8.85 (0.56)	17.60 (2.81)
C	**16.53 (0.29)**	10.05 (0.09)	12.97 (0.70)	10.60 (3.32)
D	**11.22 (0.17)**	11.33 (0.11)	4.97 (0.40)	19.10 (0.40)

All of the analysis were performed in triplicate. Loss on drying, total ash and total hydroxycinnamic derivatives are based on the dry weight of the leaf.
*EC_{50} means the effective concentration providing 50% effect. Concentration µg of dried Melissae folium extract/ml (final concentration)

In another study, the contents of hydroxycinnamic acid derivatives of *Melissa* samples were determined according to the assay methods instructions of the European Pharmacopeia 2008. The determined values ranged between 7.4 and 15.5% (Krüger, Schütze, Lohwasser & Marthe, 2010, Kittler et al., 2018).

For antioxidant activity, the methanolic extracts were analyzed by the DPPH method. The methanolic extracts of the samples were found to have DPPH radical scavenging activity with EC_{50} values ranging from 10.60 to 19.10 µg/mL (Table 1).

In the present study, the methanolic extract of Melissae folium showed a potent effect on scavenging the DPPH radical with a EC_{50} value similar to the results of previous studies on methanolic extracts of *M. officinalis* such as 13.74 µg/mL (López et al., 2007) and 24.3 µg/mL (Pereira et al., 2009)

Compatible results were obtained in antioxidant activity determinations on different extracts obtained from *Melissae officinalis* by using the DPPH method. In one study, an EC_{50} value of 9.76 dried sample mg/ml was calculated for the aqueous methanol (80%) extract of the plant (Karadağ, 2019).

In another study, the EC_{50} value for Melissae folium aqueous ethanol extract (70%) was calculated as 65.1 µg/mL (Benedec et al., 2015), while the EC_{50} value for aqueous ethanol extract (70%) was 512 mg trolox equivalent (TE)/g dw (dried weight) (Franco, Pugine, Scatoline, & Melo, 2018).

Low EC_{50} values indicate higher radical scavenging activity and therefore higher antioxidant activity. Phenolic acids are generally responsible for antioxidant activity. In the assay, for antioxidant activity, ascorbic acid was used as a standard (EC_{50} 3.31 µg/mL).

CONCLUSIONS

To the best of our knowledge, this is the first quality control study on commercial samples of Melissa folium sold in Turkey, revealing the quality of *Melissa officinalis* grown in our country and the herbal drug Melissae folium (its effective compounds, its antioxidant activity potential and quality properties such as moisture, ash). In the assays for total ash and quantitative determination of total hydroxy cinnamic acid derivatives, all of the examined commercial samples were found to be compatible with standards in the European Pharmacopoeia 6th and 8th. In contrast, the moisture contents of the samples were found to be higher than the values recorded in the European Pharmacopoeia 8th except one of the samples examined. This finding indicates that the sample was either not well dried or later absorbed moisture during packaging and transportation. Therefore, this study also pointed out that attention should be paid to moisture in the preparation and storage of herbal drugs.

Among the tested samples, sample B (a sample sold in pharmacy) seems to have the best quality with regard to the standards in the European Pharmacopoeia 6th and 8th .

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Original Article

Evaluation of herbal medicine use in the obstetric and gynecology department

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ABSTRACT

Background and Aims: Pregnancy is a sensitive period for all synthetic and herbal treatment options. This study aims to classify the herbal medicines used during pregnancy and to determine their usage rates, side effects, and risks, according to safety conditions.
Methods: This study was conducted using survey questions directed to pregnant women receiving treatment in the Obstetrics and Gynecology Department.
Results: 194 people participated in the study. 63 people (32.4%) used 31 different herbal medicines during pregnancy. Commonly used medicines were: *Mentha* sp. - *Citrus limonum, Tilia* sp. and *Matricaria chamomilla*. Herbal medicines were classified in four groups according to their effects, risk and safety during pregnancy. Accordingly, nine herbal medicines were classified as potentially harmful for use in pregnancy at a usage rate of 20.43%. These are mainly *Jasminum* sp. and *Foeniculum vulgare.*
Conclusion: The findings of this study showed that the use of herbal medicines is common during pregnancy and that potentially harmful herbal medicines are used at high rates during this time. Patients get information about herbal medicines used during pregnancy from unreliable sources. Health care providers should have information about the potential benefits/harms of herbal medicines when used during pregnancy.

Keywords: Herbal medicine, pregnancy, Obstetrics and Gynecology, safety

INTRODUCTION

The history of treating health issues with herbs is as old as human history. Despite the development of new treatment methods today, people are looking for different options when it comes to health care, due to the influence of developing communication networks and various environmental factors. These alternative options mostly include herbal medicines, which the public considers natural and harmless. Declining confidence in synthetic drugs, the idea that synthetic drugs are harmful and that medicines obtained from plants are completely harmless, and the media offering unreliable information are the main reasons for the increasing use of herbal medicines (Mosihuzzaman & Choudhary, 2008). However, due to the chemicals they contain, plants can affect many systems in the body, changing biochemical events and causing undesirable results (Dülger, 2012; Patel & Gohil, 2007; Skalli, Zaid, & Soulaymani, 2007).

While pregnant women are careful about the potential risks of conventional drug consumption, they have little information about the harmful effects of herbal medicines. Information on the potential harm of many herbal medicines in pregnancy is

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limited. Some herbal medicines are teratogenic in human and animal models, so pregnant women are at risk from the use of herbal medicine. Based on this information, it is recommended to avoid herbal medicines during pregnancy (Ernst, 2002; Tabatabaee, 2011).

Herbal medicines are any plant-derived medicines taken in any form, as a preventive or a healing treatment (e.g. leaves, roots, flowers) (Muñoz Balbontín, Stewart, Shetty, Fitton, & McLay, 2019). Herbal medicines can come in the form of raw drugs, teas and pharmaceutical formulation medicines obtained from plants, according to the WHO (World Health Organization, 2004). Although herbal medicines have been used by people in Asia, Africa and Latin America for centuries, it has been noted that their use has also increased in the Western world in recent years (Koren, Randor, Martin, & Danneman, 1990).

Women are cited as the main users of herbal medicines for both health protection and the treatment of disease (Eardley et al., 2012; Hunt et al., 2010). This widespread use continues during pregnancy. In Africa, Australia, Europe, England and the United States, 10% to 74% of pregnant women are reported to be using herbal medicines (Adams et al., 2009; Kennedy, Lupattelli, Koren, & Nordeng, 2016; Laelago, Yohannes, & Lemango, 2016). Such medicines are widely used as a method of treatment during pregnancy. In a study in which the use of herbal medicines was found to be 89% in 2,673 pregnant women, it was stated that 126 herbal medicines were used. According to the risk groups, 27 of these 126 plants used during pregnancy are classified as contraindicated, 28 are safe, and the rest should be used carefully during pregnancy (Kennedy et al., 2016). In a study conducted in a hospital in Iran, it was reported that 30.8% of 513 pregnant women used herbal medicine. Most herbal medicine use occurs in the first trimester. It was found that 87.3% of herbal medicine recommendations were made by the family. Based on these results, it was emphasized that doctors should be concerned about the use of herbal medicines in pregnant women and the possible side effects and harm of these uses (Tabatabaee, 2011).

In a study evaluating the use of herbal medicines among pregnant women in Turkey, it was found that 47.3% of 366 pregnant women used at least one herbal medicine. Linden, mint-lemon and ginger are the first three plants used in the treatment of colds and influenza. More than half of pregnant women started using herbal medicines without consulting anyone, and the rate of consultation with health care professionals was low. Herbal medicines are frequently used in the treatment of nausea, vomiting, constipation, pain control and colds, but there is insufficient data on their effects on the health of the mother and baby during pregnancy (Kıssal, Çevik Güner, & Batkın Ertürk, 2017).

An increase in the use of herbal medicines has been observed all over the world. The use of herbal medicines is especially of concern in pregnant women due to safety and lack of adequate data. Although the current data does not support herbal use during pregnancy, the use of such medicines in pregnant women is present at varying frequencies. There are many underlying reasons for choosing these medicines, such as lack of knowledge, difference in level of education, widespread advertisements in the media, and lack of information about the subject by health professionals.

There are few studies in Turkey on herbal medicine use rates, safety data and other issues during pregnancy. It is important to determine the rates of use of such medicines during pregnancy and to ensure that the consulted health professionals have information about the effects and side effects of these medicines. There are several ways to obtain information about herbal medicines that are not based on evidence. This study aims to determine which herbal medicines are used in pregnancy, and what their usage rates are. It also aims to examine the effects and risks of such medicines on pregnancy, and to classify them according to safety conditions and evaluate the sources of thought and information about herbal medicines.

MATERIALS AND METHODS

This study was conducted to get information about the use of herbal medicine during pregnancy in women who have had at least one child or who were given pre- and postnatal care in the Department of Obstetrics and Gynecology (OGD). It is a descriptive study conducted by means of a survey directing questions to pregnant women. The place where the study was conducted is a private hospital with 72 beds, 10 of which belong to the OGD.

The definition of herbal medicines was briefly explained to the participants prior to the survey. After the study was announced, oral or written enlightened consent was obtained from the women who agreed to participate. Data were collected through face-to-face interviews with pregnant women together with the researcher and service representative midwife. Survey questions consisted of three parts. The first part of the survey included questions about the patients' chronic illness, allergies, smoking, and alcohol habits. The second section of the survey included questions for those who had used herbal medicine during pregnancy, including questions to obtain information about the duration of use, where the medicine had been obtained, the cost of the medicine, and whether there had been any side effects or not. In the last part, a total of 48 questions were included, along with sociodemographic questions. The survey was prepared by the researchers in the light of the literature review. Average time to complete the survey was 10-15 minutes. The study began after receiving approval from the Istanbul Medical Faculty Clinical Research Ethics Committee No:21 dated 22.12.2017. The survey began on January 15, 2018, and when a sufficient sample size was obtained data collection ended on March 15, 2018. The study population was calculated based on the number of patients in the 2017 OGD, obtained from hospital records. For the 2-month study, the study population was determined as 348. The following values were calculated: Confidence level: 95%, Confidence Interval: 5, Population: 348. The required number of samples was calculated as 183 (https://www.surveysystem.com/sscalc.htm). 194 people were in-

cluded in the study. Pregnant women over the age of 18 with at least one child and staying in the OGD were included in the study. Those who could not answer the questions were not included in the study.

The SPSS 15.0 for Windows Evaluation Version program was used to evaluate statistical data. All data obtained in the study were coded through programs, and statistical information such as frequency, descriptive information, and inter-variable relationships were evaluated. Ordinal and nominal data are given in n (%). A chi-square test was used in the analysis of categorical variables. The results were evaluated at 95% confidence intervals and significance levels of $p<0.05$.

Various reference sources were used to classify the safety of identified herbal medicines. The following resources were used as primary resources: Herbal Medicines in Pregnancy & Lactation (Mills, Dugoua, Perri, & Koren, 2006), Botanical Safety Handbook (Second Edition American Herbal Medicines Association's), PDR for Herbal Medicines (Gruenwald et al., 2000), Kennedy et al., 2016 and Ahmed, Hwang, Choi, & Han, 2017. Other references not listed in the above mentioned sources were scanned for herbal medicines.

In case of incompatible information in the sources, newer studies on the safety classification of herbal medicines used during pregnancy (Kennedy et al., 2016 and Ahmed et al., 2017) were used as primary reference sources, followed by the Herbal Medicines in Pregnancy & Lactation (Mills et al., 2006), Botanical Safety Handbook (Second Edition American Herbal Medicines Association's) resources. If any of the herbal medicines were not listed in the reference sources, PubMed/MEDLINE was consulted.

Evidence from human studies was used when classifying the safety of herbal medicines. If a herbal medicine consisted of two or more plants, each plant was evaluated separately and the herbal medicine classified as a single medicine. Based on two recent studies, herbal medicines were divided into four groups (Ahmed et al., 2017; Kennedy et al., 2016). The definitions of the categories in this classification are presented in Table 1. In this study, herbal medicine names were evaluated according to the patient's declaration and the scientific name of the herbal medicine was determined through the Turkish name. For species known with the same name without species distinction, only the genus name is given.

RESULTS AND DISCUSSION

Today, herbal medicines are very popular in the world. However, as in many areas, there is a lack of information on the use of herbal products about the prevalence, safety, and potential negative effects during pregnancy. This study examined the perspectives of pregnant women on herbal medicines, the prevalence of use during pregnancy, fact-based information on the use of herbal medicines according to risk and safety warnings, the sources from which patients received information and other factors related to to the use of herbal medicines.

A total of 194 pregnant women participated in this survey. The age range was mainly between 25-30 (38.6%). The median age±SD was 28.2±5.49. Participants were mostly primary school graduates (43.8%), most of whom were housewives (76.2%) with health insurance (92.8%). The stated preference of 66% of the participants when they got sick was to go to hospital. Further socio-demographic information about the participants is given in Table 2.

Table 2: Socio-demographic information of the participants.

	Frequency	Rate (%)
Gender		
Woman	194	100
Age		
Average±SD	28.2±5.49	
Education Status		
Not literate	4	2.1
Primary school	85	43.8
High school	72	37.1
University	33	17
Social Insurance Status		
Yes	180	92.8
No	14	7.2
Working Status		
Not working	148	76.3
Working	46	23.7
Habits		
No	152	78.4
Smoking	39	20.1
Alcohol	3	1.5
Health Institution Preference		
Family doctor	128	66
Hospital	64	33
Self Treatment	2	1

Table 1: Safety classification of herbal medicines used during pregnancy.

Classification	Description
Safe to use in pregnancy	Available human evidence suggests the herb can be safely used in pregnancy
Use with caution	Available human evidence for the herb is limited so it should not be used without consulting a qualified health care practitioner
Potentially harmful to use in pregnancy	Available evidence has shown adverse impacts on pregnant woman or fetus following the use of the herb
Information unavailable	No reference was found regarding use of the herb in pregnancy

According to our results, 63 (32.4%) of 194 pregnant women used 31 different herbal medicines during pregnancy and these herbal remedies were used frequently. The use of herbal remedies during pregnancy varies by country or region. A survey of 400 Norwegian women shows that 36% of pregnant women have used herbal medicines during pregnancy and the average was 1.7 products (Nordeng & Havnen, 2004). In a similar study, this percentage was found to be 27.8% in Italy (Cuzzolin et al., 2010). In a study in Iran, 30.8% of 513 pregnant women were shown to use herbal medicine (Tabatabaee, 2011). Another study gives a meta-analysis showing that more than a quarter (28.9%) of the participants had used some herbal medicines (Kennedy, Lupattelli, Koren, & Nordeng, 2013). Another study involving 2,729 participants from Asian countries showed that 1,283 women (47.01%) used one or more herbal medicines during pregnancy. (Ahmed et al., 2017). Among 557 mothers who gave birth in a hospital in Scotland, the rate of herbal medicine use was 61.4% (Pallivalapila et al., 2015). A study conducted in Turkey found that 47.3% of 366 pregnant women used at least one herbal medicine (Kıssal et al., 2017).

We evaluated the sociodemographic and other characteristics of pregnant women who used and who did not use herbal medicines during pregnancy with the Chi-square test. For people who have social insurance (p: 0.016) or prefer to use herbal products in self-treatment ($p<0.001$), or have a habit of using herbal products in their life at times other than pregnancy ($p<0.001$), the rate of using herbal products during pregnancy is higher than for others, and it was found that there was a statistically significant difference between these. There was no statistically significant difference between the use of herbal medicines and the level of education, age, employment status and profession ($p>0.05$). Other details are shown in Table 3.

A study in Turkey shows that the level of education, employment status and family culture represent a statistically significant difference (43.7%) in the use of herbal medicines during pregnancy ($p<0.05$). However, the relationship between age, social security, income, number of pregnancies and use of herbal medicines during pregnancy was not statistically significant (Kıssal et al., 2017).

In a study of 400 pregnant Norwegian women a statistically significant difference was only found in the age group of 36 years and younger for the use of herbal medicines during pregnancy. (p: 0.048) (Nordeng & Havnen, 2004). A study of 392 pregnant women in Italy found statistical significance between those who used herbal medicines and those who did not, in terms of living in the countryside (p: 0.001) and being in the 31-40 age range (p: 0.009) (Cuzzolin et al., 2010). A study of 530 pregnant women in Iran found that the relationship between the use of herbal medicines during pregnancy and age, occupation and place of residence has no statistical significance. (Tabatabaee, 2011). A multinational study found statistical significance between the use of herbal medicines during pregnancy and the education level and sources of information or recommendations ($p<0.05$), but there was no statistical significance between age and occupation (Kennedy et al., 2016).

As mentioned in various studies, there are many variables that lead to statistically significant differences in the use of herbal medicines during pregnancy. In addition to the median age of the participants, factors such as region, as well as cultural and knowledge levels, may also influence this difference. According to the statistically significant differences observed in this study, it can be concluded that pregnant women who have a habit of using herbal medicines outside the pregnancy, who have a habit of self-medication and those who prefer herbal medicines in self-medication, continue these habits during pregnancy. There is no data in the literature related to these conditions, which were determined significantly different in this study. In some countries the gestational age is higher than in our country. In this study the median age of pregnant women is lower, which may explain the reason why age is not a significant factor. Most studies point to profession as a factor, but we concluded in our study that profession does not make a significant difference in the use of herbal medicines during pregnancy. All these results make it difficult to summarize the related factors. In this study, we found that participants used 31 different herbal remedies during pregnancy. The most commonly used herbal medicines during pregnancy are: *Mentha* sp., *Citrus limon* (12.2%), *Tilia* sp. (12.2%), *Matricaria chamomilla* (6.6%), *Caryophyllus aromaticum* (6.6%) and *Jasminum* sp. (6.6%). Other herbal medicines and the percentage of use are shown in Table 4. The most used plants during pregnancy, determined in a study conducted in Norway, are echinacea (22.9%), iron-rich herbal remedies (11.9%), ginger (10.4%) and chamomile (9%), ginger (10.4%) and chamomile (9%) (Nordeng & Havnen, 2004). In a study in Italy, chamomile (44%), licorice (13.8%), and fennel (11.9%) were listed as the most commonly used herbal medicines during pregnancy (Cuzzolin et al., 2010). 258 women who used herbal medicines during pregnancy stated that the herbal medicines they used were ginger (55.8%), garlic (69.8%), and eucalyptus (11.6%) (Laelago et al., 2016). As a result, 31 different herbal medicines were identified in a study involving eight cross-sectional studies from seven different Asian countries (2729 participants). Accordingly, *Mentha piperita* (22.8%), *Pimpinella anisum* (14.7%), *Boswellia sacra* (12.9%), *Descurainia sophia* (12.2%) and *Zingiber officinale* (11.5%) are mentioned as the most commonly used herbal medicines (Ahmed et al., 2017). In a study conducted in Turkey, linden (23.2%), mint-lemon (20.2%), and rosehip (6.3%) are the three most commonly used herbal medicines during the first and second trimesters of pregnancy. During pregnancy, 4.9% of women were found to use ginger, 3.3% chamomile, 1.9% cranberries, 1.6% blueberries and 1.4% raspberries. Echinacea and tutrix (0.3%) were the least used during pregancy (Kıssal et al., 2017).

As seen in the studies mentioned above, the order and scope of use of herbal medicines used during pregnancy varies. In this study, *Mentha* species-*Citrus limon*, *Tilia* sp., *Matricaria chamomilla* were shown to be widely used as herbal medicines during pregnancy.

Physical symptoms associated with pregnancy are usually nausea/morning sickness, heartburn, constipation, frequent urination, back pain and headache (Skouteris et al., 2008). Herbal

Table 3: Comparison of attitude and demographic characteristics of participants according to the use of herbal medicines during pregnancy

Features	Those Who Do Not Use Herbal Medicine During Pregnancy	Herbal Medicine Users During Pregnancy	p value
Total	131 (67.6%)	63 (32.4%)	
Do you treat yourself when you get sick?			<0.001
No	56	8	
Yes	75	55	
Do you prefer herbal medicines for self treatment?			<0.001
No	59	4	
Yes	72	59	
Do you know that herbal medicines can be harmful?			0.598
No	78	40	
Yes	53	23	
Are herbal medicines more useful than other medicines?			0.122
No	46	15	
I do not know	49	33	
Yes	36	15	
Do you use herbal medicines besides at times other than during pregnancy?			<0.001
No	74	3	
Yes	57	60	
Education Status			0.422
Not literate	4	0	
Primary school	59	26	
High school	48	24	
University	20	13	
Working Status			0.068
No	105	43	
Yes	26	20	
Social insurance			0.016
No	11	3	
Yes	120	60	
Profession			0.143
Housewife	102	43	
Self-employment	22	12	
Teacher	1	1	
Other	6	7	
Age			0.633
18-25	37	15	
26-30	48	31	
31-35	27	13	
> 35	19	4	

medicines used during pregnancy are probably intended to treat these symptoms. The fact that many herbal medicines are both affordable and readily available also increases their use in the treatment of pregnancy-related symptoms. Studies report a wide variety of symptoms for which herbal medicines are used by women during pregnancy. Many different symptoms, such as nausea/vomiting, abdominal pain, respiratory problems, cold and flu and bloating are mentioned as the most common reasons for using herbal medicines (Ahmed et al., 2017). The most commonly reported symptoms in the study in Norway are colds and respiratory disease (20.4%), nutritional disorder (14.2%) and skin diseases (13.3%). The most common symptoms of pregnancy are increased nausea and uterine tone (Nordeng & Havnen, 2004). A study in Ethiopia found that 258 people were indicated as suffering from nausea (31.8%), vomiting (16.3%), abdominal pain (24.8%), colds (71.7%), and malaria (3.1%) (Laelago et al., 2016).

Table 4 : Herbal medicines used during pregnancy and number of people using them.

Herbal medicine	Frequency*	Rate (%)
***Mentha* sp.- *Citrus limonum* (mint-lemon)**	13	12.26
***Tilia* sp. (linden)**	13	12.26
***Matricaria chamomilla* (chamomile)**	7	6.6
***Caryophyllus aromaticum* (clove)**	7	6.6
***Jasminum* sp. (jasmine flower)**	7	6.6
***Ceratonia siliqua* (carob molasses)**	6	5.66
***Sambucus nigra* (elderberry)**	6	5.66
***Camellia sinensis* (green tea)**	6	5.66
***Foeniculum vulgare* (fennel)**	5	4.71
***Zingiber officinale* (ginger)**	4	3.7 7
***Citrus limonum* (lemon)**	4	3.77
***Cinnamomum* sp. (cinnamon)**	4	3.77
***Nigella sativa* (black seed oil)**	2	1.88
***Petroselinum crispum* (parsley)**	2	1.88
***Stipites cerasorum* (cherry stalk)**	2	1.88
***Calendula officinalis* (calendula flower)**	2	1.88
***Salvia* sp. (sage)**	2	1.88
***Hibiscus sabdariffa* (hibiscus)**	1	0.94
***Lycopodium clavatum* (matchstick)**	1	0.94
***Aesculus hippocastanum* (horse chestnut seed)**	1	0.94
***Centella asiatica* (gotu kola)**	1	0.94
***Persea americana* (avocado)**	1	0.94
***Ananas comosus* (pineapple)**	1	0.94
***Linum usitatissimum* (flaxseed)**	1	0.94
***Malus communis* (apple tea)**	1	0.94
***Capsella bursa-pastoris* (shepherd's purse)**	1	0.94
***Platanus* sp. (sycamore leaves)**	1	0.94
Mirtillin Mulberry Leaf Herbal Mixture Capsule**	1	0.94
Asparagus Forte**	1	0.94
Ezicof Syrup**	1	0.94
Garliva Black Garlic Tea**	1	0.94
Total	106	100

*Some women have used more than one herbal medicine.
****Mirtillin Mulberry Leaf Herbal Mixture Capsule:** Mulberry Leaf, Black Seed, Goatscottle, *Momordica charantia*, Black Chicory, Blueberry Leaf, Cinnamon (https://www.lokmanavm.com/miritilin-dut-yaprakli-bitkisel-kapsul-atasagun Access Date: 14.10.18).
****Asparagus Forte:** *Asparagus* sp. includes (https://tr.pinterest.com/pin/444871269417102751/ Access Date: 01.06.2020)
****Ezicof Syrup:** *Glycyrrhiza glabra, Adhatoda vasica, Hysopus officinalis, Ephedra vulgaris, Abrus precatonus, Viola odorata, Ocimum tenuiflorum, Piper nigrum, Morus nigra* (https://phytocon.com.pk/index.php/project/syrup/ Access Date: 14.10.18).
****Garliva Black Garlic:** Each tablet contains 500 mg *Allium sativum* extract (http://garliva.com/garliva-urunleri.html Access Date. 14.10.18).

This study shows that pregnant women mainly used herbal medicines for cold and flu symptoms (28%), digestive disorders (nausea, vomiting, diarrhea, constipation) (19%), weight loss (14%) or migraine (11%). Other uses are shown in Figure 1. As mentioned in other studies, the most common reasons for the use of herbal medicine during pregnancy were nau-

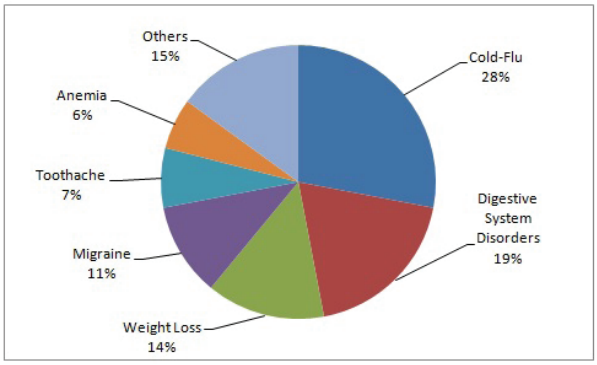

Figure 1. The main indications for herbal medicine use during pregnancy.

sea, vomiting, respiratory system disorders and colds (Kennedy et al., 2013). In this study, we found different usages for similar symptoms. Unlike other studies, weight loss and migraines were identified as reasons for the use of herbal medicines. Pregnant women used herbal medicines mostly (74%) as herbal tea once a day (61%). The cost of herbal medicines varied between 25-50 Turkish lira (44%). Other properties of the herbal medicines used are shown in Table 5. Almost half of the herbal medicines are in the range of 25-50 TL, which makes these medicines more affordable and increases their widespread use. The opinion that herbal products are cheap and accessible is a common thought among pregnant women (Ahmed et al., 2017). Another interesting situation is that the form in which herbal medicines are taken (tea, tablet, etc.) was not questioned in detail in some studies (Kennedy et al., 2013).

According to our study, 63% of those who used herbal medicines during pregnancy (40 people) did not know that herbal medicines have possible harmful side effects just like pharmaceutical drugs. The percentage of those who believe that herbal medicines are more effective than other medicines is 23.8%. The sources of information regarding herbal medicines are mainly the TV (30.2%) and the internet (28.9%). Other sources were identified as pharmacists (15%), neighbors (13.1%) and doctors (5.2%). The participants in our study tend to purchase these medicines from the pharmacy (42%) and herbalist shop (40%). The percentage of research (87%) regarding the herbal medicines they use is high. It is estimated that 57% of pregnant women who use herbal medicines during pregnancy inform their doctor. They said they benefited from the medicines they used (77%), believed in the treatment power of plants at a moderate level (46%) and did not suffer from any side effects (89%). Other important findings from the survey questions are shown in Table 5.

In a study conducted in Turkey, the thoughts of pregnant women about the use of herbal medicines were evaluated. According to this study, a high percentage (58.7%) stated that they used the medicine without obtaining the advice of anyone, and 25.7% stated that they used it on the advice of friends or relatives. Mostly, they received information from clinicians (68.3%) and nurses (22.4%). The percentage of those who thought that herbal medicines were safer than drugs was 36.1%, and the percentage of those who thought that herbal

Table 5: Opinions and behaviors of those who use herbal medicines during pregnancy.

Finding	Rate (%)
Herbal medicine use during pregnancy	63 (32%)
Do herbal medicines have harmful side effects like some other medicines	
Yes	37%
No	63%
Information/suggestion sources about herbal medicines	30.2%
TV	28.9%
Internet	15%
Pharmacist	13.1%
Neighbor	5.2%
Doctor	7.6%
Other	
The place where herbal medicines are supplied	42%
Pharmacy	40%
Herbalist shop	18%
Market	
Have you done research on the herbal medicines used?	87%
Yes	13%
No	
Is the doctor aware that the patient is using herbal medicines?	
Yes	57%
No	43%
Thoughts about the herbal medicine they use	
I saw benefit	77%
I do not know	12%
Other	11%
Therapeutic power of herbal medicines used	
Low	11%
Moderate	46%
High	43%
Do you experience any side effects from the herbal medicine you use?	11%
Yes	89%
No	
Frequency of using herbal medicine	
1 per day	61%
2 per day	33%
Other	6%
When do you use the herbal medicine?	
Every day	45%
When I get sick	55%
What is the cost of the herbal medicine used?	
0-25 Turkish liras	43%
25-50 Turkish liras	44%
50-75 Turkish liras	13%
Form of herbal medicine used*	
Herbal tea	74%
Tablet	14%
Syrup	7%
Other	5%

*Some women expressed more than one herbal medicine form.

medicines could be harmful to both the mother and the baby was 54.6% (Kıssal et al., 2017). Most countries have easy access to herbal medicines without a prescription. For this reason, many women use herbal remedies without consulting anyone or on the recommendation of family and/or friends. The level of use of herbal medicines on the recommendation of a health specialist is quite low (Kennedy et al., 2016). In a study showing the distribution of sources of advice on the use of herbal medicines by region (Europe, America, Australia), 28.6% of women used herbal medicines on their own initiative, while the percentage of those who consulted family and friends was 16.8%. Other informal sources of advice in this regard were the Internet (11.3%) and magazines and the media (3.3%). Medical doctors (21.6%) were listed as the second highest source of information (Kennedy et al., 2013).

In different countries, the use of herbal medicines on the recommendation of a medical doctor was observed as very low in this study. In Turkey, the proportion of doctors who prefer to prescribe herbal medicines as a way of treatment is low (Renda et al., 2018). For this reason, it is seen that medical doctors are lacking in sufficient information and recommendations regarding the use of herbal medicines. In addition, lack of information and communication difficulties of consulted health professionals are possible causes. Depending on cultural differences, information/advice sources are also found to be family, relatives, neighbors (82.9%), but this study shows that these sources of information have a lower percentage (13.1%) than was observed in another study (Laelago et al., 2016). The places where herbal medicines are distributed are mostly markets (41.5%) but one study shows that they are also distributed in pharmacies (36.7%) (Cuzzolin et al., 2010; Laelago et al., 2016). The preference of pharmacies is important both in terms of health counseling and in terms of trust. In this study, it was seen that more than half of the pregnant women informed their doctors about the use of herbal medicines. In another study, 51.8% reported this use to their doctors (Harrigan, 2011). As noted in this study, those who used herbal medicines in pregnancy stated that they benefited heavily (74.3%) and did not experience an unexpectedly harmful effect (91.9%) (Cuzzolin et al., 2010; Laelago et al., 2016). In the light of these results, pregnant women should be informed about the possible harmful effects of herbal medicines. Healthcare professionals need to create the necessary follow-up and raise sufficient awareness in this regard. Herbal medicines, which were found to be used by patients during pregnancy in this study, were classified according to the potential risks and warnings mentioned in the literature. Our study aimed to raise awareness about the prevention of possible side effects with this classification created about the safety of herbal medicines during pregnancy. Since there is not enough data regarding how safe such medicines are for use during pregnancy, it is recommended that pregnant women avoid risky herbal medicines and use safer herbal medicines under the supervision of a qualified healthcare specialist.

In this study, we evaluated the data of 31 different herbal drugs used in any period of pregnancy. The herbal medicines used during pregnancy were divided into four groups according to the safety data of pregnancy. Out of 31 different herbal medicines used during pregnancy, five (16.1%) were "safe to use in pregnancy", five (16.1%) showed the need to "use with caution", nine (29%) were "potentially harmful to use in pregnancy" and 12 (38.8%) came in the category of "information unavailable". Rates of use for all these categories were as follows: Safe to use in pregnancy (29.36%), Use with caution (8.60%), Potentially harmful to use in pregnancy (20.43%), Information unavailable (41.60%). Descriptions and details of the herbal medicines used are shown in Table 6 and Figure 2.

Table 6: Safety classification of identified herbal medicines.

Classification	Description	References
Safe to use in pregnancy	Available human evidence suggests the herb can be safely used in pregnancy	
Camellia sinensis	Pregnant women who consume 375 mg or more of caffeine per day may have an increased risk of spontaneous abortion. In those who consumed eight or more cups during their pregnancy, the stillbirth rate is 2 times higher than those who did not use it in pregnancy. Pregnant women who consume 600 mg or more of caffeine daily have a higher risk of low-weight birth. It can be used safely as a medium amount of tea.	(Kennedy et al., 2016; Mills, Dugoua, & Perri, 2006)
Garliva Black Garlic Tea	*Allium sativum*; There is no definite information about spontaneous abortion, risk of malformation, teratogenicity to the fetus. It has emenagogue effect.	(Ahmed et al., 2017; Demirezer, Ersöz, Saraçoğlu, & Şener, 2011; Ernst, 2002; Mills et al., 2006; Yıldırım, Desdicioglu, & Kara, 2016)
Matricaria chamomilla	It can cause various allergic reactions. It can be safely used in moderate amounts as tea, but it can function as a uterine stimulant, so high doses should be avoided. Long-term use is associated with early narrowing of the fetal ductus arteriosus .	(Ahmed et al., 2017; Demirezer et al., 2011; Kennedy et al., 2016; Shinde, Patil, & Bairagi, 2012)

Table 6: Continue.

Classification	Description	References
Mentha sp.	It is one of the herbs used in the treatment of nausea. When used as a tea, it did not show any harmful effects to the mother or fetus. Overdose should not be taken due to emenagogue effect.	(Demirezer et al., 2011; Gardner & McGuffin, 2013; Gruenwald et al., 2000; Kennedy et al., 2016; Mills et al., 2006)
Zingiber officinale	It has been stated that it has no teratogenic, embryotoxic, effects and is unlikely to cause spontaneous abortion.	(Ahmed et al., 2017; Demirezer et al., 2011; Gruenwald et al., 2000; Kennedy et al., 2016; Mills et al., 2006)
Use with caution	Available human evidence for the herb is limited so it should not be used without consulting a qualified health care practitioner.	
Ananas comosus	It may have an uterotonic effect.	(Monji et al., 2016)
Centella asiatica	It is stated to be low constructive and change the menstrual cycle. It should not be used during pregnancy due to the emenagogue effect. It should not be used during pregnancy without medical advice.	(Ernst, 2002; HMPC, 2010)
***Cinnamomum* sp .**	There is insufficient information about its benefits and risks. It should not be used during pregnancy. Animal evidence suggests the possibilty of fetal malformation of essential oil.	(Gardner & McGuffin, 2013; Kennedy et al., 2016; World Health Organization, 1999)
Linum usitatissimum	It has emenagogue effect. It has estrogenic/antiestrogenic effects. Causes low birth weight. Due to its estrogenic effect, it is considered as a potential antifertil plant. It is not recommended for use since it has insufficient safety knowledge about its use in general.	(Demirezer et al., 2011; Ernst, 2002; HMPC/EMA, 2006; Mills et al., 2006)
Nigella sativa	There are not enough scientific reports in humans about use and safety during pregnancy. Consumption of hot extracts of fruit in large amounts has a low constructive effect in pregnant women.	(Ahmed et al., 2017; Paarakh, 2010)
Potentially harmful to use in pregnancy	Available evidence has shown adverse impacts on pregnant woman or fetus following the use of the herb.	
Aesculus hippocastanum	It is toxic to use in its raw form, it can be lethal. Various clinical studies have been conducted on the use of horse chestnut extract in pregnant women. No adverse effects on fetal development have been reported when doses ranged from 480 to 600 mg (standardized to 100 mg aescin) daily for 2 to 4 weeks.	(Gardner & McGuffin, 2013; Mills et al., 2006; Nordeng & Havnen, 2004)
Capsella bursa-pastoris	It is emenagogue and low constructive. It is not recommended to use as there is no clear data on remedicineive toxicity and use in pregnancy.	(Ernst, 2002; Kennedy et al., 2016; Wharf & Kingdom, 2011)
Calendula officinalis	It is emenagogue and low constructive. It should not be used in pregnancy due to there is no information about its teratogenicity.	(Edwards, da Costa Rocha, Williamson, & Heinrich, 2015; Ernst, 2002; WHO Monographs On Selected Medicinal Plants Vol. 2., 2002)
Ezicof Syrup	*Glycyrrhiza glabra* can cause premature birth. It is not recommended during pregnancy due to possible changes in hormone levels and a relationship with preterm birth. *Ephedra* sp. Is reported to cross the placenta, stimulating the uterus.	(Ahmed et al., 2017; Demirezer et al., 2011; Ernst, 2002; Gruenwald et al., 2000; Mills et al., 2006)
Foeniculum vulgare	May cause allergic reactions, uterine contractions, miscarriages or premature births. It has emenagogue effect. Although its teratogenic effect has not been proven, it is more appropriate not to use it because of its estrogenic effects. It also has hypoglycemic , hypolipidemic and hypothyroid properties.	(Ahmed et al., 2017; Ernst, 2002; Gardner & McGuffin, 2013; Kennedy et al., 2016; Rahimi & Ardekani, 2013; Shinde et al., 2012)
***Jasminum* sp.**	It should not be used in pregnancy due to its uterine stimulating and emenagogue effects.	(Evans, 2009)

Table 6: Continue.

Classification	Description	References
Mirtillin Mulberry Leaf Herbal Mixture Capsule	There is no known problem with the use of the *Taraxacum officinale* during pregnancy. *Vaccinium myrtillus* should not be used during pregnancy. *Momordica charantia* has emenagogue and abortion effects.	(Demirezer et al., 2011; Ernst, 2002; Mills et al., 2006; Nordeng & Havnen, 2004)
Petroselinum crispum	It is an estrogenic, uterine stimulating and emenagogue herb with a risk of abortion. It can cause birth defects.	(Mills et al., 2006; Mills & Bone, 2005; Shinde et al., 2012)
***Salvia* sp.**	It should not be used in pregnancy due to its potential toxicity and low constructive effect. It has emenagogue effect.	(Ahmed et al., 2017; Ali-Shtayeh, Jamous, & Jamous, 2015; Demirezer et al., 2011; Edwards et al., 2015; Ernst, 2002; Kennedy et al., 2016)
Information unavailable	No reference was found regarding use of the herb in pregnancy.	
Caryophyllus aromaticum	There is no information regarding its use and safety conditions during pregnancy.	
Ceratonia siliqua	There is no information regarding its use and safety conditions during pregnancy.	
Citrus limonum	There is no information regarding its use and safety conditions during pregnancy.	
Hibiscus sabdariffa	It is not recommended for use during pregnancy due to the fact that its safety cannot be determined and sufficient information cannot be found.	(Edwards et al., 2015)
Asparagus Forte	There is no information regarding its use and safety conditions during pregnancy.	(Gardner & McGuffin, 2013)
Lycopodium clavatum	There is no information regarding its use and safety conditions during pregnancy.	
***Malus communis* (tea)**	There is no information regarding its use and safety conditions during pregnancy.	
Persea americana	There is no information regarding its use and safety conditions during pregnancy.	
***Platanus* sp. (leaves)**	There is no information regarding its use and safety conditions during pregnancy.	
Sambucus nigra	Its use is not recommended during pregnancy, since its safety cannot be determined.	(Edwards et al., 2015; Nordeng & Havnen, 2004; WHO Monographs On Selected Medicinal Plants Vol. 2., 2002)
Stipites cerasorum	There is no information regarding its use and safety conditions during pregnancy.	
***Tilia* sp.**	There is not enough data for use in pregnancy. In limited use in women, no increase in malformation or other harmful effects on the fetus.	(Demirezer et al., 2011; HMPC, 2012; Mills & Bone, 2005)

In a study that investigated the use of herbal medicines in 2673 pregnant women, it was stated that 126 herbal medicines were used. The study showed that 27 of 126 plants used during pregnancy were classified as contraindicated, 28 of them were safe and the rest were used carefully during pregnancy (Kennedy et al., 2016). In another study involving 2729 participants, 13 of the 33 herbal medicines identified were safe for use, five were reported as needing to be used with care, 8 were potentially harmful during pregnancy and 7 were information unavailable about safety in pregnancy pregnancy and 7 were not available in the literature on (Ahmed et al., 2017).

In our study, the distribution of herbal medicines used in pregnancy according to the risk and safety classification was found to be close to the usage rates in the literature. As in other studies, herbal medicines in the "Potentially harmful to use in pregnancy" (20.43%) category are highly used. Most of the herbal medicines used are classified in the "Information unavailable" (41.60%) category due to insufficient data on herbal medicine use during

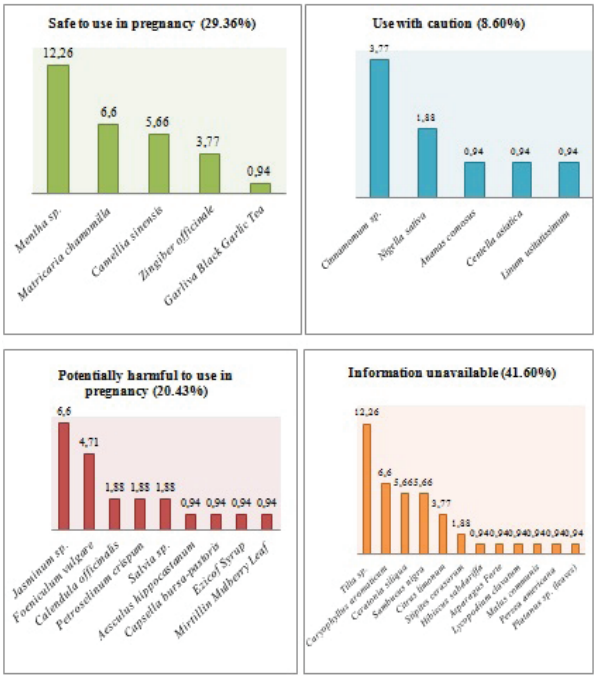

Figure 2. The ratio of herbal medicines used in pregnancy by the number of people using it.

pregnancy. When compared with studies from different regions, our study also shows the differences in herbal medicines by means of categories. In this study, the most used medicine in the "Potentially harmful" category is *Jasminum* sp. (6.6%), but in another study *Salvia* sp. (12.2%) was shown to be the most used herbal medicine in this group (Ahmed et al., 2017).

While studying the use of herbal medicines during pregnancy, it was found that the information in the current literature is insufficient. To maintain safety in pregnant women, it should be recommended that the use of these drugs be completely prevented during pregnancy or not used without the supervision of a qualified medical professional, as information is limited.

Due to pregnancy being a time of possible risk for some mothers, the use of potentially harmful herbal medicines at 20.43% requires an even more attentive approach to this issue. Medical doctors, pharmacists and other health professionals should constantly inform pregnant women in this regard, and they should constantly be monitored and given sufficient information . During the follow-up process, the use of herbal medicine should be questioned.

The limitation of the study is to determine the scientific names of the plants according to the information provided by the patients and to give the names of the species according to the Turkish names. Latin names are included in the text of the study, with the Turkish names indicated by the patients in parentheses, but it should be noted that many genera and species names in this study can also be used for other species and genera names. The genus name has been used to describe species named with the same names. The Turkish names of the commonly used plants are indicated as the species name. Due to the fact that this study was a survey study, no information was obtained about the possible side effects of the plants used by patients during pregnancy. In future studies, these deficiencies can be eliminated by paying attention to this issue. Another limitation of the study is that it does not question in which trimester of pregnancy herbal medicines were used.

CONCLUSION

Our study determined that approximately one in three pregnant women used herbal medicines during pregnancy. The study shows that the herbal medicines most used in pregnancy are placed into three categories, namely, 'safe', 'potentially harmful' and 'insufficient data'. When we evaluated the effects of herbal medicines on pregnancy, we concluded that potentially harmful herbal medicines are used at a high rate and most herbal medicines do not have sufficient safety data. While the sources of information and advice about herbal medicine are predominantly social media such as TV and the internet, which are not evidence-based, their use rates in pharmacy and doctor consultancy are low. Due to insufficient information about the use of herbal medicines in pregnancy, herbal medicines that are used based on unofficial sources become more harmful. Recently, herbal medicines have been advertised through the TV and the Internet as "miracles", "natural", "harmless" and "cheap" and such advertising does not reflect the reality that these medicines could threaten people's health. Information sharing that does not reflect such facts should be prevented and users should be informed by official health professionals. Although much research has been done on herbal medicines, the fact that one in three pregnant women use herbal medicines reveals that the right sources cannot be reached.

This study has contributed to studies in a small number of these issues in Turkey. We investigated and evaluated the use of herbal medicines in pregnancy from a multi-faceted perspective such as prevalence, risk classification, thoughts, and factors affecting use. As can be seen in this study, there is a lack of information about the possible risks, related issues, and the prevalence of herbal medicine use in pregnancy. These deficiencies can be overcome with more detailed, controlled, clinical studies.

Note: The editor in chief was not involved in the evaluation, peer-review and decision processes of the article, and these processes were carried out by the associate editors.

Ethics Committee Approval: This study was approved by the Clinical Research Ethical Committee of the Istanbul University, Istanbul Faculty of Medicine (No: 21 dated 22.12.2017).

Informed Consent: Written consent was obtained from the participants.

Peer-review: Externally peer-reviewed.

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Original Article

Exploring the effects of individual innovativeness dimensions on performance: a study with pharmaceutical managers*

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ABSTRACT

Background and Aims: Innovation is a crucial factor for individuals and businesses, particularly in the period of Industry 4.0. The objectives of the present study are to explore the factor structure of the Individual Innovativeness Scale (IS) in a manager sample, and then to investigate the dimensions' impact on performance.
Methods: A questionnaire was administered to managers in the pharmaceutical industry. Factor structure of the Individual Innovativeness Scale was determined *via* exploratory and confirmatory factor analyses. Afterwards, impacts of the dimensions on performance were assigned.
Results: After exploratory and confirmatory factor analyses, three dimensions emerged: resistance to change, openness to experience, and opinion leading. The regression model showed that opinion leading and openness to experience were significant predictors of performance.
Conclusion: The IS is three dimensional in the pharmaceutical manager sample. Performance of pharmaceutical managers are affected from opinion leading and openness to experience. This paper has both theoretical and practical contributions.

Keywords: Individual innovativeness, openness to experience, opinion leading, resistance to change, performance

*This article is extracted from the Ph.D. thesis of the corresponding author.

INTRODUCTION

Innovativeness is becoming increasingly important especially in the Industry 4.0 era. Kagermann et al. (2013) reported that Industry 4.0 is generally referred to as the fourth industrial revolution (Grzybowska & Łupicka, 2017). It leads to new technologies, processes, and important changes in business life (Horváth & Szabó, 2019). Kovács (2017) stated that technical feasibility and social acceptability have an impact on the success of Industry 4.0 (Horváth & Szabó, 2019). The pharmaceutical sector has rapid growth, more qualified managers are needed in the fourth industrial revolution (Grzybowska & Łupicka, 2017), and innovation is an important growth factor for the pharmaceutical industry (Schuhmacher, Germann, Trill, & Gassmann, 2013).

Innovation means generating new knowledge and ideas, as well as adopting external practices and processes and applying them (Scott & Bruce, 1994; Gu, Duverger, & Yu, 2017). According to Rogers (2002), innovation is "*an idea, practice, or object that is perceived as new by an individual or other unit of adoption*" and innovativeness is "*the degree to which an individual or other unit of adoption is relatively earlier in adopting new ideas than other members of a social system*".

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Human resources is a vital parameter in ensuring the dynamism of businesses, mainly in the pharmaceutical industry (Babapour, Gholipourb, & Mehralian, 2018). The success or failure of a business is directly associated with the success of employees, and employee performance is effective in achieving business goals (Gümüştekin & Öztemiz, 2005). However, employee performance is a challenging issue and every business cares about improving performance and increasing competitiveness (Inuwa, 2017; Wu & Lee, 2011). It has been stated that healthy perfectionists show innovative behaviors while achieving goals (Chang, Chou, Liou & Tu, 2016). The management process is also important and companies need open-minded, creative leaders to implement and standardize new technologies (Horváth & Szabó, 2019). Additionally, managers contribute to companies' innovation capabilities (Wang & Dass, 2017). It can be said that adopting innovation is essential to stay ahead of the competition. Industry 4.0 forms the basis of Pharma 4.0 (Kumar, Talasila, Gowrav, & Gangadharappa, 2020) and Pharma Industry 4.0 technologies enables pharmaceutical firms to provide a competitive advantage in the long term (Ding, 2018). In this context, pharmaceutical manager innovativeness and performance are crucial factors for this changeover.

In existing literature, the IS has been generally used for students (Ertuğ & Kaya, 2017; Özden, Cevik, & Sarıtaş, 2019), student teachers (Celik, 2013), teachers (Yilmaz & Bayraktar, 2014) or consumers (Pallister & Foxall 1998; Chao, Reid, & Mavondo, 2012). In addition, this scale has been developed in teacher and student samples (Hurt, Joseph, & Cook, 1977) and adapted into Turkish in student sample (Kılıçer & Odabaşı, 2010). Considering the developments, the measurement of employee innovativeness is important. The present study aims to fill this gap by investigating applicability of IS in business life, and exploring how individual innovativeness dimensions influence working performance. For these purposes, firstly the factor structure of the IS is investigated in a manager sample, after that the dimensions' impact on performance are determined.

MATERIALS AND METHODS

Innovativeness was measured with the IS developed by Hurt et al. (1977). The IS was adapted into Turkish by Kılıçer and Odabaşı (2010) in a student sample. It contains 20 items (twelve positive and eight negative) and four dimensions, which are resistance to change, risk taking, openness to experience and opinion leading. The performance scale (consisting of four items) was used to measure performance. This scale was adapted from Kirkman & Rosen (1999) by Sigler & Pearson (2000), and adapted into Turkish by Çöl (2008). Both of them were five point Likert-type scales, and items were ordered from (1) strongly disagree to (5) strongly agree.

Population size of the present study consisted of department managers in the pharmaceutical industry that are members of Association of Research-Based Pharmaceutical Companies, Pharmaceutical Manufacturers Association of Turkey and Pharmaceutical Industry Association of Turkey. The associations were informed about the study and their support was requested. Determining the total number of department managers was not possible so sample size was calculated considering the population size as being unknown. Participants were informed about the study by phone call. After their approval, the questionnaire was sent *via* e-mail. The questionnaires were administered between April and December 2017. The Ankara University Ethical Committee approved this study with permit number 89 (13.03.2017). The minimum sample size was evaluated as 96; on a 0.05 relevance level, d: 0.1 and p: 0.5. 126 managers replied to the questionnaire. Four of them were excluded from the research due to errors and inconsistencies. In total, 122 surveys were included in the analysis.

LISREL 9.2 (Jöreskog & Sörbom, 2015) and SPSS version 24.0 (IBM Corp. 2016) were used for confirmatory factor analyses and other analyses, respectively.

RESULTS

46.7% of the participants were female, and 53.3% were male. More than half of the managers (54.1%) were aged between 30-40, 16.4% of them were under 30 and 29.5% were over 40 years old. Nearly half of the managers had been working for five or fewer years in their current department. 32% of the participants were from the sales and/or marketing department (Table 1).

Table 1: Characteristics of the participants.

	Frequency (%)
Gender	
Female	46.7
Male	53.3
Age	
<30	16.4
30-40	54.1
>40	29.5
Working Years	
≤5 years	49.2
6-10 years	23.8
>10 years	27
Departments	
Sales/Marketing	32
Research&Development	10.7
Regulatory Affairs	8.2
Supply Chain/Logistics	7.4
Human Resources	6.6
Quality	6.6
Medical	5.7
Others	23

Kaiser-Meyer-Olkin (KMO) values were determined as 0.809 and 0.795 for IS and Performance Scale (PS), respectively. Also, Bartlett's test of sphericity was significant for both ($p<0.001$). These values exhibit that the sample size was sufficient, and the data were suitable for exploratory factor analysis (EFA).

Principal component analysis with varimax rotation was used to conduct EFA. According to the EFA results after removing nine items, the IS consisted of three dimensions: resistance to change (RC), openness to experience (OE), and opinion leading (OL), and the performance scale emerged as unidimensional. The means of each item and factor loadings are shown in Tables 2 and 3. The Cronbach's alpha of the PS was 0.828 and the explained variance 66.312%. Table 4 shows Cronbach's alpha values and variance ratios of IS. Confirmatory factor analyses (CFA) were performed to determine construct validity of the scales. Path diagrams of CFA models are shown in Figures 1 and 2. Rotated factor loadings were over 0.5 for all of the items. Various goodness of fit indices such as chi-square/degree of freedom (χ^2/df), p-value, root mean square error of approximation (RMSEA), comparative fit index (CFI), non-normed fit index (NNFI) and standardized root mean square residual (SRMR) were used to examine the fit of model and dataset (Arslan & Şar, 2018; Tarhan, Arslan & Şar, 2017; Arslan, Tarhan, & Şar, 2017). CFA results of IS and PS are shown in Table 5. Goodness of fit indices of three dimensional innovativeness and unidimensional performance scales are: χ^2/df= 1.19; p value=0.184; RMSEA=0.04; NNFI= 0.98; CFI=0.98 good fit; SRMR=0.055 acceptable fit, and χ^2/df 1.82; p value=0.162; NNFI=0.97; CFI=0.99; SRMR=0.02 good fit; RMSEA= 0.08 acceptable fit, respectively, according to the intervals of the goodness-of-fit indices (Çelik & Yılmaz, 2016; Hair, Anderson, Tatham, & Black, 1998; Jöreskog & Sörbom, 1996; Raykov & Marcoulides, 2006; Schermelleh-Engel, Moosbrugger, & Müller, 2003; Yılmaz, Çelik, & Arslan, 2010). These results exhibit that the CFA models are acceptable.

Table 2: Exploratory factor analysis, factor loadings and means ($\bar{x}$) of IS.

		Factor loadings		
Factors and items	**$\bar{x}$**	**RC**	**OE**	**OL**
RC				
ii6	2.28	0.656		
ii10	2.10	0.846		
ii17	2.39	0.834		
ii20	2.12	0.724		
OE				
ii2	4.36		0.794	
ii3	4.24		0.857	
ii5	4.14		0.588	
ii14	4.17		0.600	
OL				
ii8	4.20			0.761
ii9	4.16			0.825
ii12	4.33			0.703

Table 3: Exploratory factor analysis, factor loadings and means ($\bar{x}$) of PS.

Factor items	**$\bar{x}$**	**Factor loadings**
p1	4.39	0.813
p2	4.41	0.823
p3	4.27	0.812
p4	4.30	0.810

Table 4: Cronbach's α, explained variance and cumulative explained variance values of IS.

Factors	**Cronbach's α**	**Variance (%)**	**Cumulative variance (%)**
RC	0.775	22.022	22.022
OE	0.783	21.983	44.005
OL	0.764	20.296	64.302

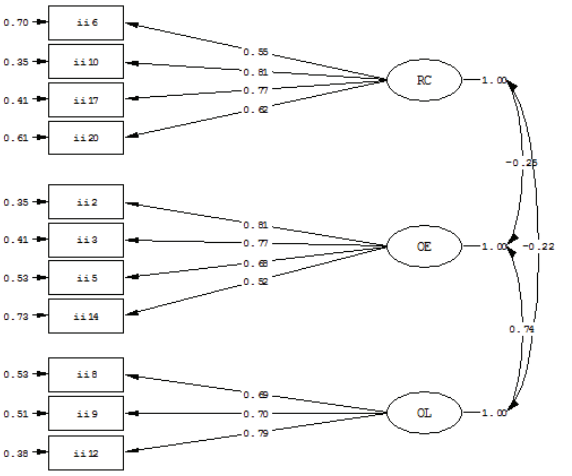

Figure 1. CFA model of the *IS*.

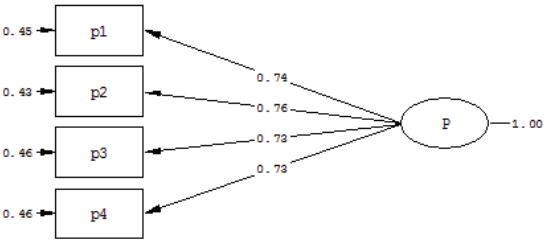

Figure 2. CFA model of the performance scale.

The CFA results supported that the IS had a three factor structure in manager sample. Pearson's correlation analysis was conducted to exhibit the relations between the variables. Significant positive correlations were found between performance and openness to experience (r=0.433; p=0.00), performance and opinion leading (r=0.195; p=0.016). However, no significant relation was identified between performance and resistance to change (r= -0.039; p=0.335). Following correlation analysis, regression analysis was performed to find out the influences of IS dimensions on performance. The regression model was significant (F=11.576, p=0.000). 22.7% of per-

Table 5: CFA results of IS and PS.

Factors and items	Standardized loadings	t-values	R^2
RC			
ii6	0.55	5.97	0.30
ii10	0.81	9.41	0.65
ii17	0.77	8.84	0.59
ii20	0.62	6.91	0.39
OE			
ii2	0.81	9.85	0.65
ii3	0.77	9.26	0.59
ii5	0.68	7.95	0.47
ii14	0.52	5.68	0.27
OL			
ii8	0.69	7.76	0.47
ii9	0.70	7.90	0.49
ii12	0.79	9.16	0.62
PS			
p1	0.74	8.75	0.55
p2	0.76	9.00	0.58
p3	0.73	8.64	0.54
p4	0.73	8.63	0.54

formance was determined by the variables (R^2=0.227) and the dimensions openness to experience (*t*=5.356, p=0.000) and opinion leading (*t*=2.411, p=0.017) were significant according to the regression results.

The independent samples t-test and one-way analyses of variance (ANOVA) were applied to assess the effects of demographics on the factors. Tukey test was conducted to determine the differences between the groups due to homogeneity of variance. According to the independent samples t-test results, no statistically significant difference was found in gender. ANOVA results showed that age of the participants had statistically significant differences in OL factor (F=3.130; p=0.047; p<0.05). Managers over forty were more opinion leaders than the participants under the age of thirty.

Concerning the working years of the managers in the departments, statistically significant differences were found in RC factor (F=2.572; p= 0.081; p<0.1). Participants with 6-10 years of working experience were high in RC than those working for more than ten years.

DISCUSSION

This study determined the factor structure of the IS in a pharmaceutical managers sample, thereafter it expressed the impacts of the innovativeness dimensions on performance, and revealed that performance is affected by openness to experience and opinion leading. In addition, the effects of gender, age and working experience on the dimensions were examined.

The present study shows that the IS consists of three dimensions: resistance to change, openness to experience and opinion leading in manager sample. Similar to this study, Gürkan & Demiralay (2017), applied IS to surgeons and found three-dimensions: resistance to change, openness to experience and opinion leadership. Besides, in studies with different samples different dimensions were emerged (Pallister & Foxall, 1998; Sarıoğlu, 2014). In the present study, the managers who had been working in the department for more than ten years had less resistance to change than the ones with between 6-10 years of working experience. Supporting this, older managers are found high in opinion leading. Similarly, Kunze, Boehm, & Bruch (2013) stated that, in contrast to widespread stereotypes, older employees exhibit less resistance to change than their younger counterparts.

Related to the gender of participants, different results occur in the literature. In a study applied to entrepreneurs, females were found to be more open to experience (Hachana, Berraies, & Ftiti 2018). On the other hand, in a study with managers and employees in the service and industrial sectors, gender was found to not affect innovation and work performance (Yıldız, Baştürk, & Boz Taştan, 2014). Similarly, in this study, gender did not have a statistically significant effect on the dimensions.

People often try to verify their opinions before reaching a decision. Opinion leaders are described as "*those individuals from whom others seek advice and information*" and influence other people's ideas (Rogers & Cartano, 1962). Opinion leaders exhibit high levels of exploratory behavior, engage in intense activities, follow developments in their interests, and have a higher tendency to assess, test, accept and adopt innovations (Chen, Weng, Yang, & Tseng, 2018; Goldsmith & Desborde, 1991). In this context, the performance of individuals with such behaviors is expected to be high. The current study shows that opinion leading affects performance positively. In the literature, studies about opinion leadership are generally examined on consumers. In a study conducted by Cho & Workman (2011), in the field of fashion, only one shopping channel is used by the consumers with low innovativeness and opinion leadership. However, more channel types are used by the participants with high innovativeness and opinion leadership. Another study conducted on consumers determined that opinion leadership increases the likelihood of subscribing to and reading computer-related journals, and going to computer stores (Shoham & Ruvio, 2008). As mentioned above, it can be thought that intense activities can improve performance.

Innovation is a critical parameter for individuals and the growth of businesses and economies. In the globalizing world, early implementation of innovations helps to gain competitive advantage (Vila, Perez, & Coll-Serrano, 2014). Interactions in the working environment affects innovative behaviors (Anderson, de Dreu & Nijstad, 2004; Zhou & Shalley, 2003; De Jong & Den Hartog, 2007) and managers have important roles in influencing innovativeness in institutions (Kılıçer & Odabaşı, 2013). Personality affects the opinions, emotions and behaviors of

employees, and also it is effective in behaviors in daily life and particularly in the working environment (Darmawan, 2017). Openness to experience has been stated to be a personality trait (McCrae & Costa, 1987; Harris, 2004). Individuals defined as 'open' are more prone to new and diverse experiences (Williams, 2004). In the present study, this dimension positively affected performance. Among the dimensions of innovativeness, openness to experience is found to be the most critical factor affecting performance. Individuals high in openness are more open to feedback and more adapted to the activities and relationships in organizations (Bartone, Eid, Johnsen, Laberg, & Snook, 2009). Darmawan (2017) conducted a study with employees from different companies, and pointed out that openness to experience has a positive effect on performance. Moreover, a study conducted with chief managers working in small and medium-sized production enterprises stated that managers' openness to experience have significant effects on the understanding of budgeting practices (Zor, Linder, & Endenich, 2019). In the literature, there are various results relating to the impact of openness to experience on performance. In a study applied to employees who make telephone sales in two large telecom firms, no relationship was found between openness to experience and performance (Klang, 2012). In another study, it was stated that openness had no effect on leader performance (Bartone et al., 2009). Openness may be an important factor affecting performance in situations where employees have to adapt to changes, but in stable situations its effect is less (Thoresen, Bradley, Bliese, & Thoresen, 2004).

In this study, the resistance to change did not have a statistically significant impact on performance. Additionally, responses to resistance to change was low. In this context, it can be said that managers working in the pharmaceutical industry do not exhibit resistance to change. Success or failure of a company depends on competent employees (Adolph, Tisch, & Metternich, 2014). Resistance to change can be considered as an undesirable condition in fields where many changes and innovations take place, such as the pharmaceutical industry. Therefore, according to the findings, it can be considered that manager traits are taken into account in recruitment and promotion situations. In addition, the managers' performance was found to be high. Low resistance to change and high-performance are expected findings, especially in the pharmaceutical industry.

CONCLUSION

Innovations and innovativeness are becoming more and more important during the period of Industry 4.0 and are expected to continue to be important as time goes on. The present study has theoretical and practical contributions in business management. IS has a three factor structure in a manager sample. The three dimensional IS can be used in recruitment and promotion stages by organizations. This study provides contributions about exploring how personality traits impact performance. Openness to experience and opinion leading are found to be performance predictors. In recruitment and promotion stages, human resource departments can give priority to the candidates who rank high in openness to experience and opinion leading. The present study sampled managers in the pharmaceutical industry. Future studies can be performed in employee samples and/or different sectors.

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Short Paper

The preliminary ethnobotanical survey of medicinal plants in Develi (Kayseri/Turkey)

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ABSTRACT

Background and Aims: In Turkey, there is a growing interest in ethnobotany due to the wealth of biological and cultural diversity. The main purpose of this study is to record and highlight the medicinal plants used therapeutically in the Develi district.
Methods: The data were obtained during different seasons of 2010-2011 through face-to-face interviews. Medicinal plants were collected with the guidance of the informants, and traditional uses was recorded. Botanical identification of all the species was made, and voucher specimens were deposited at Istanbul University, Herbarium of the Faculty of Pharmacy (ISTE).
Results: The informants reported data on 14 medicinal plants belonging to 8 botanical families. These medicinal plants are used for about 26 different purposes. The most dominant family is Lamiaceae (6 species) followed by Asteraceae (2 species). Various plant parts such as leaf, fruit, flower, root, wood and fruity branch are used; the aerial part is consumed more frequently than other plant parts.
Conclusion: Despite the modernization and cultural change, many people still benefit from the plants distributed in the Develi district. This study offers valuable information on the traditional knowledge of medicinal plants, which could form a basis for future phytochemical and pharmacological researches.

Keywords: Develi, ethnobotany, Kayseri, medicinal plants, Turkey

INTRODUCTION

Turkey has a rich flora with over 11000 taxa, approximately 34% of these are endemic. Our country gets ahead of all European countries in terms of the number of endemic plants (Güner et al., 2018; Güner, 2014). This floristic richness and cultural heritage from the past constitute a great deal of ethnobotanical knowledge which should be revealed (Yeşilada, 2013; Kendir & Güvenç, 2010).

As known, many ethnobotanical practices such as making tools or using medicinal plants are generally learned *in situ*, as children work alongside parents or elder siblings. However, technological developments and modernization change the socio-cultural structure of the society, and it is becoming more difficult to conduct ethnobotanical studies or access to information about ethnobotanical practices. Therefore, ethnobotanical data which is about to disappear attracts scholarly attention and many researchers have begun to document this academic value (Güner, 2014; Yeşilada, 2013; Kendir & Güvenç, 2010; Cotton, 1996).

In Turkey, ethnobotanical studies have become increasingly recognised as a valuable source of information on the use and conservation of many plants, particularly after the 1980s. Many systematic field studies have begun to record traditional medicine carefully, and thus become an important resource for drug discovery research (Yeşilada, 2013; Baytop, 1999). Throughout recent years, numerous ethnobotanical studies have been published in Turkey concerning the traditional use

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of medicinal plants and many studies have been conducted in Central Anatolia (Sağıroğlu, Topuz, Ceylan, & Turna, 2013; Gençler Özkan & Koyuncu, 2005; Sezik et al., 2001; Doğan, Başlar, Ay, & Mert, 2004; Bağcı, 2000; Ertuğ, 2000).

Kayseri province is located in the central Kızılırmak section of Central Anatolia. Its eastern part is situated in upper Kızılırmak River while the southern part is in the Mediterranean region. General characteristics of the Irano-Turanian vegetation types prevail in this region, these vegetation elements such as *Consolida orientalis* (J.Gay) Schrödinger, *Glaucium flavum* Crantz, *Fumaria officinalis* L., *Isatis glauca* Aucher, *Capsella bursa-pastoris* (L.) Medik., *Silene conica* subsp. *subconica* (Friv.) Gavioli, *Peganum harmala* L., *Melilotus officinalis* (L.) Pall., *Cirsium arvense* (L.) Scop., *Sideritis lanata* L., *Salvia multicaulis* Vahl, *Euphorbia orientalis* L., *Cynodon dactylon* (L.) Pers. can be seen in various areas of Kayseri province (Türkmen, 2006). This floral and also cultural richness forms a basis for noteworthy ethnobotanical knowledge. Although there are some prior ethnobotanical studies that have been carried out in various regions of Kayseri (Sağıroğlu et al., 2013; Gençler Özkan & Koyuncu, 2005; Sezik et al., 2001; Bağcı, 2000), traditional uses of medicinal plant in the Develi district have not been reported. The aim of the present study is to provide information about the ethnobotanical properties of medicinal plants which are used traditionally by the local people of the Develi district.

MATERIAL AND METHODS

Study area

Develi, which is one of the biggest districts of Kayseri, is situated in the central part of Turkey. Develi was established 6 km from the south of Mount Erciyes (38°23'18.6"N, 35°29'31.3"E), is surrounded by Tomarza District and Tufanbeyli District of Adana Province in the east, Yahyalı District and Saimbeyli-Feke Districts of Adana Province in the south, Yeşilhisar District in the west and İncesu District in the northwest (Figure 1). Develi

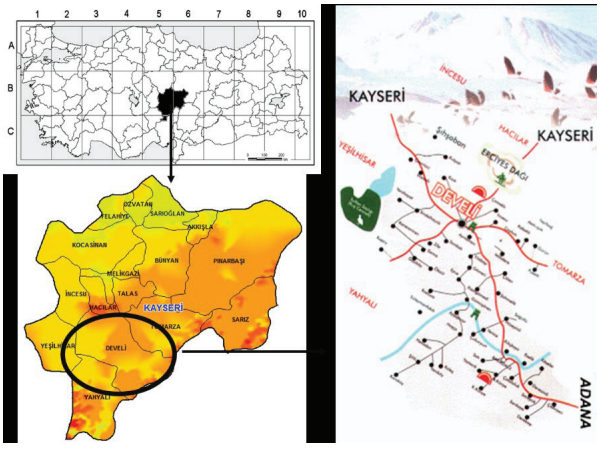

Figure 1. The geographical location of the study area.

is included in Irano-Turanian flora region and falls within the B5 grid square according to the Grid classification system used in the Flora of Turkey (Davis et al., 1988; Davis, 1965-1985).

The surface area of the district is 1887 km^2, and the altitude of the central district is 1150 m above sea level. The total population is 65,745 according to the population census in 2019. The most important mountains of the region are Erciyes Mountain, Karasivri Mountain, Büyük Kartın (Peri Kartın), Göktepe, Sümengen (Süvegen) and Bakır Mountain. The reedfield Sultan Sazlığı, which is located at the lowest part of the Develi plain, contains important floral and faunal communities. Develi is one of the least precipitation areas of our country. The average annual rainfall varies between 300 and 500 mm, and it usually falls in spring. The annual average temperature is calculated as 10.6°C. Develi has hosted various civilizations for centuries. Although the first settlement date could not exactly be determined, some ruins of the Hittite period were found in the region. After the domination of Phrygians, Persians and Seljuks, the district passed to the Ottoman administration in 1474. Develi was accepted as the district of Kozan province until 1926. When the status of Kozan changed, Develi became Kayseri district (Türkmen, 2006).

Data collection, plant materials

A field study was carried out during different seasons of 2010-2011 in the Develi district of Kayseri. After the local people were informed about the study, face-to-face interviews were held in order to document ethnobotanical knowledge. Related data were obtained from local people by using interview questions including information on local plant names, ailments and diseases treated, therapeutic effects, parts of plants used. All plant specimens were collected together with accompanied informants. Plant specimens were numbered and pressed in the field (Figures 2-5).

Figure 2. *Lavandula stoechas* L.

Figure 3. *Crataegus meyeri* Pojark.

Figure 4. *Hypericum perforatum* L.

Collected plant specimens were identified by two of the authors (Rıdvan Çolak, Şükran Kültür) using references, i.e. Flora of Turkey and the East Aegean Islands (Davis, 1965-1985; Davis, Mill, & Tan, 1988; Güner et al., 2000) and Türkçe Bitki Adları Sözlüğü (Baytop, 1994), and compared with specimens stored in Istanbul University, Herbarium of the Faculty of Pharmacy (ISTE). The voucher specimens were deposited in the ISTE Herbarium. The names of plant families were listed in alphabetical order. Scientific names of identified taxa were updated according to The Plant List (http://www.theplantlist.org/). Ethnobotanical data were screened to reveal whether the plants used have literature records or not.

Figure 5. *Verbascum* sp.

RESULT AND DISCUSSION

A total of 14 plant species with ethnobotanical importance belonging to 8 botanical families were found out and documented in this research. Each species is presented with its corresponding botanical identity, herbarium sample number, family name, vernacular name, part used and ethnobotanical usage.

***Alcea pallida* (Willd.) Waldst. & Kit., R. Celik 08, Malvaceae**

Vernacular name: Gülhatmi, Gülfatma

Used parts: Leaves, roots

Ailments treated/Therapeutic effect: Asthma, cough, expectorant, diuretic (leaves)

Previous ethnobotanical literature records: Cough (Kurnaz Karagöz & Serteser, 2017; Tetik, Civelek, & Cakılcıoğlu, 2013; Demirci & Özhatay, 2012; Keskin, 2011; Saday, 2009; Karataş, 2007; Bağcı, 2000), bronchitis (Tetik et al., 2013; Saday, 2009; Demirci & Özhatay, 2012), stomach diseases (Bağcı, 2000),

ophthalmia (Sağıroğlu et al., 2013), toothache (Sağıroğlu et al., 2013), ornamental plant (Keskin, 2011), respiratory disorders (Kurnaz Karagöz & Serteser, 2017; Keskin, 2011; Karataş, 2007), gingivitis (Oral, 2007), painkiller, kidney stone (Karataş, 2007), smoothing throat (Keskin, 2011)

***Ajuga chamaepitys* subsp. *laevigata* (Boiss.) P.H.Davis, R. Celik 05, Lamiaceae**

Vernacular name: Karın ağrısı otu, Bodur otu
Used parts: Aerial parts
Ailments treated/Therapeutic effect: Abdominal pain, severe diarrhea (especially in animals), wound healing
Previous ethnobotanical literature records: Diabetes (Polat & Çakılcıoğlu, 2018), removing fear, constipation (Kaya, Dağlı, & Celik, 2020)

***Crataegus meyeri* Pojark., R. Celik 13, Rosaceae**

Vernacular name: Alıç
Used parts: Fruits, wood
Ailments treated/Therapeutic effect: Vasodilator, kidney and urinary tract purification
Previous ethnobotanical literature records: Antidiarrheal (Yeşil & Akalın, 2009), diabetes (Şenkardeş, 2014; Yeşil & Akalın, 2009), asthma, hemorrhoids (Tetik et al., 2013), cardiovascular diseases (Şenkardeş, 2014; Gençler Özkan & Koyuncu, 2005), rheumatism (Gençler Özkan & Koyuncu, 2005), kidney stone, constipation, foodstuff, fuel, tool (Şenkardeş, 2014)

***Echinophora tenuifolia* subsp. *sibthorpiana* (Guss.) Tutin, R. Celik 09, Apiaceae**

Vernacular name: Çörtük, Çörtlük
Used parts: Aerial parts
Ailments treated/Therapeutic effect: Hand, foot and mouth wounds
Previous ethnobotanical literature records: Foodstuff (Şenkardeş, 2014; Ertuğ, 2000), soup, seasoning, drinking (Doğan et al., 2004)

***Echinops ritro* L., R. Celik 14, Asteraceae**

Vernacular name: Deve dikeni
Used parts: Aerial parts
Ailments treated/Therapeutic effect: Stomachache
Previous ethnobotanical literature records: Foodstuff (Güneş, Savran, Paksoy, Koşar, & Çakılcıoğlu, 2018; Özüdoğru, Akaydın, Erik, & Yeşilada, 2011), fodder (Ertuğ, 2000), stomach complaints (Özüdoğru et al., 2011)

***Helichrysum plicatum* DC., R. Celik 06, Asteraceae**

Vernacular name: Altın otu
Used parts: Aerial parts
Ailments treated/Therapeutic effect: Intestinal inflammation, hemorrhoid
Previous ethnobotanical literature records: Cholesterol management, cancer (Güneş, Savran, Paksoy, Koşar, & Çakılcıoğlu, 2017), kidney and stomach ailments, depreciatory (Yeşil & Akalın, 2009), kidney stones (Demirci & Özhatay, 2012; Yeşilada et al., 1995; Yeşilada et al., 1993), diarrhea, abdominal pain, diabetes (Özdemir & Alpınar, 2015), hypertension, hypercholesterolemia (Kocabaş & Gedik, 2016), nephralgia (Sargın, 2015), stomach ulcer, diuretic (Özüdoğru et al., 2011), wound (Sezik et al., 2001; Tetik et al., 2013), ear ache (for baby) (Demirci & Özhatay, 2012), jaundice, dysuria, snake repellent (Yeşilada et al., 1995), antifungal, urinary dysfunction (Karaman & Kocabaş, 2001), constipation (Çömlekçioğlu & Karaman, 2008)

***Hypericum perforatum L.*, R. Celik 12, Hypericaceae**

Vernacular name: Sarı kantaron
Used parts: Aerial parts, flowers
Ailments treated/Therapeutic effect: Rheumatic pain, stomachache, kidney inflammation
Previous ethnobotanical literature records: Ulcers, allergies (Özüdoğru et al., 2011), burn, wound (Sargın & Büyükcengiz, 2018; Sargın, Selvi, & Buyukcengiz, 2015; Yeşilada et al., 1993), gastric ulcer, antibiotic, immune system booster (Sargın & Büyükcengiz, 2018), hemorrhoids (Han & Bulut, 2015; Akaydın, Şimşek, Arıtuluk, & Yeşilada, 2013), constipation, prostatitis, diabetes, hypertension, urinary infections, diaper rash, rheumatism, osteoporosis (Akaydın et al., 2013), stomachic, scorch cure (Demirci & Özhatay, 2012), analgesic, gastrointestinal pains, depression (Sargın, 2015), pyrosis, reflux (Sargın et al., 2015), diarrhea, colic (Özdemir & Alpınar, 2015, rheumatic pains, nervous disorders (Kocabaş & Gedik, 2016), sedative, foodstuff, fuel, dye (Akan & Bakır Sade, 2015), stomachache (Yeşilada et al., 1995; Yeşilada et al., 1993), cardiovascular diseases (Tuzlacı & Şenkardeş, 2011), spazmolytic, antiseptic, hepatitis, hemostatic, vulnerary, cholagogue, hepatoprotector, anti inflammatory, orexigenic, digestive (Everest & Öztürk, 2005), gastric diseases, muscle relaxant rheumatism, gastric pain (Uzun & Kaya, 2016), tonsillit, diuretic, expectorant, shortness of breath (Karataş, 2007), tuberculosis, cold, anthelmintic (Ezer & Avcı, 2004), relaxation, insomnia (Akgül et al., 2016), gastrointestinal system disorders (Bağcı, Erdoğan, & Doğu, 2016), contusion and burn, snake bite and scorpion bite (Çömlekçioğlu & Karaman, 2008), appetizing (Karaman & Kocabaş, 2001)

***Lavandula stoechas* L., R. Celik 03, Lamiaceae**

Vernacular name: Karabaş otu
Used parts: Aerial parts, flowers
Ailments treated/Therapeutic effect: Headaches, smoking cessation (as an aid)
Previous ethnobotanical literature records: Cancer (Sargın & Büyükcengiz, 2018; Akan & Bakır Sade, 2015), sedative (Sargın & Büyükcengiz, 2018), pains, smoking cessation (Akan & Bakır Sade, 2015; Sargın, 2015), cardiovascular disease (Güneş et al., 2017; Sargın, 2015; Çömlekçioğlu & Karaman, 2008), vasodilator, asthma, bronchitis, headache, brain diseases, ulcer, hypertension, insomnia (Sargın, 2015), stomachache (Güneş et al., 2017; Abay & Kılıç, 2001), analgesic (Sargın et al., 2015), epilepsy, nervosity, anodyne, stethalgia, blood stimulant, hypertension, throat disorders, digestive, obesity (Everest & Öztürk, 2005), nervous disorders (Abay & Kılıç, 2001), hypercholesterolemia (Çömlekçioğlu & Karaman, 2008)

***Marrubium anisodon* K.Koch, R. Celik 07, Lamiaceae**

Vernacular name: Elbir otu
Used parts: Aerial parts

Ailments treated/Therapeutic effect: Kidney stones, female infertility, regulates the blood pressure
Previous ethnobotanical literature records: Not reported.

***Melissa officinalis* L., R. Celik 01, Lamiaceae**

Vernacular name: Melisa, Oğul otu
Used parts: Leaves
Ailments treated/Therapeutic effect: Cough, asthma, sedative
Previous ethnobotanical literature records: Arteriosclerosis (Paksoy, Selvi, & Savran, 2015), sedative, stress, vasodilator, soporific, female sterility (Sargın, 2015), headache, cardiovascular diseases (Demirci & Özhatay, 2012), insomnia, scorpion bite, bath, heart palpitation (Akan & Bakır Sade, 2015), tranquillizer, antidepressant, anhypnia, gastrit, angiemphraxis, epilepsy, fainting, allergy, digestive, cardiotonic, hearth stimulant, carminative, spasmolytic, diaphoretic, disinfectant (Everest & Öztürk, 2005), cholesterol, hypertension (Oral, 2007), foodstuff (Koçak & Özhatay, 2013), anemia, diabetes, memory (Akgül et al., 2016), thyroid, sleep disturbances, stress, digestion system (Saltan & Özaydın, 2013), antiseptic, cold (Karaman & Kocabaş, 2001)

***Peganum harmala* L., R. Celik 11, Nitrariaceae**

Vernacular name: Yüzellik, Güzellik otu
Used parts: Fruity branches, fruits
Ailments treated/Therapeutic effect: Protect against the evil eye, allergic asthma
Previous ethnobotanical literature records: Amulet (Özüdoğru et al., 2011), sedative, hemorrhoids, vermifuge, menstrual diuretic (Özdemir & Alpınar, 2015), evil eye, ornaments (Öztürk, 2004), expectorant, stimulating nervous system, hair loss, eczema, malaria, dye (Akan & Bakır Sade, 2015), Parkinson, insomnia (Yaşar, Koyuncu, Turan Koyuncu, & Kuş, 2019; Akan & Bakır Sade, 2015), emmenagogue, narcotic, sedative, nutritive, cephalalgia, anti hysteria, notalgia, ophthalmalgia, omalgia, hand-tremble (Everest & Öztürk, 2005), anthelmintic, stomachache (Tugay et al., 2005), headache, hypertension, carminative (for babies) (Oral, 2007), shortness of breath, stomach ulcer, hair dandruff, hair care (Şenkardeş, 2014), foodstuff (Hakverdi & Yiğit, 2017); dermal diseases (Vural, Karavelioğulları, & Polat, 1997).

***Sideritis libanotica* subsp. *linearis* (Benth.) Bornm., R. Celik 04, Lamiaceae**

Vernacular name: Dağ çayı
Used parts: Aerial parts
Ailments treated/Therapeutic effect: Smoothing throat, vasodilator, diabetes
Previous ethnobotanical literature records: Cold (Demirci & Özhatay, 2012; Yeşil & Akalın, 2009), flu (Demirci & Özhatay, 2012), as a tonic (Yeşilada et al., 1993), foodstuff (Doğan et al., 2004), throat and flu infections (Oral, 2007)

***Teucrium chamaedrys* subsp. *syspirense* (K.Koch) Rech.f., R. Celik 02, Lamiaceae**

Vernacular name: Kısamahmut otu
Used parts: Aerial parts
Ailments treated/Therapeutic effect: Hemorrhoid, intestinal fungal infections, antidote
Previous ethnobotanical literature records: Diabetes (Dalar, 2018), foodstuff (as tea) (Aksakal & Kaya, 2008)

***Verbascum* sp., R. Celik 10, Scrophulariaceae**

Vernacular name: Yalancı sığır kuyruğu
Used parts: Fruits, leaves
Ailments treated/Therapeutic effect: Allergic asthma
Previous ethnobotanical literature records: draining inflammation, cough, pain of abarticulation or broken bones (Akaydın et al., 2013), asthma, expectorant (Sargın & Büyükcengiz, 2018), pruritus, shortness of breath, hair loss, dyestuff, uterine inflammations (Özüdoğru et al., 2011), urinary inflammations, fissures on hand, abdominal pain (Gençler Özkan & Koyuncu, 2005), warts (Savran et al., 2008), sinusitis, nepatitis, hemorrhoids (Tuzlacı & Şenkardeş, 2011), bronchitis (Saday, 2009)

The Lamiaceae family (6 species) is the most commonly used and species-rich family in this study. It is followed by Asteraceae (2 sp.), Hypericaceae (1 taxon), Malvaceae (1 taxon), Rosaceae (1 taxon), Scrophulariaceae (1 taxon), Apiaceae (1 taxon) and Nitrariaceae (1 taxon), by their number of species. Aerial parts were primarily used for ethnobotanical uses by local people. Other used plant parts were found as leaf, fruit, flower, root, wood and fruity branch, respectively (Figure 6). During this study, a total of 26 traditional usages were recorded. Medicinal plants were used for both human and animal health. *Ajuga chamaepitys* subsp. *laevigata* (Boiss.) P. H. Davis was not used only in humans but also in animal treatment. Besides the treatment of diseases, people benefited from plants for different purposes. They believed that *Peganum harmala* L. protects them from the evil eye.

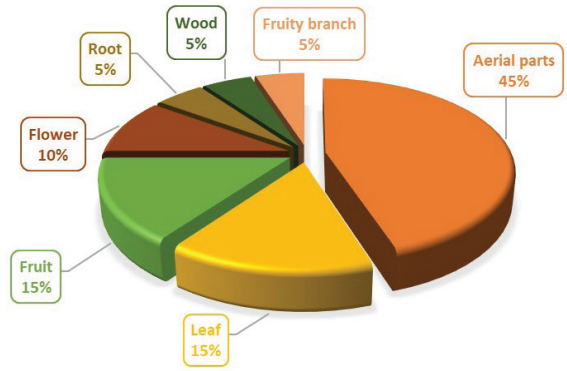

Figure 6. Plant parts used for ethnobotanical purposes ranked by frequency of use.

Recorded traditional uses in the study area were compared with those reported in ethnobotanical studies of Central Anatolia and its environs, and strong affinity was found with the ethnobotanical use. As a result of this comparison, although no medicinal use of *Teucrium chamaedrys* subsp. *syspirense* (K.Koch) Rech.f. and *Ajuga chamaepitys* subsp. *laevigata* was found, ethnobotanical data from distant regions present several utilizations. *T. chamaedrys* subsp. *syspirense* was used for diabetes in Van Province and (Dalar, 2018) *Ajuga chamaepitys* subsp. *laevigata* was used for diabetes; removing fear and constipation in Bingöl (Polat & Çakılcıoğlu, 2018) and Şanlıurfa Provinces (Kaya et al., 2020). Previous ethnobotanical data screening in Turkey revealed that the medicinal use of *Marrubium anisodon* K.Koch

was shown for the first time in our study. In addition, some new medicinal uses were found out in this paper: Aerial parts of *Ajuga chamaepitys* subsp. *laevigata* for abdominal pain, severe diarrhea (especially in animals) and wound healing; aerial parts of *Teucrium chamaedrys* subsp. *syspirense* for hemorrhoid, intestinal fungal infections and as an antidote; fruits and wood of *Crataegus meyeri* Pojark as vasodilator and for kidney and urinary tract purification; aerial parts of *Echinophora tenuifolia* subsp. *sibthorpiana* (Guss.) Tutin for hand, foot and mouth wounds. Comparing this study with the studies from Aladağlar, Yahyalı and Pınarbaşı districts of Kayseri (Sağıroğlu et al., 2013; Gençler Özkan & Koyuncu, 2005; Bağcı, 2000), *Alcea pallida* (Willd.) Waldst. & Kit., *Peganum harmala*, *Crataegus meyeri*, *Verbascum* sp. and *Hypericum perforatum* L. were detected as five shared taxa used. In addition to similarities in uses, some different uses are also noticeable. For instance, while *C. meyeri* was used in the treatment of cardiac disorders and rheumatism in Pınarbaşı region (Gençler Özkan & Koyuncu, 2005), people in the Develi district have benefited from its vasodilator and kidney - urinary tract purification properties.

Some aforementioned medicinal plants contain toxic constituents that adversely affect human health, for this reason, overdose or side effects of these plants can be dangerous according to the literature. *Teucrium chamaedrys* subsp. *syspirense* is known as a hepatotoxic plant, therefore, it should not be administered without the recommendation of authority in proper use. Photosensitization of *Hypericum perforatum* in humans is associated with excessive intake of the plant (Gruenwald et al., 2000). Special attention should be paid to the use of these plants, even though there is no notification from the local people.

CONCLUSION

The present study documented the traditional uses of 14 plant species belonging to 8 families of medicinal plants by local people. These plants are used either as a remedy for various diseases or for any other purposes in the Develi district of Kayseri. The medicinal uses of *Marrubium anisodon* were recorded for the first time in this study. It is noteworthy that local people mainly use aerial parts of the plants not underground parts (rhizomes, roots and bulbs). They make contributions to the conservation of the species in the region by preventing the indiscriminate cutting or removing the entire plant.

Since the ethnobotanical knowledge of local plants is mainly possessed by elder family members and transmitted from generation to generation, more studies should focus on compiling as much information as possible before the eventual elimination of traditional knowledge. It is believed that this study provides valuable information on medicinal plants, reinforcing the importance of continuing with ethnobotanical research in Turkey, which can lead to the development of new pharmaceuticals.

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Review Article

Examining the basic principles of quality by design (QbD) approach in analytical studies

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ABSTRACT

Although the first application of Quality by Design (QbD) concept started for product development studies, the number of studies regarding its application to analytical development has been increased recently. Basically, QbD strategy in both formulation development and analytic studies are identical logically and conceptually, but they have somedifferences in terms of its terminology and application. Essential terminology and approach differences in this concept are; the determination of the analytic target profile, critical method characteristics, critical process parameters, and the determination of the method study area. However, the risk evaluation method which is necessary for the appropriate application of quality by design is also an inseparable part of the analytical quality by design. Despite those terminological differences, developing a quality-based method with the analytical design that contributes to research with an appropriately applied risk-based design quality approach and provides multiple advantages that will be noticed each and every time, will be useful both for researchers and authorities who investigate license documentation and changes. Therefore, the terminology which is used for analytic quality by design and appropriate risk evaluation approaches are explained in this study.

Keywords: Analitycal quality by design, risk assesment, process analytical technology, analytical target profile, critical quality atrributes

INTRODUCTION

Quality by Design (QbD) in Pharmaceutical Industry

QbD Concept means checking production through a total understanding of the process to reduce the risk for the patient and related with it, is an evaluation of the product quality control with a scientific-based approach (Zhang & Mao, 2017). This concept was brought to the pharmaceutical agenda for the first time in 2004 with a different point of view inside the ICH Q8 guideline (ICHQ8, 2014).

In the conventional approach, the quality is verified by evaluating if it is following the approved specifications in the completed pharmaceutical product. In the case of an unqualified product, the product will follow the process again or it will be canceled. The production process and parameters, specifications, and methods are determined by using a few pilot production serials which are used for authority license application and in R&D studies. All the approved process and quality parameters are determined in R&D studies for providing production consistency. Most of the production processes, in-process control and product specifications, or other changes are validated in scale-up productionor industrial production.

The essential differences between the conventional and recent quality approaches are listed in Table 1.

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Table 1. Differences between the conventional and QbD approaches (Aksu & Mesut, 2015).

Parameter	Conventional Approach	Recent QbD Approaches
Development stage	Empirical, randomly, generally processed on a variable on each time.	It is aimed to extrapolate an area of design of experiment results that are based on statistics and plans.
Production Process	Strength. Alternations are not recommended. Process validation is based on the first industrial production serials.	Constant validation of small variations of the protocol for discovering the effects of product quality based on life cycle.
In-Process Control	Tests for progress pause decisions. Offline analysis.	Using Process Analytic Technology (PAT) for real time feedback and forward.
Quality Control	In-Process and batch releasing tests for supervision and specification.	Real time releasing. Validates quality as a part of risk-based control strategy.
Authority License Application Documents	Through data of pilot scale production serials	Based on process perception, product information and performance requirements.
Life Cycle	Reactive with corrective actions. Necessity of variation applications after authority approval of product.	Preventive actions is possible using the design area with continuous recovery. The process and quality parameters approved in the design area do not need to be changedafter the validation of process.

Although QbD concept and guidelines are created to involve primarily the product development process, including the formulation development stage, the quality control of the product and the production process of different strengths, , it also include its application and contribution to the analytic process,which is called as Analytic Quality of Design (AQbD) (Reid et al., 2013).

Process Analytical Technology (PAT)

Process Analytic Technology (PAT), is defined as 'devices and systems which are using real time or fast measurements during the process compatible with process quality and performance features are used for providing information to produce the final product having consistency with the finished product'. - Qualified quality and performance standards ICH Q9 (ICHQ9, 2015) and ICH Q8 (ICHQ8, 2014), defines PAT usage for providing the process to be in a certain Design Area.

Looking in a perspective of PAT, it is considered as well understood, when the factors mentioned below are provided;

(1) All the variable sources are defined and explained;
(2) Variability is directed by the process; and
(3) Features of product quality can be foreseen safely and true (Patwardhan, Asgarzadeh,& Dassinger, 2015; Rao, Moreira & Brorson, 2009).

A systematic and an effective insight that combines with an inference system is essential due to the complexity of pharmaceutical product manufacturing process design. Real-time observation devices are becoming more and more attractive to pharmaceutical manufacturers. The constant production and real-time observation have been mostly used for the tablet production process until now. It is expected that PAT devices in the production of other dosage forms will be used soon as well as the successful application in tablet dosage forms. Spectroscopic techniques like Raman spectroscopy, UV-VIS spectroscopy and Nuclear Magnetic Resonance (NMR) are commonly used. Besides, other PAT analytical methods like Near Infrared spectroscopy (NIR), Focused Beam Reflection Measurements (FBRM), Manometric Temperature Measurement (MTM), Tunable Dirt Laser Absorption Spectroscopy (TDLAS) are also commonly used in pharmaceutical industry. They play a major role in the real-time observation of production files and process, as summarized in Table 2 (Zhang & Mao, 2017).

Analytical Quality by Design Studies (AQbD)

ICH guidelines covering analytical studies;

- ICH Q1: Stability
- ICH Q2: Analytical Method Validation
- ICH Q3: Impurities
- ICH Q4: Pharmacopoeias
- ICH Q5: Quality of Biotech Products
- ICH Q6: Specifications
- ICH Q7: Good Manufacturing Practice

In addition to existing guides, guides to be taken into account in analytical studies within the framework of QbD;

- ICH Q9: Quality Risk Management
- ICH Q10: Pharmaceutical Quality System
- ICH Q12: Life Cycle Management
- ICH Q14: Analytical Procedure Development and Revision of Q2 (R1) Analytical Validation

It is observed that 69% of the pharmaceutical companies are using the AQbD approach according to a questionary study performed on among the pharmaceutical companies. This rate is about 83% for big pharmaceutical companies (Argentine et al., 2017). The first stage of the AQbD procedure for which care should be taken, is the true evaluation/determination of the

Table 2. Representatives of some monitoring tools used in pharmaceutical processes (2011-2015).

Process	Observation Device	Measured Feature	Important Outputs
Co-Precipitation Process	Lasentec particle view microscopy system PVM 819 (Mettler-Toledo AutoChem, Columbia, MD)	Nuclearization and crystal magnification	Provides direct information about both size and morphology of precipitates
Culture process of mammalian cell	RamanStation spectrometer (AVALON Instruments Ltd., Belfast, NI, now acquired by Perkin-Elmer, USA)	Glycoprotein product efficiency	Determination of which small scale serials are proper for large scale serial production, mostly recovering process efficiency
China hamster Ovary (CHO) Cell feeding- with ball	Fluorescence stimulation-emission matrix (EEM) spectroscopy (Cary Eclipse (Varian, now Agilent, USA))	Key fluorophores (Ex. Tyrosine, Tyriptophane, etc)	Recombinant glycoprotein production quantitative predicted analysis
Fluidized bed granulation	Microwave resonance technology (MRT) (Döscher & Döscher GmbH (Hamburg, Germany))	Determination of moisture, temperature	Gives information about the last status of granule and density of granules size
Pan covering Process	New real-time monitoring tool (PyroButtons) (Philedelphia, USA)	Real time record and data	Gives information about Thermodynamic condition (micro-environment)
Constant direct Print Tablet Production process	Near infrared (NIR) spectroscopy, OPUS (Bruker) software is used to operate the NIR sensor (USA).	Powder mixture mass density	NIR spectrums, mixture density and medication concentration are sent to a real time estimation model through NIR calibration models and estimation device (OLUPX) and produce signal for real time control of variables

analysis. An Analytical Target Profile (ATP), which is appropriate for the analysis and the method, is determined after deciding the analysis (amount determination, impurity, content monotony, etc.) and the method (Spectroscopic, chromatographic, etc.), subsequently. CQA's are detected through an ATP. Risk evaluation is done and risky parameters are revealed. The Design Area is decided according to that evaluation (Mosford, 2018).

When we search Procedure Stages schematically;

Figure 1. Analytical QbD Flow Diagram.

Defining ATP

AQbD is primarily related to the forms of ATP and CQA in the file of medication. In analytic studies, ATP substitutes QTPP in product design (Peraman, Bhadraya & Padmanabha Reddy , 2015a) and defined as a simple device for the determination of requirements of method development. Although the general method requirements are shared in an ICH guidlines, the requirements of the method are determined in this stage (Sangshetti, Deshpande & Zaheer, 2017). Therefore, by considering both requirements stated in the regulations and requirements of the method, ATP's are detected here. Different from pharmaceutical product development, here Critic Method Attributes (CMA) and Critic Method Parameters (CMP) substitutes CMA and CPP.

Risk assessment

Risk Evaluation is a scientific/ statistical process used for the determination of the ATP. The evaluation of process should involve all the stages including control, communication and revision. Generally, the methods for the determination of risk definition and evaluation are arranged in ICH Q9 (ICHQ9, 2015). The Methods and definitions used in the risk evaluation in the presentation called 'Design Quality in Analytical Methods' (2011) (Tang, 2011) are stated below;

Riskfactor=Severity x probability of occurrence x Detectability (Eq. 1)

Severity means the effect on the patient in terms of safety and efficiency except for the problems that are faced during production.

Occurrence possibility, means alternations that may occur in an existing process or uncertainty in a new process and includes information about the product and process and controls.

Detectability means the capacity and appropriateness of the analytical method and includes the importance of sampling design.

Although the method shared by the FDA presentation is evaluation of the method Risk factor with the FMEA method, it should be considered that other risk methods can also be used. The important thing is to choose the most appropriate method for the study. Other risk evaluation approaches in ICH Q9 are listed below (Aksu, 2013, Aksu, 2015);

- Failure mode, effects and critical analysis (FMECA): This is an approach which is developed from modification of the FMEA method. Different from the FMEA method, the critical status of the process or material also creates an effectiveness factor.
- Failure Tree analysis (FTA): Determination of all the root causes of the situation that cause failure of the product or the process with graphics or symbols.
- Hazard Analysis and Critical Control Points (HACCP): This is preferred mostly in situations that require food safety. It is possible to use this approach for both development and life cycle.
- Hazard and Operability Analysis (HAZOP): The main problem is evaluated as 'deviations' for this approach. Possible problems are followed as a list.
- Risk ranking and filtering (RR&F): Risk factors are evaluated by dividing it in to compounds in this methodology.
- Preliminary hazard analysis (PHA): This approach evaluates future problems by considering previous experiences.
- Fishbone Diagram: A statement type on a diagram for the factors that may cause possible problems (Ex: see Figure 2).

Method Operable Design Area

Method Operable Design Area takes place in a design area intended for the product in analytical studies [10]. Method Operable Design Area is the operating range for inputs in Critical Quality Attributes (like CQA) which produce results that meet the targets determined by the ATP consistently (Peraman, Bhadraya & Reddy,2015b).

Control Strategies

Product control strategy should be defined for the determination of the analytical methods which should be used in AQbD studies and in which stage they should be used. In other words, the development of the control strategy includes the entire process which the method is started (Amri, 2019). A good control strategy is obtained by evaluating the data which is obtained during method development and confirmation stage by considering ATP criteria (Reid et al., 2013).

Constantly Development

Constant development is applied as a result of the definition of the method validation process and additional critical attribute and obtaining additional data (LoBrutto, 2013), which includes the observation of method performance and recovery as possible as it can (Chatterjee, 2013).

Procedure stages that should be done for an AQbD study are listed below when all those definitions are considered;

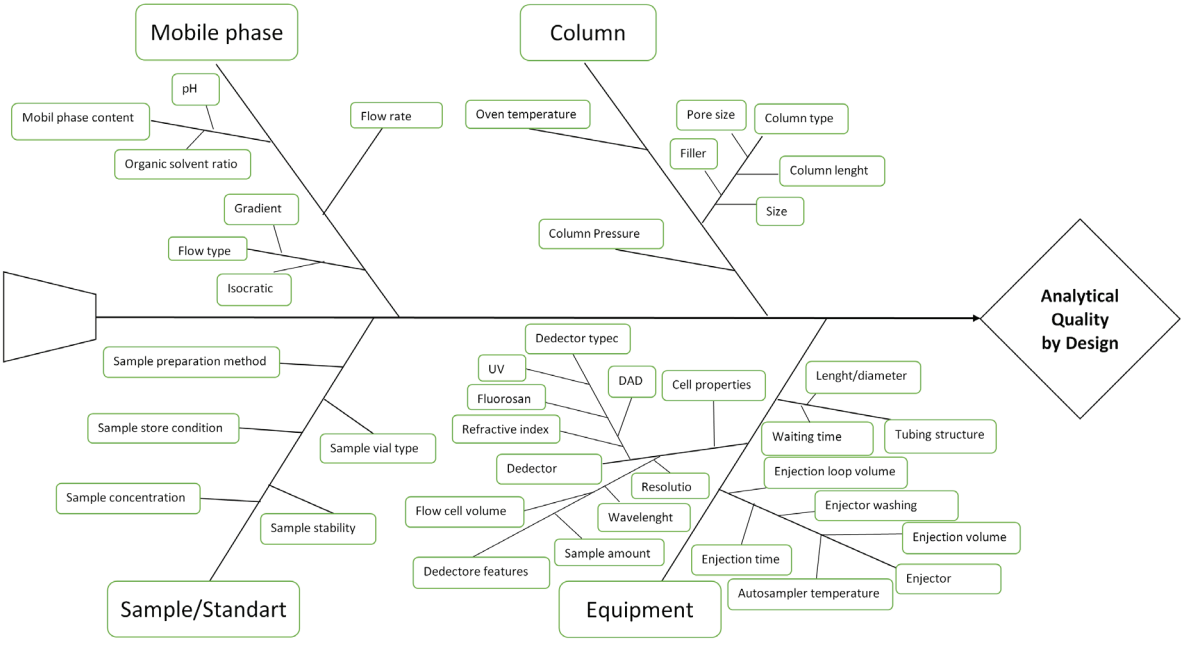

Figure 2. Fishbone diagram for HPLC AQbD studies.

1. Primarily QTPP should be created for the product to be developed. Finally, the analytical method that will be developed will affect the realization of product quality due to the purposes of this product.
2. CQA of pharmaceutical product should be detected.
3. ATP's should be detected for critical analysis determined by pharmaceutical product CQA.
4. The analytical method its decided with the detected ATP's.
5. CMA and CMP should be determined by evaluating the risk.
6. Parameters that affect the performance of the method that will be evaluated and the response of the analytical method are detected.
7. MODR areas are created.
8. Control strategy and consistent recovery is performed during and after method development.

Literature Examples

1) In a study reported in 2018 by Zacharis and Vastardi (Zacharis & Vastardi, 2018), Aprepitant's impurity p-toluenesulphonates HPLC method intending detection was developed by AQbD approach. The HPLC system was Shimadzu (SPD-20A PDA detector) (Kyoto, Japan).

Primarily, ATP's were detected;

The main purpose of the study was to develop solid, fast, selective and sensitive analytical method to determine of the impurity of the aprepitant.

In the study, the Plackett-Burman scanning design is performed to define Critic method parameters (CMP's) and search the effect of chromatographic parameters which were selected at the beginning on CMA.

As CMA;

- Resolution and separation activity between critical impurity peaks,
- Analysis process was selected.

The Fishbone method was used with risk evaluation to determine CMA's.

Tried on 12 study minimum (-1) and maximum (+1) values.

As CMP;

· Flow rate
· Gradient ratio
· Acetonitrile amount which was given at the first stage for gradient elusion was determined.

MODR;

Respectively Plackett-Burman and Box-Behnken was used for creation of design areas.

The most appropriate differentiation conditions were determined as requested criterion.

In statistical evaluations; ANOVA analysis, F value, p-value, Lack of Fi (LoF) and R^2 value were applied as essential methods.

2) Nompari and colleagues, in their study which published in 2018 (Nompari, Orlandini & Pasquini, 2018), they developed with UHPLC (Nexera X2 method scouting UHPLC series 30, Shimadzu (Kyoto, Japan)) within the scope of the AQbD approach intended to analyse the Bexsero meningococcal group B vaccine (Nompari et al., 2018);

As CMA;

- The capacity factor of Neisseria Heparin Binding Antigen
- Peak resolution of antigen
- Size of antigen peak area were selected.

Subsequently appropriate UHPLC study conditions were determined by preparing a preliminary test study. Critical method parameters were determined by using a Fish-bone diagram with a risk evaluation.

As CMP's detected with Quality Risk Management;

· Flow rate
· Gradient rate
· Acetonitrile amount given at the first stage of gradient elusion.

MODR;

Plackett-Burman and Box-Behnken designs were conducted in order.

The most appropriate differentiation condition in the method is determined as requested criteria.

3) Abdel-Moety and colleagues (2021) developed a new analytical method by using the AQbD approach (Abdel-Moety, Rezk & Wadia, 2021). In the study, the HPLC (Agilent HPLC-DAD system (1260 infinity II series), USA) system was used as analytical equipment. They aimed to analyse the content uniformity of the mixture of the Tamsulosin/Tadalafil and the mixture of the Alfuzosin/Solifenacin in the pharmaceutical dosage forms.

Critical quality attributes (CQA) were;

- Peak resolution
- USP tailing factor

As CMA and CMP;

- The Ethanol concentration (%) in the mobil phase
- Buffer solution pH value
- Type of buffer solution
- Addition of TEA (0,1 %) in the mobil phase
- Flow rate.

were chosen by using a Risk assessment method (Fishbone diagram). And, the Placket- Burmen method was chosen as the screening method.

4) Kopp and colleagues (2020) developed a new reverse phase liquid chromatography (Dionex UltiMate 3000, Thermo Scientific, USA) method to analysis protein quantification (Kopp, Zauner & Pell, 2020).

The CMPs were;

- Gradient time
- Column temperature.

2x2 full factorial design was applied.

CONCLUSION

As observed from the samples, it is recommended that AQbD fault evaluation and risk management should be applied widely in all analytical processes that apply statistical approaches and develop related analysis methods. With the applied AQbD approach it is aimed to develop the method's analytic response to be solid, fast and selective for the problems both related with the method and equipment.

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Review Article

The role of compaction simulator equipment in formulation design

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ABSTRACT

The assessment of the compaction performances of pharmaceutical powders is an important aspect of tablet product design, development and manufacturing. Compaction simulators have potential applications in pharmaceutical research and development in terms of studying basic compaction mechanisms, troubleshooting, various process variables, compaction data library creation, scale-up parameters, and fingerprinting of new active pharmaceutical ingredients (APIs) or excipients. The upscaling of the compaction process between early R&D and production can be time-consuming and costly, resulting in a long time-to-market and shorter commercial lifecycles. Due to the limited availability and high price of a new APIs during early drug development phases, compaction simulators have proven highly valuable as the dwell time and punch speed can be set accurately to mimic bottom and upper punch movement on a rotary tablet press.

Many issues inherent in the formula ingredients or acquired from previous processes can be avoided or reduced if applied correctly. However, if adequate attention is not paid to understanding the compaction behaviour of what is being pressed, this process may also be the source of several other problems. Pharmaceutical scientists now use a variety of instrumented presses to produce robust tablet formulations. They allow scientists to conduct experiments for in-depth analysis of compaction characteristics of pharmaceutical materials with great efficiency in terms of time, expense, and knowledge gained. The ability to use simulators for anything from early formulation studies to manufacturing troubleshooting makes them invaluable, particularly in light of the recent Process Analytical Technology (PAT)/Ouality by design (QbD) phenomenon.

Keywords: Compaction, compaction simulator, tablet press simulator

INTRODUCTION

Compaction is one of the most crucial unit operations in the pharmaceutical industry because it determines physical and mechanical properties of tablets such as strength and density (friability/hardness). The tablet compression process has an effect on dosage form integrity and bioavailability. Tablet compact production is a complicated process involving several variables and a variety of engineering principles, and a thorough understanding of compression physics is still a work in progress (Mohan, 2012).

There are several types of equipments that carry out powder compaction in the pharmaceutical area, in particular, these include the single-press, the rotary-press and the compaction simulator (Çelik & Marshall, 1989). Instrumented tablet punching devices, instrumented punches/dies, and compaction simulators can all be used to investigate mechanical aspects of tablet formation.

Compaction simulators have potential applications in pharmaceutical research and development in the study of basic compaction mechanisms, troubleshooting, various process variables, compaction data library creation, scale-up parameters, and fingerprinting of new APIs or excipients.

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Description and types of compaction simulator

Compaction simulation is an integrated compaction research system, which has been of scientific interest since the 1970s, with the first compression simulator described in 1976. The aim of compaction simulation is to be able to simulate industrial compression – that is, to be able to predict which parameters to apply to industrial equipment to obtain the desired properties in a tablet (Moulin & Kowalski, 2019).

Compaction simulators are defined as devices capable of mimicking in real-time, the exact cycle of any tablet press and recording the parameters. It enables a new approach in tableting research and is used to study powder compaction behaviour and fundamental material characterization using different compression parameters such as compression force and punch displacement (Reugger & Celik, 2016).

They are multifunctional equipments that can assist in all phases of the pharmaceutical industry's drug development and production (Celik, 2016; Celik and Marshall, 2010; Michaut et al., 2010).

Advantages and disadvantages of compaction simulators

Compaction simulators (Figure 1) have many benefits, including the ability to evaluate the following: tablet properties (strength, disintegration, and dissolution) under identical manufacturing conditions (since each individual tablet's compaction history is known); the ability to evaluate small amounts of material; basic compaction mechanisms; scale-up parameters; fingerprinting of actives, excipients, and formulations; build-up effects such as adhesion problems (much easier with mechanical rotary machine due to number of tablets produced per hour); the effect of process variables (speed, etc.); the effect of tooling variation.

The following are some of the most well-known drawbacks of all compaction simulators: While the available systems have paddle feeding systems, they cannot directly replicate the effect of the force feeder on a rotary tablet press; while basic machine operation is simple to learn, full machine use necessitates expertise in data manipulation and analysis; the effect of bulk flow on the rotary tablet press cannot be simulated; the centrifugal force caused by the turret rotation cannot be simulated (Celik 2016).

Types of compaction simulators

Compaction simulators are mainly divided into two categories:

1. Hydraulic compaction simulators
2. Mechanical compaction simulators

a. Mechanical linear compaction simulators

b. Mechanical rotary cam compaction simulators (Medelpharm, 2021)

Mechanical rotary cam compaction simulators (Stylcam)

The mechanical rotary cam compaction simulator (Stylcam, Medelpharm) is set up as a single station eccentric tablet press with two punch holders, with both punches following a programmable electronic cam that simulates a rotary tablet press. A revolving cam guides the movement of each punch holder. During the pre-compression and compression stages, the upper and lower punches are designed to travel in a symmetrical manner. Strain gauges in the upper and lower punch holders track the pressure on the upper and lower punches. The measurement of punch movement is made simpler by two displacement captors positioned on both the upper and lower punch holders (Medelpharm, 2021).

The two cams are mechanically synchronized and powered by a programmable motor that accelerates or stops to adapt the punches' movement. The Compaction Simulator can simulate the compression timings of any rotary press (including relaxation and, for most presses, ejection timing) and record all important parameters throughout the cycle (Celik & Marshall, 1989). Moving the upper and lower cams together or farther apart, similar to the rolls on a rotary press, adjusts the main compression force or tablet thickness (Celik, 2016).

Figure 1. Image of Stylcam 200R; mechanical rotary cam compaction simulator (Jiwa, 2020).

The device can run at a constant speed or the cycle's speed can be modulated during operation. This adaptability enables the simulation of various rotary tablet presses. No parts are needed to be changed to simulate various presses because the tablet press profiles are built into the device. Filling height, ejection height, pre-compression force, main compression force, and speed can all be adjusted. A transducer mounted on the feeding shoe senses the force needed to separate the tablet from the punch surface and can be used to measure tablet take-off force. Its ability to produce small batches of tablets in an automated mode adds to its versatility (Celik, 2016).

Using a compaction simulator, tablets are prepared under restricted conditions, for instance, the punches can be controlled and varied considerably. There are various applications that can be served through such machine. For example, the sensitivity of the drug to such variations (such as force) can be investigated. In addition to, the loading pattern of production presses can be mimicked in order to predict any future scale-up obstacles that may be present by using only small quantities of the materials needed (Jain, 1999).

Benefit of compaction simulator in the pharmaceutical area

Some benefits of the use of compaction simulator in the pharmaceutical area are as follows (Celik & Marshall, 1989):

- The effect of process variables (speed, etc.) can be determined.
- The effects of tooling variations, such as tooling type and punch shape can be studied.
- Scale-up parameters, such as type of press can be investigated.
- Build up effects such as adhesion problems.
- Basic compaction mechanisms can be determined, and the robustness of the formulation can be tested at high speeds (for different tablet presses).
- Tablet properties (strength, disintegration, dissolution) under identical manufacturing conditions can be tested (since the compaction history of each individual tablet is known).
- Fingerprinting of actives, excipients and formulations if possible.
- The effects of drug substance, excipient, or process change can be predicted.

Practical applications of compaction simulators

Compaction simulators can be used in practical applications in different phases of product development and manufacturing in the pharmaceutical area as seen in Figure 2 (Celik & Marshall, 1989):

Evaluation of compaction simulator data in the pharmaceutical area

Simulators have the ability to reproduce upper and lower punches displacement profiles in order to get information about powder compressibility (Celik, 2016). Compressibility profiles, which are functions of solid fraction *versus* applied pressure, are used to explain the fundamental mechanical behaviour of powders during compaction. These functions, which are collected during compression (in-die) or post ejection (out-of-die), show how much pressure is required to compress a given powder formulation to a given density or thickness. Several studies have been completed using a compaction simulator to evaluate powder compressibility by evaluating tensile strength values at different compaction forces (Al-Karawi et al., 2002; Arida and Al-Tabakha, 2008; Dudhat, Kettler & Dave, 2017; Muzíková & Zvolánková, 2007; Ruegger and Celik 2000; Wang, Wen, & Desai, 2010; York & Pilpel, 1973).

Data acquisition software (ANALIS, Medelpharm) allows you to construct comprehensive individual analyses, operating with single data points during a compression period or exploiting average values during batch processing, using standard reporting parameters like Heckel, force hardness ejection force, and, others. Studies on ejection force values produced by compaction simulator give insight into lubricant efficiency and optimization of lubricant concentration which is beneficial for formulators (Khan & Rhodes, 1976; Paul & Sun, 2018; Salpekar & Augsburger, 1974; Sun, 2015). A compaction simulator has the ability to perform Heckel Analysis which assists in characterizing the deformation behavior of materials using a simulator which gives better understanding of pharmaceutical excipients (Ozalp, Onayo, & Jiwa, 2020; Sun & Grant, 2001). Elasticity/plasticity, work of compaction, and time-dependent deformation behaviour of pharmaceutical powders are described using mathematical models, force-distance (De Blaey & Polderman, 1971; Moulin & Kowalski, 2019; Ragnarsson & Sjögren, 1985; Tay, Sun, & Amidon, 2019) force-time, (Leitritz, Krumme, & Schmidt, 1996; Yliruusi, 1997) and die-wall force parameters of tablet manufacturing produced by compaction simulator data (Hoblitzell, & Rhodes, 1990).

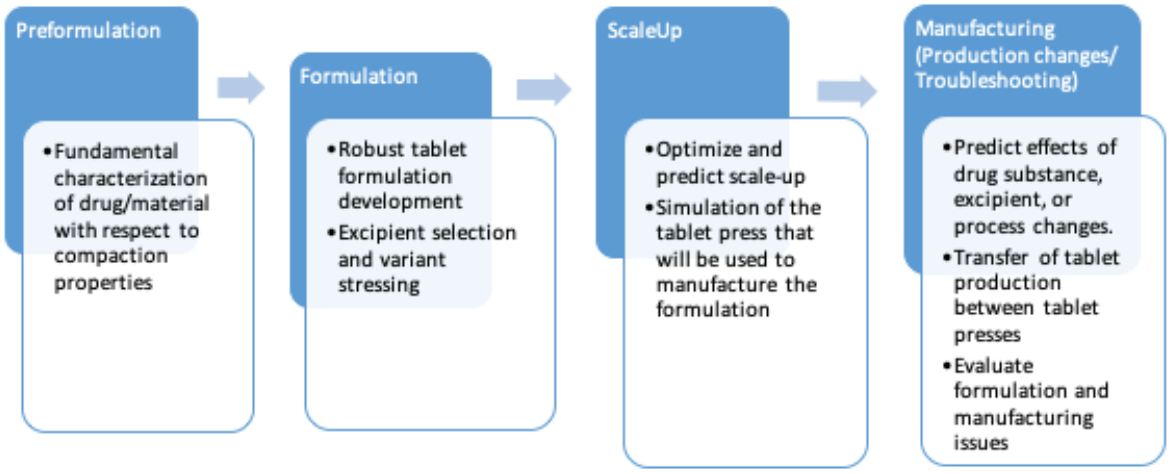


Figure 2. Illustration of practical applications of compaction simulators in the pharmaceutical area.

A compaction simulator produces force-displacement (F-D) curve data in order to evaluate the amount of energy used during the tabletting process, as seen in Figure 3 below. Due to variations in packing characteristics of individual formulation powders, the work of compaction provides a thorough assessment of the characteristics of tableting parameters, as shown by the F-D curve. Different plastic and elastic deformational properties, as well as different packing characteristics, absorb different quantities of energy, as shown by variations in compaction energy (Ozalp, Chunu, & Jiwa, 2020).

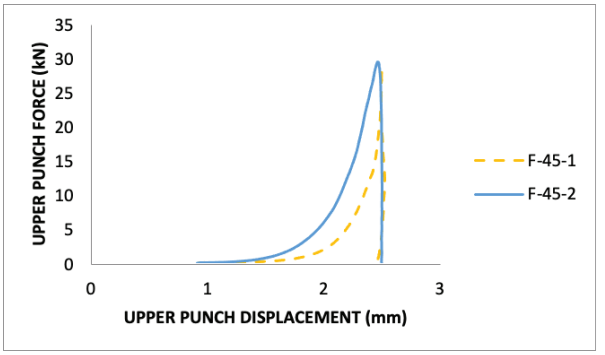


Figure 3. F-D curve for two different formulations, produced by compaction simulator (Ozalp et al., 2020).

The porosity and pressure are determined using an instrumented tableting presses or a compaction simulator, which allows the punch displacements to be measured simultaneously with the compressive forces (Celik & Marshall 1989; Cook & Summers, 1990; Duberg & Nyström, 1985; Jiwa, 2020; Newton & Grant, 1974; Nordström et al. 2013; Van der Voort Maarschalk & Bolhuls, 1999; Van Veen et al., 2000).

Figure 4 shows porosity plots of two pure fillers at varying compaction pressures produced by compaction simulator data. In-die porosity measurements give insight into powder compactability and volume reduction during the compaction process, which assist formulators in selecting suitable excipients (Jiwa, 2020).

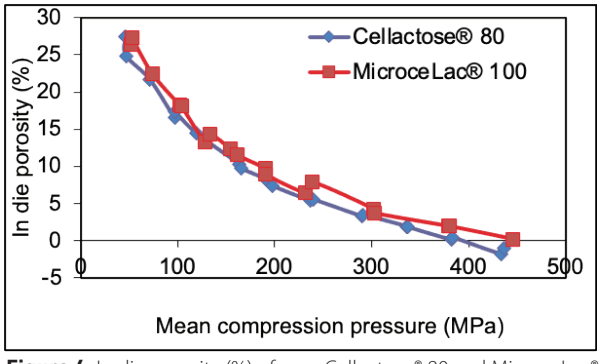


Figure 4. In die porosity (%) of pure Cellactose® 80 and MicroceLac® 100 at compression pressures between 5MPa and 450MPa (Jiwa, 2020).

Excipient research and fundamental material characterization with respect to compaction properties

The compaction simulator is a good tool to test the functionality and performance of excipients as well as characterise tabletting properties of powders. Simulators can be used to compare the compaction behaviour of different materials (excipients, APIs, formulations) in addition to evaluation of compressibility, deformation mechanism of powders both as pure materials and in formulations to determine the characteristics of the material (Busignies et al., 2006; Busignies et al., 2012; Heinz et al., 2000; Muzíková, & Zvolánková, 2007).

A recent study was carried out to characterize the behaviour of two fillers by observing the ejection force data produced by compaction simulator at compaction pressures between 50-450MPa (Figure 5) (Jiwa, Aksu, & Ozalp, 2020).

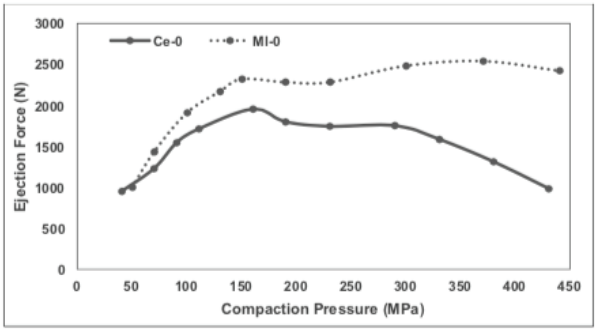
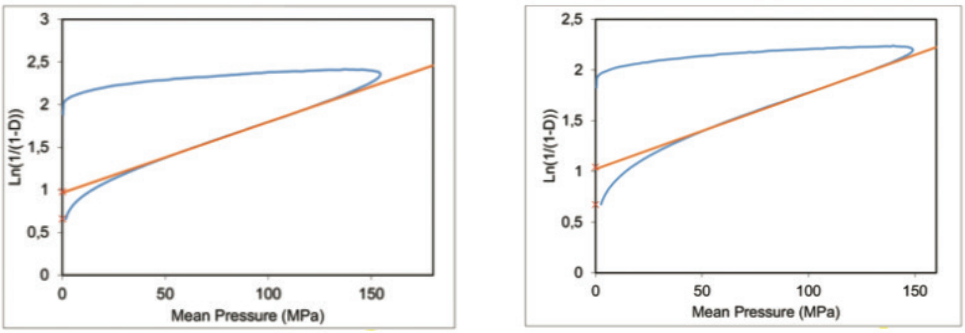


Figure 5. Comparison of ejection force data of pure Cellactose®80 (Meggle) and MicroceLac® 100 (Meggle) produced by a compaction simulator (Nailla, 2020).

Some models are used for compact production to classify tablet excipients. The predominant behaviour of powder densification and deformation (plastic, brittle and elastic) can be determined using simulators (Picker, 2000). Data from the Heckel study can be obtained using two methods, the tablet-in-die method (at pressure) and the ejected tablet method (at zero pressure) (Fell and Newton 1971; Heckel 1961). As seen in Figure 6, the compaction simulator readily produces in-die Heckel plots in order to evaluate powder densification and deformation characteristics of excipient. This gives insight for formulators when selecting excipients (Ozalp et al., 2020).

Formulation research and robust tablet formulation development

Simulators are used to develop formulation rules for selection of excipients. They assist formulators in the assessment and study of the effects of different methods for massing, drying, and blending as well as adjusting process parameters to obtain a robust formulation. New and robust formulations for manufacturing can be developed using compaction simulator in order to optimize the excipients, as well as amounts used. A study was completed in order to improve the compressibility and tablet characteristics of Paracetamol using both filler and binder using a compaction simulator. Results of tensile strength for different formulations seen in Figure 7 give insight into compactability of excipients at different compaction pressures (Ozalp et al., 2020).



(a) (b)

Figure 6. In-die Heckel plot for pure (a) StarLac® and (b) FlowLac®10 compressed at 150 MPa (Ozalp et al., 2020).

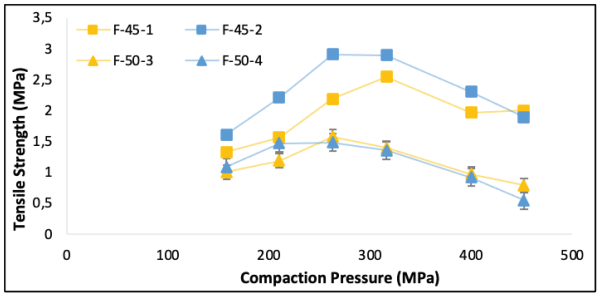


Figure 7. Tensile strength of formulations containing different concentrations of excipient combinations at different compaction pressures (Ozalp et al., 2020).

During formulation, a formulator should keep in mind that the speed and amount of pressure applied will almost certainly affect the formulation's compaction behaviour. As a result, for a given model of a given press, the formulation should ideally be run at a variety of speeds and pressure levels. Simulators are extremely useful for determining the impact of punch velocity on the compaction properties of powders in order to develop a robust formulation (Michaut et al., 2010; Tye & Sun, 2015). For problematic formulations in industrial production, compaction simulator is used as a troubleshooting tool in order to better understand and solve tabletting problems (e.g., capping, lamination, lubrication etc.) (Sun, 2015).

Simulation of the tablet press that will be used to manufacture the formulation

The compaction simulator has the ability to establish whether a particular formulation has scale-up potential. It is used to evaluate the transferability of product properties (such as tensile strength and porosity) from a compaction simulator to various scales of rotary presses for common pharmaceutical ingredients. To smoothen the transfer process, crucial process and system parameters can be defined and clarified. (Heinz et al., 2000; Natoli et al., 2017; Wünsch et al., 2020).

Transfer of tablet production between tablet presses

Manufacturers occasionally require tablet production to be transferred between machines as they aim to increase production or replace older equipment with newer models. Due to the complicated powder mechanics involved in tabletting, any changes in process that increase speed, duration, or compression force will cause a tablet formulation to fail to operate successfully on new equipment. Even minor changes to tooling design may affect powder compaction properties, leading to tablet failure due to capping or lamination (Dudhat et al., 2017). Simulators have the ability to evaluate changes in compatibility that can occur when transferring a substance from one machine to another, as well as decide alternate ingredients that may be used in a formulation (Celik, 2016).

CONCLUSION

Continuous manufacturing technologies deliver major advantages in terms of time and cost savings, flexibility, efficiency, and environmental impact during the manufacture of oral solid dosage forms. As a result, these technologies have piqued the attention of the pharmaceutical industry. Compaction simulators are one of the most integrated and versatile tabletting research tools available; they allow scientists to conduct experiments for in-depth analysis of compaction characteristics of pharmaceutical materials with minimal time, expense, and knowledge gained. The ability to use simulators for anything from early formulation studies to manufacturing troubleshooting makes them invaluable, particularly in light of the recent PAT/QbD phenomena.

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Review Article

Histone deacetylase inhibitors providing an epigenetic treatment in cancer

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ABSTRACT

Cancer is among the leading causes of death worldwide and is therefore one of the diseases in which there have been major medical advances and which is the focus of researchers. Drugs used in cancer treatment affect rapidly proliferating normal cells as well as cancer cells. In recent years, targeted therapy has been provided by identifying specific pathways in cancer cells. Epigenetic mechanisms are among the targeted therapies in cancer treatment. Epigenetic regulators ensure the continuity of the normal process by inducing epigenetic changes through epigenetic mechanisms such as DNA methylation, histone post-translational modifications, and non-coding RNA regulation. Histone deacetylases (HDACs), which are involved in transcription-independent events such as DNA repair and mitosis, are enzymes that remove acetyl groups attached to the lysine residue in the amino terminal tails of histones. Histone deacetylase inhibitors (HDACIs) that provide epigenetic treatment of cancer, which play a key role in the balance between acetylation and deacetylation of histone, have been extensively studied by researchers. Today, there are four HDACIs on the market approved by the FDA (Food and Drug Administration) and combinations of these drugs with agents that show anticancer activity by different mechanisms are being studied. Promising results have been obtained from these combinations, and further studies are ongoing on hybrid derivatives of certain HDACIs in various stages.

Keywords: Cancer, epigenetic mechanisms, histone deacetylase, histone deacetylase inhibitors

INTRODUCTION

Epigenetics is the study of inheritable changes in gene expression or phenotype that do not result from changes in the DNA sequence (Dupont, Armant, & Brenner, 2009). It has also been expressed as a bridge between phenotype and genotype (Korkmaz et al., 2011). These changes are called epigenetic changes and are crucial to the normal development processes of cells. Epigenetic regulators ensure the continuity of the normal process by inducing epigenetic changes through epigenetic mechanisms such as DNA methylation, histone post-translational modifications, and non-coding RNA regulation (Cao & Yan, 2020; Handy, Castro, & Loscalzo, 2011; İzmirli, 2013; Küçükoğlu, 2013). The presence of an abnormal epigenetic regulation in these epigenetic mechanisms causes many diseases such as cancer, autoimmune disorders, neurological diseases (Parkinson, Alzheimer, schizophrenia), inflammation, and metabolic disorders (Arrowsmith, Bountra, Fish, Lee, & Schapira, 2012; Moosavi & Ardekani, 2016). Therefore these mechanisms are therapeutic targets for researchers.

Post-translational modification is an important process in which certain groups such as acetyl and phosphate are transferred between proteins. These modifications include acetylation, phosphorylation, glycosylation, hydroxylation. Binding or separation of groups such as acetyl, hydroxyl, or phosphate to proteins affects cellular processes such as cell division, chromatin modification,

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gene silencing, protein-protein interactions, DNA replication, and apoptosis (Hitosugi & Chen, 2014; Karve & Cheema, 2011; Shukla & Tekwani, 2020). Histones examples of such proteins that undergo post translational modification in this way. As a result of acetylation, which is one of the post-translational modifications of histones, the chromatin structure changes and plays an important role in gene expression (Eckschlager, Plch, Stiborova, & Hrabeta, 2017), and HDACs are enzymes that remove acetyl groups attached to the lysine residue in the amino terminal tails of histones. As a result of the electrostatic interaction between the negatively charged DNA and the positively charged histone in the deacetylated state, a more compact chromatin structure is formed. In this state, the chromatin structure is "closed" and transcription is suppressed. When the histone is acetylated, the "open" chromatin structure is observed and transcription is active. Acetylation of histones and the level of transcription is controlled by the HAT (histone acetylase) and HDAC enzymes and is reversible (Gürel, Feyda Nursal, & Yigit, 2016; Meng et al., 2016). Additionally, HDACs are involved in transcription-independent events such as DNA repair and mitosis, and histones are not their only substrates. It deacetylates non-histone proteins involved in various physiological events such as cell proliferation and gene expression. It causes the accumulation of deacetylated forms of non-histone protein substrates such as DNA repair enzymes, inflammation mediators and transcription factors. For this reason, HDACs have been seen as drug targets due to their active role in transcription and transcription independent physiological events. Treatment with HDACIs result in acetylation of substrates, which is associated with transcriptional activation, but this treatment induces transcriptional suppressors, resulting in gene suppression. HDACIs are used in cancer treatment by causing apoptosis, growth arrest, inhibiting migration, invasion and angiogenesis. In addition, normal cells are more resistant to treatment with HDACIs than transformed cells and HDACIs have low toxicity in cancer treatment. This is one of the reasons why they are seen as drug targets (Martínez-Iglesias et al., 2008; Xu, Parmigiani, & Marks, 2007). HDACs can also influence transcription-independent events such as mitosis or deoxyribonucleic acid (DNA). The chromatin structure that changes with the addition and removal of the acetyl group to the chromatin structure via HAT and HDAC enzymes is shown in Figure 1 (Zuma & De Souza, 2018).

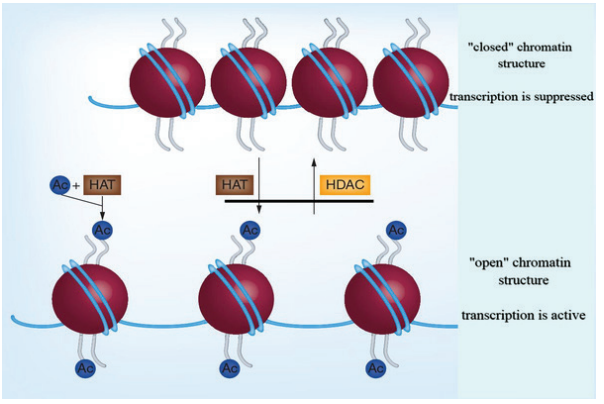

Figure 1. The "closed" and "open" chromatin structure formed by the transfer of the acetyl group via HAT and HDAC enzymes (Zuma & De Souza, 2018).

Structural components of typical histone deacetylase inhibitors

HDACIs consist of four main structural components: the zinc binding region, the linker, the surface recognition region (cap group) and the connecting unit between linker and surface recognition region. The zinc binding zone is located at the bottom of the 11 A° long enzyme pocket, and polar functional groups in the molecule chelate with the zinc ion therein. These functional groups can be hydroxamic acid, the first derivative, or nonhydroxamic acid derivatives, thiol, sulfone, carboxylic acid, boronic acid, trifluoromethylketone, and alpha substituted ketoamide.

The linker of hydrophobic character occupies the 11 A° long channel. The groups here can be linear, cyclic, saturated or unsaturated, and the residues in the channel change in different HDAC isoforms. This difference enables more selective inhibitors to be developed. The surface recognition region has groups to interact with residues at the entrance of the enzyme channel. These groups are aromatic heteroaromatic groups that are hydrophobic in nature. Many studies consider the connecting unit (or polar linker) to be within the surface recognition region and some as a separate pharmacophore (Bertrand, 2010; Ganai, 2019; Pontiki & Hadjipavlou-Litina, 2012).

As a result of the research, an internal cavity with a hydrophobic character and a length of 14 A° was discovered right next to the internal cavity. Since the size of this cavity differs between isoforms, it is seen as a target to achieve a selective effect (Pontiki & Hadjipavlou-Litina, 2012). In Figure 2, the enzyme sites where the inhibitors bind are observed in the X-ray crystal of bacterial histone deacetylase-like protein and Trichostatin A (TSA) (Finnin et al., 1999).

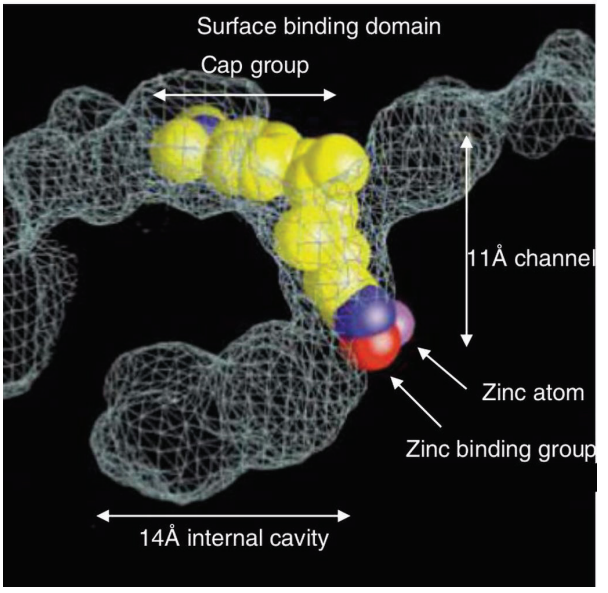

Figure 2. X-ray crystal of TSA in the active site of bacterial histone deacetylase-like protein (Finnin et al., 1999).

FDA approved histone deacetylase inhibitors

HDACIs have gained importance in cancer treatment due to their low toxicity and cancer cells being more sensitive to these inhibitors than normal cells (Martínez-Iglesias *et al*., 2008).

In treatment with conventional chemotherapeutic agents, changes occur in the tumor cell through epigenetic mechanisms and as a result, resistance to treatment begins (Fardi, Solali, & Farshdousti Hagh, 2018). Because of their effect on one of the epigenetic mechanisms, the use of HDACIs is promising as monotherapy in cancer treatment or in combination with traditional chemotherapeutic agents. Combination therapies of HDACIs with radiation therapy, kinase inhibitors (sorafenib and imatinib), and topoisomerases are in clinical trials. HDACIs, which are considered as chemosensitizers in combined therapy, show a synergistic effect by increasing the effect of other agents in combined therapy. In combination therapy with HDACIs, it has been shown that the response to traditional chemotherapeutic agents is increased, as well as the decrease in resistance to these agents (Fardi et al., 2018; Küçükoğlu, 2013; Martínez-Iglesias *et al.*, 2008; Suraweera, O'Byrne, & Richard, 2018).

Vorinostat (SAHA)

Vorinostat (suberoylanilide hydroxamic acid), also known as SAHA, was approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma (CTCL) patients and is an orally used HDACI. As emphasized by the name SAHA, the drug is a derivative of hydroxamic acid. (Cappellacci, Perinelli, Maggi, Grifantini, & Petrelli, 2018; Mann, Johnson, Cohen, Justice, & Pazdur, 2007). The effect of DMSO (dimethyl sulfoxide) on murine erythroleukemia cells led to the discovery of HMBA (hexamethylene bisacetamide). A series of bishydroxamic acids derivatives have been synthesized due to HMBA's inhibitory effect on cell growth in altered cells. The best activity among them was observed with vorinostat. The similarity of vorinostat to TSA allowed the discovery of its HDACI effect (Marks & Breslow, 2007). The chemical structures of vorinostat, HMBA, DMSO and TSA are shown in Figure 3.

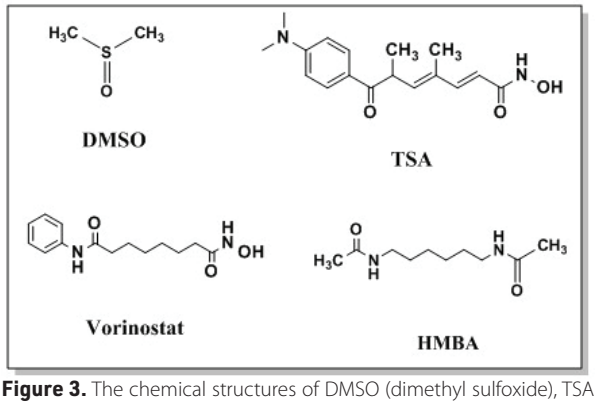

Figure 3. The chemical structures of DMSO (dimethyl sulfoxide), TSA (Trichostatin A), HMBA (hexamethylene bisacetamide) and vorinostat.

Vorinostat has an inhibitory effect on HDAC I and II enzymes at nanomolar levels (Marks & Dokmanovic, 2005). The compound, which binds to the zinc ion in the catalytic region of the enzyme, acts as an inhibitor in this way and shows an anticancer effect by preventing the deacetylation of histone protein like other HDACIs (Yoo & Jones, 2006).

Patients with nonsmall cell lung cancer (NSCLC) and hepatocellular carcinoma (HCC) do not respond to treatment with a single therapy with tyrosine kinase inhibitors due to the development of resistance over time. The combination of vorinostat with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) gefitinib or a multi-target kinase inhibitor sorafenib was investigated *in vitro* and *in vivo* study. Vorinostat has been found to have the potential to restore the reduced sensitivity of the cancerous cell due to the development of resistance and to lower the concentration required for cell death (Jeannot et al., 2016). Moreover, hybrid molecules such as CUDC-101 have been developed as a result of the synergistic effect of HDACIs and EGFRIs together and the sensitizing effect of HDACIs. CUDC-101 is a multi-target inhibitor that inhibits not only EGFR and HDAC but also human epidermal growth factor 2 (HER2) (Lai et al., 2010; Parag-sharma et al., 2021). The chemical structure of CUDC-101 is shown in Figure 4.

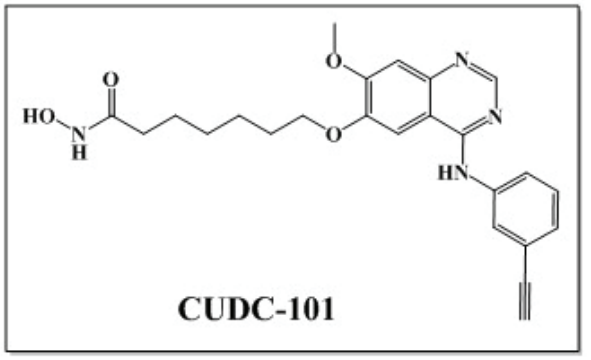

Figure 4. The chemical structure of CUDC-101.

Combined treatment of vorinostat with belinostat and dexamethasone against multiple myeloma is new because vorinostat, which is HDACI, and belinostat, which is a proteasome inhibitor, show anticancer effects through different mechanisms. This triple combination therapy against multiple myeloma has yielded good results in the phase II trial. However, a toleration problem has been observed. Research suggests that more specific HDACIs with different toxicity profiles may provide more tolerance (Brown et al., 2021).

Various effects of vorinostat on cervical cancer have been demonstrated, such as inhibition of proliferation, migration and invasion. Natural killer (NK) cells are part of the immune response against tumor cells. Increased MICA (major histocompatibility class I related chain A) in cervical cancer cells affects the ability of NK cells to recognize and kill them. MICA induced by vorinostat increases the ability of NK cells to kill and recognize cervical cancer cells, so vorinostat is considered as an immunotherapeutic enhancer (Xia, He, Cai, & Liang, 2020).

Panobinostat (FARYDAK, LBH589)

Panobinostat is an orally used inhibitory drug that targets HDAC I, IIa, IIb and IV enzymes (Park, Terranova-barberio, Zhong, Thomas, & Munster, 2017; Raedler, 2016). The drug was approved by the FDA in 2015 to treat people with multiple myeloma who have previously had at least 2 standard regimens. This standard regimen includes bortezomib and immunomodulatory drugs. Panobinostat is effective at the nanomolar

level. The drug, which reaches its maximum concentration approximately 2 hours after oral ingestion, is eliminated in the liver by the CYP3A4 enzyme (Moore, 2016; Raedler, 2016). Like other histone deacetylase inhibitors, it removes acetyl groups attached to lysine residues in histone or nonhistone proteins. Removal of the acetyl group from the lysine residue leads to relaxation of the chromatin structure and, consequently, transcriptional activation occurs. As a result of the accumulation of these acetylated proteins, apoptosis and cell cycle arrest are observed in abnormal cells (Raedler, 2016). The chemical structure of panobinostat is shown in Figure 5.

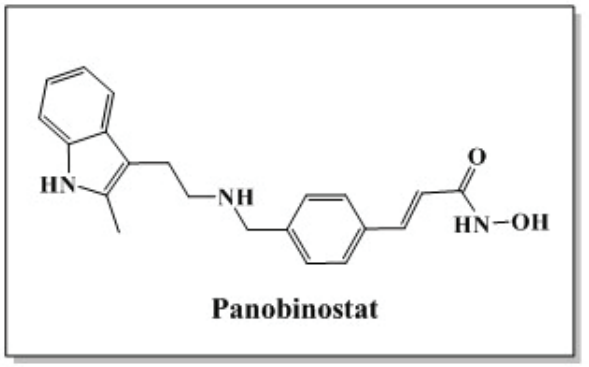

Figure 5. The chemical structure of panobinostat.

It has been suggested that HDACIs have a therapeutic effect against glioblastoma multiforme (GBM) using various mechanisms such as inhibition of angiogenesis or cell cycle arrest. Thereupon, the anti-GBM efficacy of the combined treatment of HDACI panobinostat and dual PI3K / mTOR inhibitor BEZ235 was investigated. This binary combination has been shown to synergistically induce apoptosis, inhibit cell growth and proliferation in GBM cells. In addition, this combination shows these effects through various mechanisms such as increasing caspase 3/7 activity (Meng et al., 2019). The chemical structure of BEZ235 is shown in Figure 6.

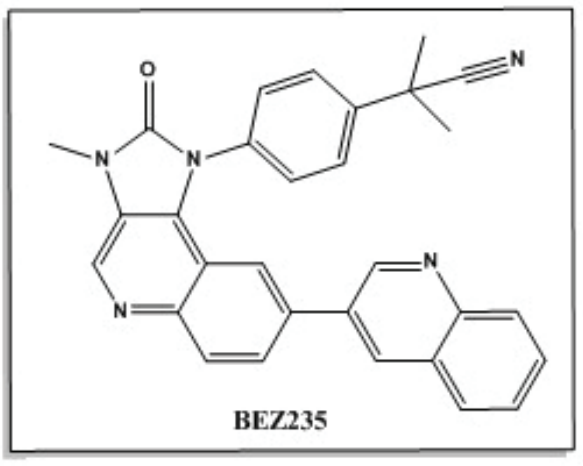

Figure 6. The chemical structure of BEZ235.

Various studies have shown that some HDACI drugs, such as vorinostat and panobinostat, have sensitizing effects against ovarian cancer. These HDACIs block the removal of acetyl groups in a number of histone and nonhistone proteins as mentioned earlier. The increased acetylation level of heat shock protein 90 (HSP90) by panobinostat leads to a decrease in chaperone activity. Furthermore, resistance to drugs that cause DNA damage such as cisplatin develops. Priming with HDACIs such as panobinostat has been shown to sensitize ovarian cancer cells to HSP90 inhibitors and cisplatin, but triple therapy with HDACI, HSP90 inhibitor and cisplatin has no advantage (Moita et al., 2020; Ozaki et al., 2008).

In a phase I study conducted with 12 menopausal metastatic breast cancer patients, it was concluded that the combination therapy of panobinostat with an aromatase inhibitor letrozole is an effective potential therapy, especially in patients who have been evaluated to have chemotherapy and endocrine resistance (Tan et al., 2016). The chemical structure of letrozole is shown in Figure 7.

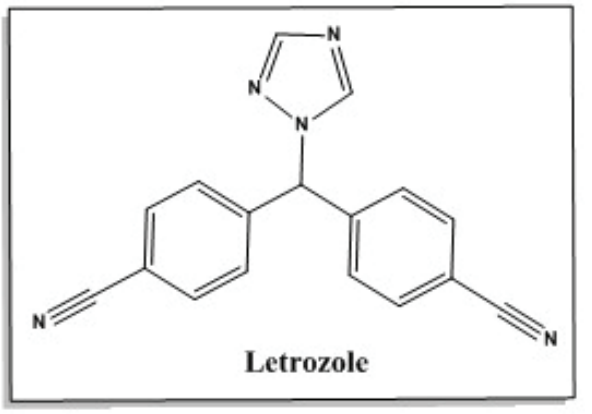

Figure 7. The chemical structure of letrozole.

Belinostat (PXD-101)

Belinostat is an inhibitor of HDAC I, II and IV enzyme isoforms, approved by the FDA in 2014. FDA approval was based on the results of a trial conducted with 120 patients and the drug has been approved for the treatment of patients with relapsed or refractory peripheral T-cell lymphoma (Campbell & Thomas, 2017; Poole, 2014). The chemical structure of belinostat is shown in Figure 8.

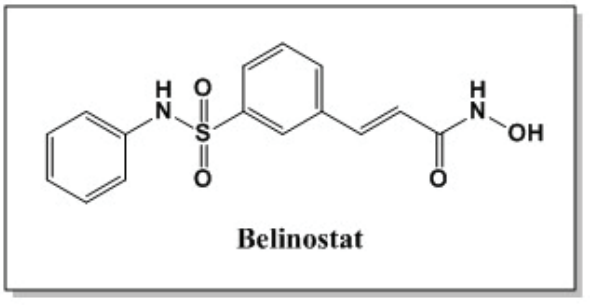

Figure 8. Chemical structure of belinostat.

In a phase II study of belinostat in women with epithelial ovarian cancer (EOC), belinostat was tested and found to be well tolerated in platinum-resistant EOC patients. However, it was concluded that belinostat as a single agent did not show

enough efficacy for further research (Mackay et al., 2010). In another study, a synergistic effect of belinostat with paclitaxel and carboplatin on ovarian cancer was observed, whereupon a phase 1b/2 clinical trial was conducted to further investigate the synergistic effect of belinostat with carboplatin and paclitaxel in female patients with EOC. In this phase 1b/2 trial, women with ovarian cancer had previously received platinum therapy, and patients were evaluated as platinum sensitive or platinum-resistant according to the time elapsed after the platinum treatment. In this trial, it was shown that the overall response rate (ORR) to treatment was 43% and which was due to the heterogeneous distribution of the patients compared to the previous platinum treatment. It was not possible to evaluate the benefit of belinostat over paclitaxel and carboplatin combination therapy. Therefore, it was decided that a study in which patients were classified according to their sensitivity to platinum was required (Dizon et al., 2012).

Romidepsin (Depsipeptide or FK228)

Romidepsin is a naturally occurring compound that was first isolated in 1994 from the Gram negative bacterium *Chromobacterium violaceum*. The compound was evaluated as a novel antitumor antibiotic in 1994 and received FDA approval in 2011 for the treatment of peripheral T-cell lymphomas (PTCLs) (Barbarotta & Hurley, 2015; Shigematsu, Ueda, Takase & Tanaka, 1994; Ueda et al., 1994). Romidepsin is a prodrug that becomes active as a result of the reduction of the intramolecular disulfide bond. The thiol group formed by reduction coordinates the Zn^{2+} ion of the enzyme (Cappellacci et al., 2018; Porter & Christianson, 2019). The chemical structure of romidepsin is shown in Figure 9.

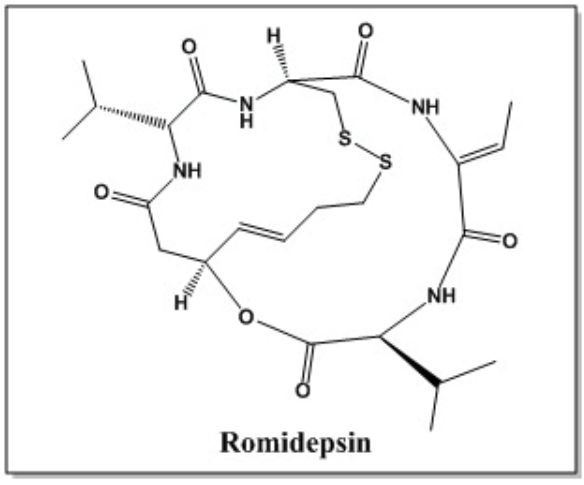

Figure 9. Chemical structure of romidepsin.

Combinations of romidepsin with various drugs such as ifosfamide, carboplatin and etoposide on PTCL were found to be more effective than romidepsin alone, but it was observed that the toxicity was higher in these combinations. For this purpose, due to the low toxicity of bendamustine, the combination of this drug with romidepsin was tried in patients with relapsed/ refractory T-cell lymphoma who had previously received treatment. Romidepsin/bendamustine combination was observed to have a greater effect on patients with relapsed/ refractory T-cell lymphoma than either drug alone. In the study, it was emphasized that the combination showed acceptable toxicity, but this clinical data belongs to a small patient group and this combination deserves further research (Nachmias et al., 2019).

Pirfenidone and nintedanib are two drugs approved for the treatment of idiopathic pulmonary fibrosis (IPF), a progressive disease. However, these drugs have undesirable side effects, and no treatment for IPF has stopped the decline in lung function. After demonstrating in a phase II study that some HDACIs such as vorinostat and panobinostat have antifibrotic properties and romidepsin has selectivity against lung cancer, the antifibrotic effects of romidepsin was investigated *in vivo* and *in vitro*. As a result of the research, the antiproliferative and antifibrotic effects of romidepsin were found to be potent and it was stated that these effects supported the evaluation of romidepsin as a new treatment for IPF in clinical research (Conforti et al., 2017).

Future Directions

Epigenetic therapy has proven its importance and success for cancer treatment. For this reason, in order to develop more effective and specific molecules and to reduce side effects, it is important to understand both the structures of enzyme isoforms and their differences from each other and the mechanism of action of these epigenetic drugs. In this way, isoform-specific molecules can be designed and more biomarkers can be discovered by understanding their mechanism of action. Thus, a mechanism-based treatment approach is provided (Küçükoğlu, 2013; Shukla & Tekwani, 2020).

Another important issue is the epigenetic profiles of the patients. The epigenetic and genetic profile of each patient is unique. The epigenetic and genetic profile of the patient should be known in order to obtain an optimum response in these combined treatments with epigenetic drugs. Otherwise, no response may be obtained from the treatment. In the future, patients can be screened with more precise methods and classified according to their genetic structure. With all these considerations, a personal, unique treatment should be provided for each patient (specific drug should be selected) in order to achieve optimal effect as well as reduce side effects and toxicity (Fardi et al., 2018; Martínez-Iglesias et al., 2008; Schwartsmann et al., 2000).

At this point, biomarkers should be determined. HDAC levels in the patient can be used as a biomarker and have prognostic importance. In this way, patient subgroups that will benefit from HDACIs can be determined and specific treatment can be provided for the patient (Stimson & La Thangue, 2009).

Another major promising topic is hybrid HDACIs. There is a paradigm shift from "one drug one target" theory to "multi-target drug". Combination of HDACIs with other anticancer drugs is being investigated in preclinical and clinical studies and many combinations are known to have synergistic effects. For this purpose, hybrid molecules have been designed to reduce both side effects and drug-drug interactions, and also to achieve this synergistic effect with a single molecule. Hybrid drugs designed with the protection of HDACIs and other

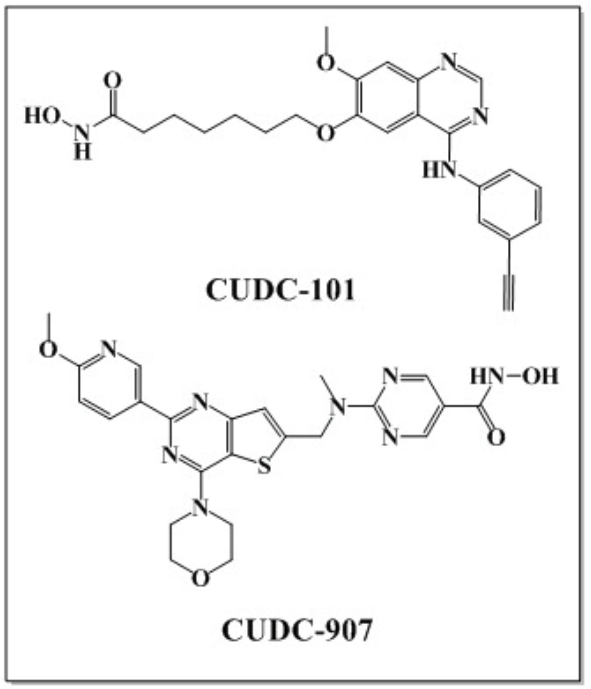

Figure 10. Chemical structures of CUDC-101 and CUDC-907.

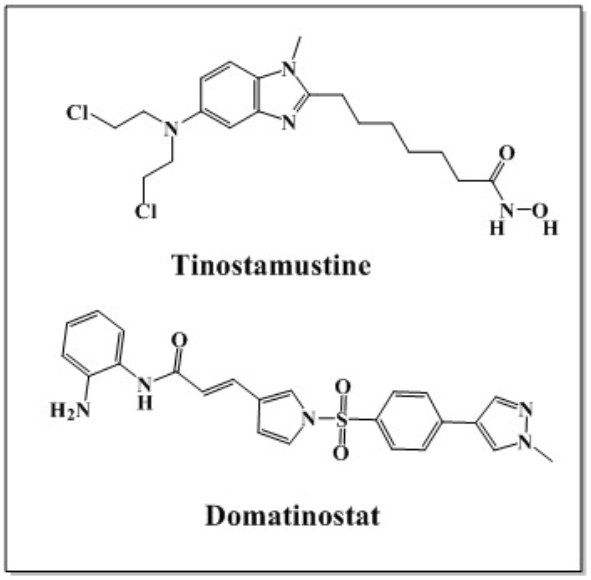

Figure 11. Chemical structures of tinostamustine and domatinostat.

anticancer drug's pharmacophore groups that interact with the enzyme can simultaneously inhibit various cancer-related targets or pathways. Hybrid molecules of HDACIs have been designed with many drug groups such as topoisomerase, protein kinase, proteasome, phosphodiesterase type 5 (PDE5) and bromodomain containing 4 (BRD4) inhibitors. Of these, CUDC-101 (a molecule mentioned earlier) and fimepinostat (CUDC-907) are hybrid molecules in various clinical trial phases for the treatment of different types of cancer (Bass et al., 2021; Vaidya et al., 2021). Fimepinostat (CUDC-907), a dual inhibitor of HDAC and PI3K enzymes, has been shown to have more tumor growth inhibiting and pro-apoptotic activity in various cancer lines than single targeted agents that inhibit these enzymes (Younes et al., 2016). The chemical structures of CUDC-101 and CUDC-907 are shown in Figure 10.

Domatinostat is a hybrid molecule that targets both HDAC and LSD1, which was investigated in the phase I trial for the treatment of patients with hematological malignancies and demonstrated good tolerability and efficacy in this study (von Tresckow et al., 2019; Wobser et al., 2019). The chemical structures of tinostamustine and domatinostat are shown in Figure 11.

CONCLUSION

HDACIs are a promising class of anticancer drugs that has caught the attention of researchers. Many studies have shown that epigenetic changes play an important role in cancer development, tumorigenesis or resistance to the anticancer drug used. There are currently four FDA-approved HDACIs, although their effects against solid tumors as a monotherapy are limited. Belinostat, vorinostat, panobinostat, and romidepsin are approved for the treatment of patients with peripheral and/or cutaneous T-cell lymphoma and multiple myeloma. In addition, the effects of these drugs in combination with antitumor drugs that affect different pathways such as protein kinases, topoisomerases, proteasome inhibitors or their effects with radiation therapy have been investigated in various clinical studies. Studies have shown the important effects of HDACIs, such as regaining reduced sensitivity with the development of resistance to these drugs and reducing the dose required for treatment. Although HDACIs are successful in combination therapy, a new approach is to rationally design hybrid molecules to minimize drug interactions and side effects. Phase studies of some hybrid molecules targeting both the HDAC enzyme and another cancer pathway are ongoing. With the discovery of specific effective and multi-targeted new drugs, it will be possible to reduce side effects and increase efficiency in cancer treatment.

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

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

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