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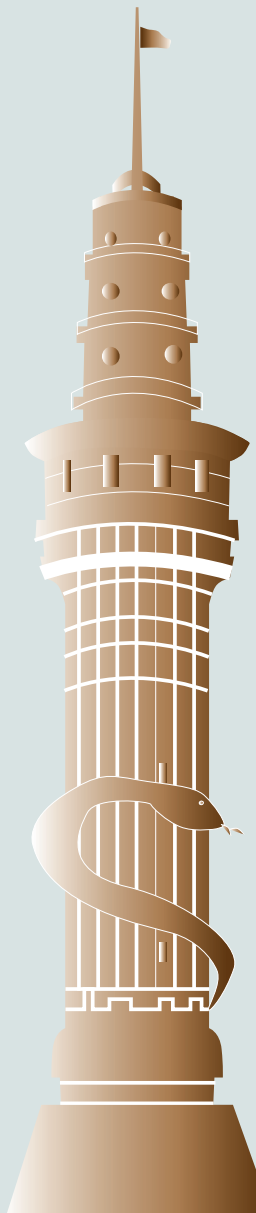

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Letters to the Editor: This type of manuscript discusses important parts, overlooked aspects, or lacking parts of a previously published article. Articles on subjects within the scope of the journal that might attract the readers' attention, particularly educative cases, may also be submitted in the form of a "Letter to the Editor." Readers can also present their comments on the published manuscripts in the form of a "Letter to the Editor." Abstract, Keywords, and Tables, Figures, Images, and other media should not be included. The text should be unstructured. The manuscript that is being commented on must be properly cited within this manuscript.

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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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Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100×100 mm). Figure legends should be listed at the end of the main document.

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 main text. The abbreviation should be provided in parentheses following the definition.

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

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

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Type of manuscript	Word limit	Abstract word limit	Table limit	Figure limit
Original Article	3500	250 (Structured)	6	7 or total of 15 images
Review Article	5000	250	6	10 or total of 20 images
Short Paper	1000	200	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	No tables	No media

Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

References

While citing publications, preference should be given to the latest, most up-to-date publications. If an ahead-of-print publication is cited, the DOI number should be provided. Authors are responsible for the accuracy of references. In the main text of the manuscript, references should be cited by the author(s) surname and the publication date. When there are more than two authors, the first author should be listed followed by "et al." Please see below for examples:

One author: (Ergenç 2000)
Two authors: (Ergenç and Rollas 2000)
More than two authors: (Ergenç et al. 2000)
More than one paper in the same year by the same author (s): (Ergenç and Rollas 2000a, b)
Listed by the earliest year first for multiple citations: (Ergenç and Rollas 2000; Ergenç et al. 2001; Ergenç 2005)

The references must be listed alphabetically in the references section. The names of the journals should be written in italics and volume numbers should be indicated in bold letters. Journal titles should be abbreviated in accordance with the ISSN List of Title Word Abbreviations.

The reference styles for different types of publications are presented in the following examples.

Journal Article: Orlacchio A, Campos-Cavieres M, Pashev I, Munn EA (1979) Some kinetic and other properties of the isoenzymes of aspartate aminotransferase isolated from sheep liver. *Biochem J* **177:** 583-593.

Book Section: Benn MH, Jacyno JM (1983) The toxicology and pharmacology of diterpenoid alkaloids. In: Pelletier SW (ed./eds.) Alkaloids: Chemical and Biological Perspectives, Vol. 1, John Wiley & Sons, New York, pp. 153-210.

Books with a Single Author: Bremer K (1994) Asteraceae: Cladistics and Classification. 1st ed, Timber Press, USA.

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Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, Ferris FL 3rd; Early Treatment Diabetic Retinopathy Study Research Group. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study Kidney Int: 2004. Report No: 26.

Dissertation (Thesis): Gürdal B (2010) Ethnobotanical Study in Marmaris District (Muğla). Unpublished MSc Thesis, İstanbul University, Institute of Health Science, İstanbul.

Manuscripts Accepted for Publication, Not Published Yet: Slots J (1974) The microflora of black stain on human primary teeth. *Scand J Dent Res.*

Article by DOI: Ermut G, Karalı N, Özsoy N, Can A (2014) New spiroindolinones bearing 5-chlorobenzothiazole moiety. *J Enzyme Inhib Med Chem* doi: 10.3109/14756366.2013.800058.

Manuscripts Published in Electronic Format: (2014) World Nuclear Association. Radioisotopes in Medicine, http://www.world-nuclear. org/info/inf55.html,www.world-nuclear.org/info/inf55. html. Accessed 13.10.2014.

Treglia G, Ceriani L, Sadeghi R, Giovacchini G, Giovanella, L. (2014) Relationship between prostate-specific antigen kinetics and detection rate of radiolabelled choline PET/CT in restaging prostate cancer patients: A meta-analysis, Cli Chem Lab Med. http://www.reference-global.com/toc/cclm/current Accessed 16.09.2014.

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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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CONTENTS

ORIGINAL ARTICLES

REVIEW

Istanbul J Pharm 49 (3): 121-124
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Original Article

The assessment of *in vitro* cardiotoxic potentials for synthetic cannabinoid, AM-2201

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ABSTRACT

Synthetic cannabinoid abuse has become more common in recent years, although knowledge about the risk of the relatively new synthetic cannabinoid molecules is not adequate. Data is limited and analytical methods and case reports related to the clinical effects of this substance are recent and new. The studies are generally related to the cardiac effects of first defined molecules rather than every molecule in the group. The cardiac clinical effects of synthetic cannabinoid abuse and its underlying mechanisms are not certain. In this regard, this study aims to investigate AM-2201, one of synthetic cannabinoids, because knowledge related to AM-2201 is less than the others in this group. The cardiotoxicity and underlying mechanisms of AM-2201 were assessed on cardiac cell culture. The half-maximal inhibition concentration (IC_{50}) values were 101.49 and 63.33 µM by WST-1 and LDH assays. AM-2201 did not induced the reactive oxygen species (ROS) levels. Correlatively, no change was observed in total antioxidant capacity (TAC) levels. As to the measurements, Annexin V-FITC and acridine orange dye, AM-2201 did not induce apoptosis and the primary cell death was necrosis. According to our results, further studies such as mechanism on cell death and cancer pathways should be investigated.

Keywords: AM-2201, apoptosis, cardiotoxicity, cytotoxicity, oxidative stress, synthetic cannabinoid

INTRODUCTION

Synthetic cannabinoids elicit cannabimimetic effects similar to Δ9-tetrahydrocannabinol (Δ9-THC) which is the primary psychoactive component of cannabis through interaction with CB1 and CB2 cannabinoid receptors. Because synthetic cannabinoids are full agonist and can bind to the CB1 receptor with an affinity greater than Δ9-THC, they are more potent and have a longer effect than Δ9-THC. So, they are associated with more severe and dangerous health effects than marijuana (Mckeever et al. 2015; Castellanos and Gralnik 2016).

Synthetic cannabinoids, which are considerably new molecules, were initially developed for research purposes as potential therapeutics; however, their abuse was firstly reported and gradually increased in the early 2000s (Debruyne and Boisselier 2015; Cooper 2016; Hess et al. 2016). This increase in synthetic cannabinoids abuse is attributed to intense psychoactive effects and lack of detectability in routine drug screening tests. Despite the molecules being synthetic and consisting of unknown mixtures of chemicals (mostly more than one type of synthetic cannabinoid or other drugs), users believe that the products are natural and harmless (Castaneto 2014; Ibrahim and Al-Saffar 2014; Mckeever et al. 2015; Castellanos and Gralnik 2016). In synthetic cannabinoid abuse, agitation or irritability, anxiety, confusion, psychosis, nausea and vomiting, shortness of breath, tremor, and seizures have been mostly reported (Castaneto 2014; Mckeever et al. 2015). Cardiovascular side effects such as tachycardia, hypertension, chest pain, and myocardial infarction have been reported in case reports (Aksel 2015; Atik et al. 2015). Most studies in this area have been

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about the effects of synthetic cannabinoids on the nervous system, whereas, knowledge about the toxic effects of synthetic cannabinoids on the cardiovascular system is limited. This study aims to define the toxic potentials and underlying mechanisms of AM-2201, synthetic cannabinoid, on cardiac cells.

MATERIALS AND METHODS

Cell culture conditions: The rat cardiomyoblast cell line, H9c2 (CRL1446™), was obtained from the American Type Culture Collection (ATCC), and cultured according to the manufacturer's instructions. AM-2201 was obtained from Lipomed (JWH-1503-1, Arlesheim-Swiss). AM-2201 (1 mg) was dissolved in DMSO (27 µL) to prepare the stock solution (100 mM).

For all tests, the cells were seeded in 6-well or 96-well plates at a density of 2.5 x 10^4 cells/mL, and cultured in 37°C for 24 h. After pre-incubation, each well was treated with 0-100 µM AM-2201 for 48 h. The assays and tests were done in triplicate.

Cytotoxicity: WST-1 (Roche Cell Proliferation Reagent, Germany) and LDH (Roche, Germany) assays were performed according to the manufacturer's instructions.

In WST-1 assay, after exposure to AM-2201, 10 µL of the WST-1 solution was added to each well. After incubation for 30 minutes at 37°C, the samples were analyzed using a microplate reader at 420 nm (Biotek, Epoch, Germany). In LDH assay, after exposure to AM-2201, 100 µL/well supernatant was removed and transferred into a clear microplate. To determine the LDH activity in the supernatants, 100 µL reaction mixtures were added to each well, and incubated for 30 minutes protected from light. Then, the absorbance of the samples were measured using microplate reader at 490 nm. The assay medium (200 µL) for background control and Triton-X solution (100 µL) for positive control were added to wells containing 1x10^4 cells/100 µL/well. The unexposed cells were evaluated as the negative control. To determine cytotoxicity, the absorbance values were measured using a microplate spectrophotometer system (Epoch, Germany). Then, the inhibition of enzyme activity observed in the cells was calculated with the absorbance values and compared to that of unexposed cells. The concentration-cell death (%) curves were used to calculate the half maximal inhibition concentrations (IC_{50}) that are responsible for the death of 50% of the cells. The cytotoxicity was evaluated with IC_{50} values.

Oxidative stress: The induction of ROS production was evaluated by 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Sigma-Aldrich, Germany) dye using flow cytometry (Acea Novocyte 1000, California, USA) at Ex/Em: 488/535 nm (Schupp et al. 2008). The controls and the exposed cells were adjusted to be 1x10^7 cells/mL. At least 1x10^4 cells in 6-well plates were analyzed, the results were shown as percent of florescence intensity. Phosphate buffer serum (PBS) was used as a negative control. In the results, the presence of ROS was expressed as the percentage of the total cell quantity.

For TAC assay, Total Antioxidant Capacity Assay Kit (Sigma-Aldrich, Germany) was used according to the manufacturer's instructions. The stock Trolox solution was prepared at 1 mM in DMSO and stored at -20°C. One part of Cu^{+2} reagent was diluted with 49 part of assay diluents for the Cu^{+2} working solution. The working concentrations of Trolox were 4, 8, 12, 16 and 20 nmol/well. After exposure to AM-2201, the Cu^{+2} working solution (100 µL) was added to every well, and incubated for 90 min protected from light. Then, the absorbance was measured at 570 nm by a microplate reader. By using Trolox standard curve, the amounts of Trolox in the samples were calculated as in the following equation:

$$TAC_{Sample} = Ts / SA$$

Ts: sample volume added in the sample wells (µL)

SA: TAC amounts in the sample well calculated from standard curve (nmol)

Apoptosis: Annexin V-FITC detection kit (Biolegend, CA, USA) was used according to the manufacturer's instructions. The fluorescence intensity was measured in FITC and PI channel (at 494/518 nm (Ex/Em) for Annexin V-FTIC and 535/617 nm (Ex/Em) for PI) by flow cytometer. Necrotic cell as Annexin V^-/PI^+, living cell as Annexin V^-/PI^-, early stage apoptotic cell as Annexin V^+/PI^-, late apoptotic cells as Annexin V^+/PI^- expressions appeared. The results were expressed as the percentage of total cell quantity.

Autophagy: Acridine orange dye (Sigma-Aldrich, Germany) was used (Mitou et al. 2009; Gözüaçık 2011). After exposure to AM-2201, the wells were washed with PBS (1X), detached with trypsin (0.2%), and then centrifuged. Then, the cells (2.5x10^5) were suspended in the cell medium, and incubated with acridine orange suspension (2%) in the dark for 15 minutes. Finally, the absorbance value was analyzed at 488/676 nm (Ex/Em) with PerCP filter by flow cytometer. The results were expressed as the percentage of autophagy seen cells to total cell quantity.

Statistical analysis

Data were expressed as mean ± standart error of mean (SEM). One-way ANOVA, Post Hoc and Dunnet t-test using SPSS version 23 for Windows (IBM Corp.; Armonk, NY, USA) was used to analyse the data. $p<0.05$ indicates a statistically significant difference.

RESULTS AND DISCUSSION

Despite the increasing use of synthetic cannabinoids, the reported serious adverse effects and limited toxicology data make their abuse an important public health issue. Some case reports have reported a large number of side effects on the different organs and systems. On the cardiovascular system, tachycardia, hypertension, dysrhythmia, chest pain, cardiac ischemia and myocardial infarction are common adverse clinical effects for synthetic cannabinoid abuse. Knowledge about the toxic effects of synthetic cannabinoids on the cardiovascular system is limited. Most of the studies are about the toxic effects of synthetic cannabinoids on the nervous system. Therefore, we aimed to investigate AM-2201, one of synthetic cannabinoids, because knowledge of AM-2201 is less than that of the others in this group. In addition, we evaluated the potential of cytotoxic, oxidative and apoptotic damage of AM-2201 on H9c2 rat cardiomyoblast cell line.

Firstly, WST-1 and LDH assays were performed for the cytotoxic effects of AM-2201. The IC_{50} values were 101.49 and 63.33 µM by WST-1 and LDH assays, respectively (Figure 1). The LDH leakage assay was observed more sensitive than WST-1 assay for AM-2201 cytotoxicity in H9c2 cell line.

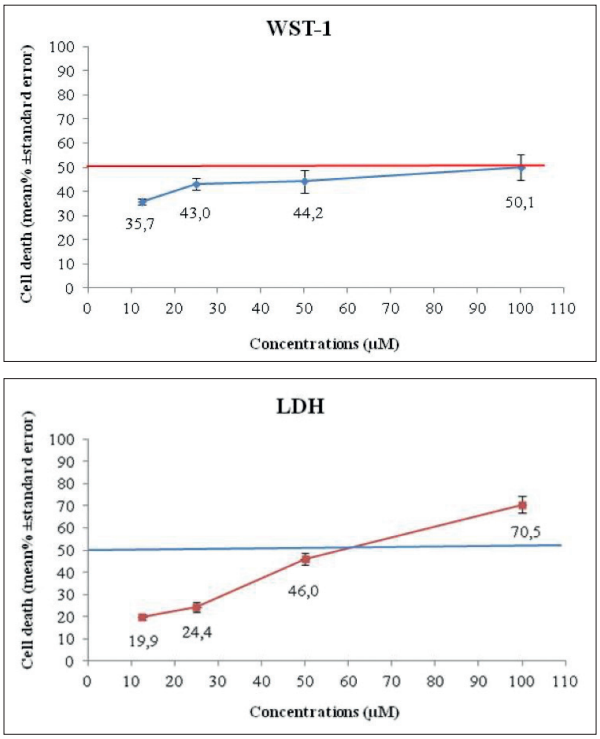

Figure 1. The effects of AM-2201 on H9c2 cell viability by WST-1 and LDH.

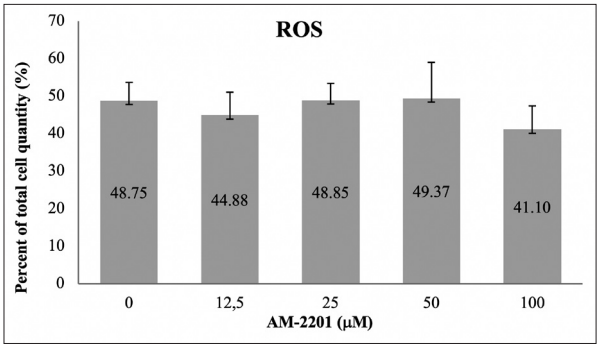

Figure 2. The ROS levels in H9c2 cells exposed AM-2201 at 12.5, 25, 50 and 100 mM concentrations.

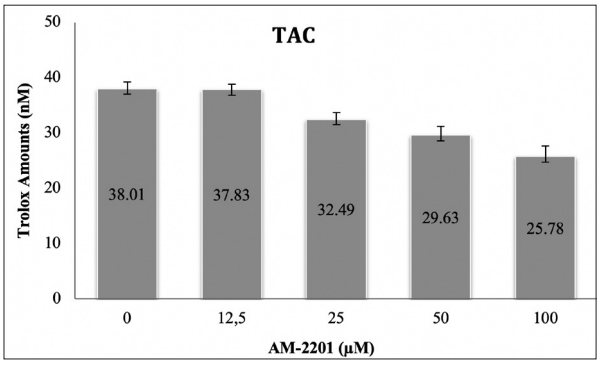

Figure 3. Total antioxidant capacity of H9c2 cells exposed AM-2201 at 12.5, 25, 50 and 100 mM concentrations.

Tomiyama and Funada (2011) reported that CP-47,497, CP-47,497-C8 and CP-55,940 were significantly cytotoxic for 2 h exposure at 30 µM. CP-55,940 caused nuclear fragmentation and condensation at 30 µM concentration. They also reported that their cytotoxic effects were significantly suppressed by the CB1 receptor antagonist AM-251, but not by the CB2 receptor antagonist AM-630. In the other study conducted by Tomiyama and Funada (2014), it was observed that eight synthetic cannabinoids (HU-210, CP-55,940, CP-47,497, CP-47,497-C8, JWH-018, JWH-210, AM-2201, and MAM-2201) induced a significantly cytotoxic effect in a dose-dependent manner on primary mouse neuronal cells. They reported that the relative cytotoxicity levels of AM-2201 were 2.4 and 2.8 at 10 µM and 30 µM AM-2201, respectively, to the control group. In comparison with our results, the primary mouse neuronal cells could be more sensitive than mouse cardiomyoblast cells to AM-2201.

In the present study, the type of cell death was determined by staining with Annexin V-FTIC-PI. At the highest concentration (100 µM), early stages of apoptosis were not observed, and late apoptosis was determined to be maximum 1.17% (data not shown). Total apoptosis results were shown in Table 1. It was observed that the necrosis ratio was maximum 12.76%. At 100 µM exposure, apoptosis and necrosis were increased 3 and 9.73 fold, respectively, compared to the control ($p>0.05$) (Table 1).

Tomiyama and Funada (2011) reported that the number of Annexin V^+ cells was significantly increased at 2 h after treatment with CP-55,940. Also, some morphological changes were induced by CP-55,940, which were reflected the necrotic or late apoptotic cells. Couceiro et al. (2016) indicated JWH-018 metabolite, N-(3-hidroksipentil), decreased the cell via necrosis on human kidney and neuroblastoma cell lines. Similarly, we observed necrotic cell death after treatment with AM-2201.

In the present study, the ROS levels were observed to decrease 15.69% at 100 µM compared to control ($p>0.05$) (Figure 2). The TAC levels also decreased depending on exposure concentration, however it was not statistically significant ($p>0.05$). The decrease of TAC was ≤32.18% at the exposure concentrations (Figure 3). However, the results should be ignored because a

Table 1. The results of apoptosis and necrosis in the H9c2 cardiomyoblast cells treated with AM-2201

Concentrations (µM)	Apoptosis (mean%± standard error)	Necrosis (mean%± standard error)
Negative control	0.31±0.03	1.03±0.08
12.5	0.36±0.04	3.94±0.07*
25	0.48±0.06	8.27±1.12*
50	0.76±0.08*	7.22±1.05*
100	0.94±0.08*	8.05±1.11*

*p>0.05, one-way ANOVA, Post Hoc and Dunnet t-test.

high cytotoxicity level was observed at these exposure concentrations.

Rajesh et al. (2010) investigated the cell death and signal transduction pathways related to CB1 receptors activated via endocannabinoid anandamide and HU-210 (synthetic agonist) in human coroner artery endothelial cells. They reported endocannabinoid anandamide and HU-210 increased ROS levels, and also CB1 receptor antagonist or antioxidants (acetylcysteine) decreased ROS levels. However, Almada et al. (2017) observed that WIN-55,212 decreased 16% of cell viability in human choriocarcinoma and primer culture of placenta cytotrophoblast cells. Also, they reported that WIN 55,212 did not induce ROS formation. Similar to Almada et al. (2017), we observed that AM-2201 did not induce ROS production. However, the results were not statistically significant ($p>0.05$) even if AM-2201 decreased ROS levels in rat cardiomyoblast cells.

Data about the effects of autophagy induction of synthetic cannabinoids is not available in the literature. In our study, the autophagic effects of AM-2201 were detected with acridine orange dye. In the rat H9c2 cardiomyoblast cells exposed to AM-2201 during 48 h, the autophagy ratio to total cell was determined to be 57.24-72.40%. The induction was ≤1.28 fold compared to control, however, the results were not statistically significant ($p>0.05$) (Figure 4).

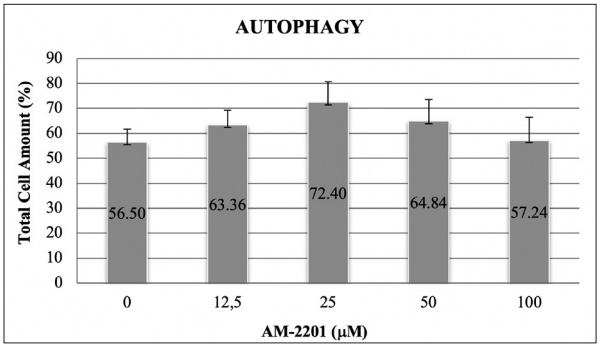

Figure 4. The autophagy-inducing potentials of AM-2201.

In conclusion, we observed necrotic cell death principally under the concentrations of IC_{50} values (<63.33 μM). Based on our results, it could be concluded that the cardiotoxic effect of AM-2201 might be due to hypertrophic and/or arhythmogenic effects rather than direct cardiomyoblast cell death effects. Therefore, it is considered that further studies should be carried out in terms of the effects of metabolites, in hypertrophic signal pathways, and cardiac conduction pathways. Besides, knowledge of most synthetic cannabinoid packages includes multiple synthetic cannabinoid types and other psychoactive ingredients. Therefore, the investigation about interactions between synthetic cannabinoids and other drugs need to be researched.

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

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Original Article

Determination of pharmacy pharmacists and pharmacy employees' knowledge of and attitudes towards magistral drug preparation in Malatya/ Turkey

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ABSTRACT

Magistral drugs are preferred when present preparations are not sufficient. Even though their usage had decreased, it is predicted that magistral applications will be used more often in the future. In this study pharmacists and pharmacy employees working in 95 pharmacies in Malatya city center agreed to participate in the study (n=203) and filled in a questionnaire which was prepared according to literature. In the analysis of the obtained data, frequency and percentages were calculated using SPSS 24.0 program. It was observed that 11.1% of the pharmacies were close to a health center and 88.0% of the participants were preparing magistral drugs. 55.2% of the participants stated that they prepared magistral medications 1-2 times a week. According to the results, although the frequency varied with the localization of pharmacies, magistral drug preparation ratios were generally similar to those found in the literature. Preferred magistral drug forms were: creams (74.7%), pomades (69.8%) and solutions (54.7%). Of the participants who declared themselves to be inadequately skilled in magistral drug preparation, 19.5% said that the prescriptions were insufficient and 33.3% reported that the doctors' prescriptions were incomplete or unreadable.

Keywords: Magistral formulas, pharmacy workers, knowledge, preparation, Malatya/Turkey

INTRODUCTION

Magistral drugs are prepared by pharmacists according to the prescriptions of doctors (Geçgil 1991). Extemporaneous, compounding, off-label and unlicensed are terms also commonly used instead of magistral (Conroy et al. 2000; Kairuz et al. 2007). The common points for all terms are: they have at least one raw material, they are produced on a non-industrial scale and they are prepared in a suitable pharmaceutical form for the individual.

Industrial developments and advances in pharmaceutical technology has reduced the need and usage of magistral drugs. With such advances, more drugs can be produced in a shorter time and in a cheaper manner (Baytop 1997). However, genetic differences in people and the psychological motivations of a person based medication have received attention to magistral preparations again (Kairuz et al. 2007a; Ilgın Ruhi 2010). In cases where readymade preparations are insufficient, magistral applications are preferred for the patient-drug-dosage equilibration (Minghettiu et al. 2000; Martin et al. 2009; Schellekens et al. 2017).

The ability to prepare magistral formulas is considered an important skill for pharmacists in many countries (Täerel et al. 2014; Kristina et al. 2017). Although the frequency of magistral drug preparation in different countries has been reported to be 1-10% in recent years, these rates vary (Kristina et al. 2017). Difficulties in finding appropriate drug forms in newborn and childhood peri-

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ods, usage of some drugs in the treatment of rare diseases lead to the prescription of magistral formulas (Staubach and Metz 2013; Garcia 2015; Pérez 2016; Staubach and Weisshaar 2016; Schellekens et al. 2017). The ability to form different forms of drugs in essential doses alongside raw material procurement is an important advantage, but pharmacokinetic issues and certain other problems are faced in magistral drug preparations (Leal et al. 2012; Sklenár et al. 2013; Pereira et al. 2016).

The increasing importance of clinical pharmacy globally and in Turkey has made research into magistral drugs mandatory. Although there many global and European studies (Conroy et al. 2000; Brion et al. 2003; Kairuz et al. 2007a; Kairuz et al. 2007b; Neubert et al. 2008; Sellers and Utian 2014; Kristina et al. 2017), there are not enough studies in Turkey (Andaç et al. 2015). There is not sufficient data regarding the current state of the magistral drugs. Research into magistral medication will be important in increasing levels of pharmacist competence (Kristina et al. 2017).

The aims of this study are: to determine the scope of magistral drug applications, to establish the frequency of magistral drug applications, to identify the problems in preparing and providing magistral drug preparation among pharmacists. Similar studies will provide important viewpoints in the future of pharmaceuticals and pharmacies.

MATERIALS AND METHODS

This research was carried out in order to determine the information/attitudes of pharmacy pharmacists and pharmacy employees towards the preparation of magistral drugs and the problems they encounter in preparing magistral formulations. The universe of the study was made up of those pharmacists and pharmacy employees working in the city center of Malatya between 2017-2018 (Malatya Eczacılar Odası, 2018). The aim of the study was to reach the entire universe without using a sample selection method. The research was completed with 203 pharmacists and pharmacy employees working in 95 pharmacies. Necessary permissions were obtained for conducting the research from the Malatya Clinical Research Ethics Committee (Ethics Committee no. 2018/7-5). After the visits to the pharmacies and the purpose-method of the study were explained, 203 pharmacists and pharmacy employees agreed to participate in the study on a voluntary basis. In the collection of data, a questionnaire form which was prepared by the researchers according to the literature was used. The questionnaire consisted of questions about the following: pharmacy-specific features, socio-demographic characteristics of the employees, the magistral drug preparation ability of the employees, magistral formula preperation frequency, knowledge and attitudes towards magistral drugs (Kairuz et al. 2007a; Kairuz et al. 2007b; Neubert et al. 2008; Zaid et al. 2012; Andaç et al. 2015). The questionnaires were completed in 5-10 minutes and the forms were collected by the researchers.

Statistical analysis

The data was analyzed using the IBM SPSS 24.0 program (IBM, Armonk, NY, USA), and the frequency and percentages were determined.

RESULTS

When the descriptive features of the pharmacies were investigated; 29.1% (n=58) were determined to be on the street, 28.6% (n=57) were on side streets, 25.6% (n=51) were near to hospitals and 11.1% (n=22) were near to health centers. The number of years the pharmacies had been operating differed from 1 to 76 and the average number of years was 14.87±13.36. No statistical significance was found when location and working years of the pharmacies were compared with magistral drug preparation status ($p>0.05$) (Table 1).

Of the pharmacy employees; 22.8% (n=46) were pharmacists, 7.8% (n=16) were technicians (associate degree), 69.2% (n=137) were male, 54.5% (n=110) were high school graduates and the average age was 34.23±10.22. When magistral drug preparation status was compared with the features of the pharmacy workers, pharmacy workers who had not graduated from university were determinated to prepare magistral drugs more ($p=0.016$) Also there was no statistical significance in working time of participants and self-sufficiency levels ($p>0.05$) (Table 2).

Among the pharmacy employees; 88.0% (n=176) stated that they prepared magistral drugs, 66.3% (n=122) pointed out that they only prepared prescribed preparations and 55.2% (n=100) said that magistral drug preparation frequency was 1-2 times a week. 19.5% (n=32) of the participants stated that they felt inadequately skilled in magistral drug preparation. While the main reasons for this status were prescription inadequacies (33.3%, n=12) and lack of adequate information resources (25.0%, n=9), 5.6% (n=2) of the participants stated that they had forgotten their knowledge on this topic.

Magistral prescriptions were determined to come from: state hospitals (75.0%, n= 138), private hospitals (72.8%, n=134), dermatology (95.3%, n=122), ear-nose and throat (ENT) (50.3%, n=69) and general surgery (17.0%, n=20) polyclinics (Table 3).

Table 1. Comparison of the magistral drug preparation status and the features of the pharmacies

		Magistral Drug Preparation Status				
		Yes		No		
		n	%	n	%	Significance
Location of the pharmacy	Not near to a health center	79	59.0	6	40.0	X^2=1.97
	Near to a health center	55	41.0	9	60.0	p=0.179
Working years of the pharmacy	1-9 years	47	43.9	4	36.4	X^2=0.23

Table 2. Comparison of magistral drug preparation status and the features of the pharmacy workers

		Majistral Drug Preparation Status				
		Yes		No		
		n	%	n	%	Significance
Working time of participants	<15 years	16	22.5	3	42.9	X2=1.42
	≥1 years	55	77.5	4	57.1	p=0.352
Education level	High school or less	61	74.4	4	36.4	X2=6.66
	University	21	25.6	7	63.6	p=0.016
Feeling self-sufficient	Yes	90	79.6	8	80.0	X2=0.73
	No	23	20.4	2	20.0	p=0.670

Table 3. From which polyclinics do frequently prescribed magistral formulas come?

Branches	N	%	Branches	N	%
Dermatology	122	95.3	ENT	69	50.3
General surgery	20	17.0	Internal medicine	21	17.2
Anesthesia and reanimation	16	11.7	Pediatrics	14	9.4
Infectious Diseases	10	6.4	Oncology	9	5.8
Neurology	7	4.7	Urology	7	4.7
Orthopedic	6	4.1	Ophthalmology	6	4.1
Transplantology	6	4.1	Gynecology	6	4.1
Psychiatry	5	3.5	Cardiology	4	2.3
Radiology	4	2.3	Gastrology	2	1.2

Table 4. Which pharmaceutical dosage forms are preferred as magistral formulations?

Pharmaceutical dosages form	N	%	Pharmaceutical dosages form	N	%
Cream	95	74.7	Ointment	96	69.8
Solution	82	54.7	Lotion	72	50.9
Shampoo	56	46.5	Powders	42	33.5
Emulsion	38	30.4	Suspension	37	28.4
Capsule-Cachet	31	25.9	Suppository	16	13.5
Pockets	14	10.7	Ovule	7	7.1
Liniment	8	5.9	Colloids	5	3.6

Table 5. Problems of pharmacy employees when preparing a prescription for magistral medication

	N	%
Non-payment of some prescriptions in the SGK system	129	82.7
Incomplete or unreadable prescription from doctors	100	67.8
Lack of raw materials	99	67.6
Inability to supply raw materials quickly	73	54.9
Unknown formula	60	45.8
Dissolution shortage	42	32.6
Calculation shortage	36	28.6
Too many pharmaceutical packaging forms	32	26.7

N: Number of respondents, %: Percentage of respondents.

The pharmaceutical forms of the desired magistral formulas were found to be: creams (74.7%, n=95), ointments (69.8%, n=96), solutions (54.7%, n=82), lotions (50.9%, n=72) and shampoos (46.5%, n=56), respectively (Table 4).

The answers to the question 'How often do you experience problems (as outlined in table 5), when preparing a prescription for a magistral drug?' were: payment problems with the Social Security Institution (SGK) system (82.7%, n=129), incomplete or unreadable prescriptions (67.8%, n=100), lack of raw materials (67.6%, n=99) and the inability to supply the raw material quickly (54.9%, n=73) (Table 5).

Pharmacy workers stated that they mostly consulted the 'Magistral Formulas Book' (14.2%, n=26) and professional experience (9.8%, n=18) in preparation of magistral formulas while 64.5% (n=118) stated they used more than one source (Table 6).

Of the pharmacy workers who said 'yes' to the question 'Is there a magistral formula that you do not want to prepare even though it is prescribed?'(19.1%), twentyseven (n=27) of them gave the reason for their answer as; technical hardware problems (33.3%, n=9), responsibilities of green and red prescriptions (22.2%, n=6) and raw material supply problems (18.5%, n=5) in order (Table 7).

Most of the pharmacies that did not prepare magistral formulas were experiencing raw material problems. The low frequency of prescriptions caused problems, both in supplying and maintaining raw materials (Table 8). Although most of the pharmacies often prepared frequently prescribed formulas, only a few pharmacies stated that they prepared rarely prescribed formulas. No one among the respondents stated that they did not trust themselves.

Pharmacy workers who said 'no' to the question 'Do you feel sufficient self-sufficiency in preparing a magistral prescription?' (19.5%, n=36) gave the reason for their answer as: insufficient prescriptions from doctors (33.3%, n=12) and lack of source books (25.0%, n=9) (Table 9).

Only one (0.6%) pharmacy employee answered the question 'What are the patients attitudes for non-prescribed magistral formulas?' as 'negative'. It was understood that patients did not usually give negative feedback on magistral formulas (Table 10).

Of the pharmacy employees, 44.7% (n=85) stated that vocational training programs in 'magistral formulas preparation' were organized, and 22.0% (n=42) said that these programs were sufficient. Of the employees, 89.5% (n=171) thought that vocational training programs should be organized in this regard.

When the thoughts of pharmacy workers regarding magistral formulas were questioned; 71.9% (n=133) stated that 'Magistral drug applications are useful applications for health professionals, pharmaceutical companies and health authorities', 62.2% (n=115) stated that 'Magistral drugs or drug mixes should not be made by employees without training', 60.5% (n=112) stated that 'More authority and responsibility should be given to pharmacists and pharmaceutics employees about magistral

Table 6. Source of information in preparation of magistral drug

	N	%
Book of magistral formulas	26	14.2
Based on professional experience	18	9.8
Books of medical formulas	7	3.8
Pharmacopeia and Codex	4	2.2
Consult with experienced colleagues	4	2.2
To consult doctor information	2	1.1
Internet	1	0.5

Table 7. The reasons for not preparing magistral formulas in pharmacies

	N	%
Technical hardware problems	9	33.3
Responsibilities in narcotic prescription and others	6	22.2
Raw material supply problems	5	18.5
Financial worries	4	14.8
Time problem	2	7.4
Distrust of patients towards magistral drugs	1	3.7

Table 8. Why do pharmacies not prepare prescriptions?

	N	%
Difficulties in supplying raw materials	12	6.1
Lack of raw materials to meet the prescription	11	5.6
Low prescription frequency, need to buy raw materials and then having to expire them due to expiration dates	9	4.5
Not enough laboratory facilities	6	3.0
Difficulties in entering the prescription in the system and being afraid of SGK interruptions	4	2.0
Difficulties in supplying, recording and preserving toxic and separate substances	3	1.5
Considering that the written magistral formulation is incompatible with the diagnosis and not wanting to take responsibility	2	1.0
Difficulties in reading prescriptions	1	0.5
Unsuitable hygiene conditions in the pharmacy	1	0.5
Finding ready drugs more reliable when compared with magistral formulations	1	0.5

Table 9. Causes of not feeling sufficient in preparing a prescription for magistral medication

	N	%
Insufficient prescriptions of doctors	12	33.3
Lack of enough information resources in pharmacies related to these prescriptions	9	25.0
Lack of enough education about pharmaceutics in university	3	8.3
Contradiction between information sources	3	8.3
Having forgotten their knowledge over time	2	5.6
Other reasons	7	19.4

Table 10. What are the patients' attitudes towards non-prescribed magistral formulas?

	N	%
No negative feedback so far	117	69.6
Although we have a few problems, we usually receive positive feedback	27	16.1
There has been no positive or negative feedback so far	23	13.7
We have received negative feedback	1	0.6

Table 11. Thoughts about magistral formulas

	N	%
Magistral drug applications are useful applications for health professionals, pharmaceutical companies and health authorities	133	71.9
Magistral drugs or drug mixes should not be made by employees without training	115	62.2
More authority and responsibility should be given to pharmacists and pharmaceutics employees regarding magistral drugs	112	60.5
Herbal and alternative medicine products should be prepared and presented as magistral in pharmacies	106	57.3
Pharmacies should be able to receive academic or direct government support for the improvement and modernization of laboratory environments	105	56.8
Clinical studies on magistral formulas should be made and approved	104	56.5
Magistral formulas are more effective in terms of patient motivation when compared with readymade drugs	70	37.8
Good pharmaceutical manufacturing practices (GMP) standards are achieved in pharmacy laboratories	58	31.4
Definitions, laws and regulations about magistral drug applications are sufficient	50	27.0
There is no disadvantage to preparing magistral formulas for children or pregnant women	44	23.8
Magistral formulas should only be applied externally or peroral	27	14.6
There should be no need for magistral formulas, all drugs should be produced by pharmaceutical companies according to certain standards	23	12.4

Table 12. Thoughts on the necessity of magistral formulas

	N	%
Magistral formulas contribute to pharmacy turnover	135	79.9
Contribution to the motivation of colleagues as a morale factor	83	57.2
Provide facility in production of special personal drugs (intermediate dose, etc.)	80	53.7
Provide preparation of personal and home care medications	74	49.7
They are functional cosmetic products and supplements	59	42.4
Provide the production of medicines with new formulas and stability problems	60	42.3
Provides the production of drugs with import problems	54	37.5
Has strategic importance in social negativities such as war and economic crises	52	35.6
Provides advantages for the preparation of orphan drugs (less frequently sold drugs)	43	30.3
Provides access to non-industrial drugs at a cheap price	33	24.3

drugs', 57.3% (n=106) stated that 'Herbal and alternative medicine products should be prepared and presented as magistral in pharmacies' (Table 11).

The pharmacy workers stated; 'Magistral formulas contribute to pharmacy turnover', 'Magistral formulas contribute to the motivation of colleague as a morale factor', 'Magistral formulas provide facility in production of special personal drugs (intermediate dose, etc.)', 'Magistral formulas provide preparation of personal and home care medications' by 79.9%, 57.2%, 53.7%, 49.7% (Table 12).

DISCUSSION

In the research, although the frequency of magistral formula preparation varied with the localization of pharmacies, the results were generally compatible with Kristina (2017) and Martin (2009)'s studies (Martin et al. 2009; Kristina et al. 2017).

According to the results of the research, no statistical significance was found when magistral drug preparation status was compared with location of the pharmacies, working years of the pharmacies, working time and ability of participants in preparing magistral formulas (Table 1, Table 2). However, magistral drug preparation status was significantly lower in university graduates when compared with graduates of high school or lower (Table 2). Ergün et al. (Ergün et al. 2010) determined that, most of the magistral drugs examined in their study were inappropriate. When this study is assessed with the research's findings, the reason for inappropriate magistral drug production may be interpreted as the result of lower education levels of the pharmacy workers who prepare magistral drug. These topics are important points to research further.

It was found that magistral prescriptions mostly came from dermatology (95.3%) and ENT (50.3%) polyclinics (Table 3). Dermatology and ENT polyclinics commonly prefer magistral formulas (Martin et al. 2009; Staubach and Metz 2013; Staubach and Weisshaar 2016; Kristina et al. 2017). The results of the research regarding this topic are generally compatible with reference studies (Martin et al. 2009; Kristina et al. 2017).

Martin et al. determined the topical and transdermal formulas preparation rate of at least once a week to be between 30% and 46% in their study (Martin et al. 2009). In this study, the rate

of magistral drug preparation (mostly topical and transdermal) was found to be as 55.2%, (Table 4). The results are compatible with reference studies. We think that the low rate in oral magistral drug preparation is due to the non-inclusion of hospital pharmacies (Gubara et al. 2016; Gubara et al. 2018).

In the study, of the participants who found themselves insufficiently trained in preparing magistral preperations, 19.5% stated that the prescriptions were inadequate and 33.3% said that the prescriptions were incomplete or unreadable (Table 9). In addition to this, a high frequency of technical equipment problems, a shortage of raw materials and supply problems (Table 7) are compatible with reference studies (Minghettiu et al. 2000; Leal et al. 2012).

In the study, the suggestion that magistral drugs should not be carried out except by pharmacists and pharmacy employees was approved by 62.2% (Table 11). This rate is close to the result of 69% from the compilation of Kristina et al. (Kristina et al. 2017).

In Martin et al.'s study regarding preparing a magistral formula, consulting 'an experienced colleague' comes first, 'reference book' and 'experience' afterwards (Martin et al. 2009). This study shows a partial similarity with Martin et al.'s study (Table 6).

Of the participants, 88% stated that they prepared magistral drugs. This rate is significantly higher than Taerel et al.'s study in Bucharest (Tăerel et al. 2014). We think that the reason for the difference is that those pharmacies that did not prepare magistral prescriptions refused to participate in the research (Neubert et al. 2008; Gubara et al. 2016; Gubara et al. 2018)

The most common problems encountered by pharmacies in preparing magistral formulas are the problems experienced with refund institutions (SGK) (82.7%), incomplete or unreadable prescriptions (67.8%) and lack of raw materials (67.6%) (Table 5).

The reasons for not preparing magistral formulas were stated as; having difficulties in supplying the essential raw materials (6.1%), having difficulties in preserving raw materials for the prescriptions (5.6%), the need for buying the raw materials each time the prescription comes because of the low prescription rates, discarding the raw materials because of the expiration dates (4.5%) and inadequate laboratory facilities (3.0%) (Table 8).

Of the participants, 89.5% think that vocational training programs should be organized for the preparation of magistral drugs, and this result is similar to the results of Martin et al's study (Martin et al. 2009).

CONCLUSION

The quality of magistral drugs is important (Kristina et al. 2017). In Leal et al.'s study concerning the control of the magistral drugs prepared in Brazil, it was reported that 70% of the required criteria could not be determined. The cause of this situation was defined as: active substance differences, raw material quality, inadequate raw material control and procedures (Leal et al. 2012). However, magistral drugs prepared under appropriate conditions show the same effects as fabricated forms (Olguín et al. 2009).

The preparation of magistral products which have not been prescribed by the physician and the lack of formulations according to the standards are also problems encountered in Turkey (Emmerton et al. 2005; Ergün et al. 2010; Özbek and Kırmızı 2016).

In this study, it is understood that pharmacies prepare magistral products, but they should be supported, with training, technical facilities and procedures, to achieve the desired quality standards. Making raw materials easier to be obtained in smaller packages and support of pharmacies in terms of laboratory facilities will help in solving these problems. Quality control research into magistral products with similar studies will contribute to further improvements in the health sector.

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Original Article

In vitro evaluation of *Rheum ribes* induced genotoxicity in HepG2 cell lines

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ABSTRACT

Rheum ribes is a perennial herbaceous plant belonging to the *Polygonaceae* family that grows more on rocky and gravelly slopes in high altitude areas of Levant and Turkey. *Rheum ribes* is consumed as food and widely used in folk medicine against nausea, constipation and for different diseases including diabetes and hypertension. Unfortunately, the research on *Rheum ribes* toxicity is insufficient. In our study, the human hepatocellular carcinoma (HepG2) cell line was used in a cytotoxicity evaluation of *Rheum ribes* water, methanol and chloroform extracts by MTT and NRU tests. Comet assay was used to investigate the genotoxicity potentials of the plant extracts. Our results show that all extracts cause cell death in a concentration dependent manner at 5-50 mg/mL concentrations. The IC_{50} values are 14.29-31.94 mg/mL by MTT and 21.15-27.66 mg/mL by NRU assay. The highest concentration (25 mg/mL) of methanol extract causes significant DNA damage (8.7-folds). In conclusion, similar to a lot of plants used in folk medicine the risk of *Rheum ribes* is still unknown. The uncontrolled use of this plant could cause harm to the patients. Our results indicate the possible cyto- and gentoxicity effects of *Rheum ribes*, these results should elevate concerns about the safety of *Rheum ribes* and other folk herbs.

Keywords: *Rheum ribes*, herbal toxicity, genotoxicity, cytotoxicity, HepG2 cells

INTRODUCTION

Mankind has been discovering the therapeutic power of plants and benefiting from the herbal power to survive and fight diseases since ancient times. In developing countries, more than 80% of the population still use traditional medical plants as the first choice in the treatment of different diseases. About 80% of the world's population is thought to be living in developing countries; which means that about 64% of the world's population uses herbal remedies (Farnsworth et al., 1990). In addition to this, approximately 25% of the drugs sold by prescription in developed countries are herb-derived chemicals (Principe et al., 1991). In developed countries a new wave of "back to nature" has affected individuals and communities leading to an increased interest in alternative medicine causing increases in the use of herbal medicines. Additionally, the high costs of pharmaceutical and health protection products are pushing a large part of the population of developing countries towards choosing traditional remedies in the treatment of their disease (Verschaeve et al., 2004).

Rheum ribes L. (known in Turkish as Ribês, Rêwas, Reweş, Uçkun, Işkın, Işgın) is a perennial herbaceous plant belonging to the Polygonaceae family (Öztürk et al. 2007; Korkmaz et al. 2015; Polat et al. 2015). It is located in Palestine, Lebanon, Armenia, Iraq, Iran and the Eastern regions of Turkey (Ağrı, Bingöl, Elazığ, Hakkari, Kars, Van and Sivas), and mainly grown on the rocky and gravelly slopes at high altitude areas (Otoom et al. 2006; Öztürk et al. 2007; Cakilcioglu et al. 2010; Polat et al. 2013).

Rheum ribes is consumed fresh, and cooked as a jam (Cakilcioglu et al. 2011; Polat et al. 2015). Also, it is assumed to be a very important herb with different uses in the folk medicine of Turkey and Iran. The root and fruit (stem part) in particular, are frequently

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used in diabetes, high blood pressure, cholesterol, cirrhosis, arthritis, Alzheimer and other diseases (Abu-Irmaileh et al. 2003; Otoom et al. 2006; Naqishbandi et al. 2009; Sayyah et al. 2009; Kasabri et al. 2011; Polat et al. 2013; Hamzeh et al. 2014; Zahedi et al. 2015). Also, it is widely consumed to combat nausea and constipation (Oktay et al. 2007; Tetik et al. 2013).

Although the therapeutic, pharmacognostic, antibiotic and some of biochemical characteristics of *Rheum ribes* have been well studied, the toxic potential studies are very few and insufficient. For this reason, in this study the cytotoxic and genotoxic potential of methanol, chloroform and water root extracts were evaluated in HepG2 human hepatocarcinoma cells used previously as models of *in vitro* conditions to study the apical uptake, metabolism and absorption of nutrients and the toxicity of chemicals and drugs (Martin et al. 1997; Brand et al. 2000; Goya et al. 2015).

MATERIALS AND METHODS

Materials

Human hepatocarcinoma HepG2 cell line (HB-8065) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell culture medium (Eagle's minimum essential medium, EMEM), Phosphate buffer solution, Fetal bovine serum (FBS), Trypsin-EDTA solution and the antibiotic solution (100 IU/mL penicillin and 100 mg/mL streptomycin) were obtained from Wisent Bioproducts (Montreal, Canada), and all the other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Dried roots of *Rheum ribes [Rhizoma Rhei ribi]* were purchased from herbalists and spice sellers in Istanbul. Plant samples were tested and identified by Prof. Dr. Emine Akalin (Istanbul University, Department of Pharmaceutical Botany).

Plant extractions

Three different extracts of *Rheum ribes* were prepared using water, methanol and chloroform. For this, the roots were pulverized, 2.5g of this powder was then treated with 25 mL methanol or chloroform for 30 minutes in a water bath shaker at 25°C. A rotary evaporator and steam from nitrogen gas (40°C) were used to concentrate and dry the extracts. After dissolving the solid residue in 1 mL of dimethyl sulfoxide (DMSO), the solutions were filtrated using 0.45 µm filters. For the water extract, the infusion method was performed. 25 mL of hot (90°C) sterile water was added to 2.5 g of root powder and stirred for 30 minutes at a fixed temperature (90°C). After cooling, the mixture was filtered with filter paper and 0.45 µm filters. The methanol and chloroform extracts concentrations were 2500 mg/mL, the water extract concentration was 100 mg/mL (Abudayyak et al. 2015).

Cell culture and exposure

Human hepatocarcinoma cells (HepG2) were cultured in EMEM medium supplied with 10% heat inactivated FBS and 1% antibiotics. The cells were incubated at 37°C, 90% humidity and 5% CO_2 (confluence 60-80%). 96-well plates were used for the cytotoxicity assays and 6-well plates for the genotoxicity assay. The cell density was $1x10^3$-$5x10^3$ cell/ well for the cytotoxicty assays and $1x10^5$ cell/ well for the genotoxicty assay. The final exposure concentrations were 0.25-50 mg/mL, the exposure period was 24 hours.

Cytotoxicity evaluation

The cytotoxic effects of *Rheum ribes* root extracts were evaluated using MTT and NRU assays. After the incubation with different extract concentrations (5-50 mg/mL), the exposed cells were treated with MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) for 2 hours, the succinate dehydrogenase enzyme in the viable cells metabolized the yellowish water soluble MTT to water non-soluble formazan violet crystals. In the NRU assay, the weak red cationic dye accumulates in integral and healthy cells by creating electrostatic hydrophobic bonds with the lysosomal matrix. The cellular enzyme activity in the MTT assay and the integrity of the cells in the NRU assay are evaluated as a sign of cells' viability. A Microplate spectrophotometer system (Epoch, Germany) at 540 nm and 590 nm was used to measure the optical densities (ODs) for NRU and MTT, respectively. The nonexposed cells and cells exposed to DMSO (1%) were evaluated as negative and solvent controls respectively. The concentration – cell death (%) curves were used to calculate the median inhibitory concentrations (IC_{50}) that were responsible for the death of 50% of the cells (Mosmann 1983; Borenfreund et al. 1985).

Genotoxicity evaluation

The Comet assay was used to evaluate the genotoxic potential of *Rheum ribes* rhizomes extracts in the HepG2 cells. The concentrations of exposure were 6.25; 12.5 and 25 mg/mL for both methanol and chloroform extracts and 0.25; 0.5 and 1 mg/mL for water extract. For this, the exposed cells were trypsinized, washed with PBS 1X and mixed with prewarmed low-melting point agarose. Cells were layered on agarose pre-coated microscope slides, covered with a cover slip and allow to solidify. After fixation on the slides, the cells were treated with lysis solution for one night. The slides were washed and incubated for 20 min in fresh a cold electrophoresis buffer before electrophoresis for 20 min, and treated with a neutralization buffer for 15 minutes. Before evaluation under a fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) the cells were stained with ethidium bromide. The Comet analysis and scoring program (Comet Assay IV, Perceptive Instruments, Suffolk, UK) was used to image and score at least one hundred cells per sample. DNA damage to individual cells was expressed as a percentage of DNA in the comet tail (mean tail intensity %) (Singh et al. 1988; Abudayyak et al. 2017). The nonexposed cells and cells exposed to DMSO (1%) were evaluated as negative and solvent controls respectively. For genotoxicity evaluation hydrogen peroxide (H_2O_2; 100 µmol/L) was used as a positive control.

Statistical analysis

Cytotoxicity assays were done in triplicate, experiments were also repeated four times on different days (n=12). The genotoxicity evaluation was done in triplicate. Data is expressed as mean ± standard deviation (SD). The significance of the differences between negative control and exposed cells was evaluated using one-way analysis of variance (ANOVA) and Dunnett t-test by The Statistical Package for the Social Sciences (SPSS) version 23.0 for Windows (IBM Corp.; Armonk, NY, USA). P values of less than 0.05 were selected as the levels of significance.

RESULTS AND DISCUSSION

Rheum ribes is used as a raw and cooked food in Middle East culture (Oktay et al. 2007; Özcan et al. 2007), it is also widely used as a medical herb (Abu-Irmaileh et al. 2003; Alaadin et al. 2007; Özcan et al. 2007; Naqishbandi et al. 2009). The research related to *Rheum ribes* has mainly focused on the ethnopharmacological relevance (Afifi et al. 2000; Abu-Irmaileh et al. 2003; Cakilcioglu et al. 2010; Nabati et al. 2012; Polat et al. 2013; 2015; Kaval et al. 2015; Korkmaz et al. 2015), pharmacognostic characterization (Munzuroglu et al. 2000; Tosun et al. 2003; Özcan et al. 2007; Andiç et al. 2009; Naemi et al. 2014; Amiri et al. 2015), therapeutic (Otoom et al. 2006; Gholamhoseinian et al. 2009; Naqishbandi et al. 2009; Sayyah et al. 2009; Sindhu et al. 2010; Kasabri et al. 2011; Hadjzadeh et al. 2013; Hamzeh et al. 2014; Zahedi et al. 2015), antioxidant (Öztürk et al. 2007; Krishnaiah et al. 2011) and antibiotic (Hudson et al. 2000; Bonjar et al. 2004; Fazly-Bazzaz et al. 2005; Nabati et al. 2012) effects of *Rheum ribes*. There are only very few works related to the toxic potential of the plant. Sardari et al. (2009) evaluated the cytotoxic effect of ethanol extracts of the herb *Rheum ribes* in different cell lines by MTT test, with the results showing IC_{50} values ranging between 11.2-67.96 mg/mL. Esmaeilbeig et al. (2015) evaluated the anti-cancer effect of different *Rheum ribes* extracts against tumor cells using MTT cytotoxicity assay – the results showed that IC_{50} was115 µg/mL in human blood (K562) cell line while 200 µg/mL concentration caused less than a 15% decrease in the viability of Hela cells. Similarly, Cinar et al. (2016) calculated the IC_{50} to be 400 µg/mL in MCF-7 breast cancer cells. An *in vivo* study evaluated the acute and sub chronic toxicity (for 60 days) of *Rheum ribes* aromatic water in the Wistar rat, an increases of some enzymes like lactate dehydrogenase, abnormality in heart with tissue hemorrhage, hypertrophy and infiltration of inflammatory cells were noticed, the not observed adverse effect level (NOAEL) was calculated to be 250 and 500 mg/kg b.w./day for male and female rats, respectively (Mojarrab et al. 2015)

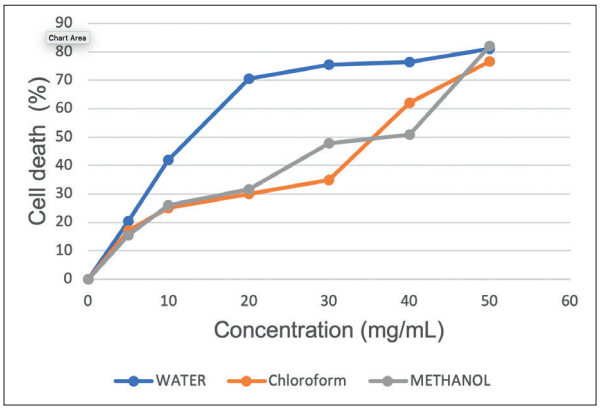

Figure 1. The cell death (%) obtained by MTT assay in HepG2 cells following the exposure to *Rheum ribes* extract.

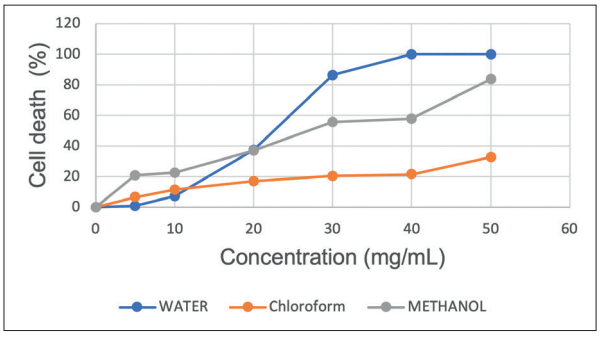

Figure 2. The cell death (%) – concentration curve obtained by NRU assay in HepG2 cells following the exposure to *Rheum ribes* extract.

In this work exposure to *Rheum ribes* extracts for 24 hours caused a decrease in the viability of HepG2 cells dependent on concentration manner. The MTT assay results show that IC_{50}

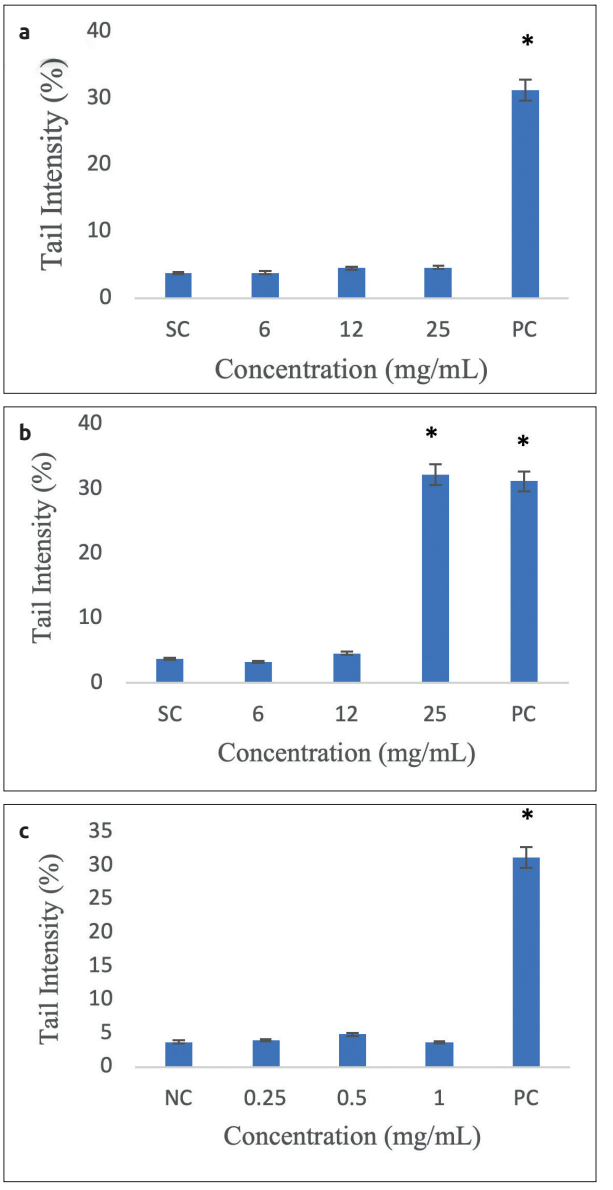

Figure 3. a-c. The genotoxic potential of *Rheum ribes* extract.

mean tail intensity obtained from comet assay in HepG2 cells following the exposure to (a) Chloroform (b) methanol (c) Water extracts of *Rheum ribes* roots. The results were presented as mean tail intensity (%) with ±SD.

*p ≤0.05 were selected as the levels of significance by one-way ANOVA Dunnett t-test.

values are 14.29; 33.67; and 31.94 mg/mL for the extracts of water, chloroform and methanol, respectively (Figure 1). The IC_{50} values for the NRU assay for water and methanol were 21.15 and 27.66 mg/mL, respectively (Figure 2). At the highest concentration (50 mg/mL) of chloroform extract, cellular death was 32.8%. The differences between the cytotoxicity assays were discussed previously and the different results obtained in these different assays were reported (Weyermann et al. 2005; Fotakis & Timbrell 2006). There were many factors including the interaction between the tested xenobiotics and the chemicals of the assay (Wang et al. 2010). In the case of NRU some chemicals decrease the lysosomes account in the cells, leading to negative false results. Additionally, some chemicals may increase the activity of succinate dehydrogenase enzymes causing false positive results in the MTT test. Similarly, chemicals affecting cellular adherence could also cause cells loss leading to false positive results. This could explain the difference in chloroform extracts between MTT and NRU assays.

To the best of our knowledge the genotoxicity of *Rheum ribes* has not been evaluated previously. Comet assay results show that *Rheum ribes* water and chloroform and the low concentrations of methanol extracts did not cause any significant DNA damages after 24 hours exposure. Only the highest concentration of methanol extract (50 mg/mL) causes significant DNA damage (8.7-folds) in HepG2 cells (Figure 3).

Unfortunately, there is very little research which discusses the difference in toxicity between different herbal extraction methods. According to this research, it is also difficult to argue that methanol extracts are more toxic than chloroform ones or vice versa. In a previous study, the toxicity of the extracts of ten herbs was evaluated with the MTT test. The results showed that the chloroform extracts of all the test herbs were more cytotoxic than the methanol extracts and none of the water extracts showed any cytotoxicity. The chloroform extracts (in general) possessed more mutagenic activity with the Ames test than methanol and water extracts (Abudayyak et al. 2015). Similar results were also found by Chan et al. (2015). In contrast, previous studies showed that the chloroform and water extracts of *Tribulus terrestris* were less cyto- and genotoxic than methanol extracts (Abudayyak et al. 2015 B). However, in this study, the results indicate that while the methanol extracts were less cytotoxic than the water and chloroform extracts, they possessed the highest genotoxicity.

CONCLUSION

Contrary to the popular belief that herbs are safe because they are natural products, some herbs can cause significant toxic effects, drug interactions, and even morbidity or mortality. It would be beneficial to evaluate at the very least the cytotoxicity, genotoxicity and carcinogenicity of these herbs in order to assess the associated risks to our health. *Rheum ribes* is one of herbs that are consumed frequently in Turkey and Middle East countries yet the data concerning the safety of *Rheum ribes* is still insufficient. There is a need for *in vivo* and *in vitro* studies to evaluate its toxic effects. Our results conclude that *Rheum ribes* can have some negative effects on human hepatocytes by causing cell death and DNA damage.

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Original Article

Risk factors associated with the frequency of antibodies to *Francisella tularensis* in two areas from Turkey

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ABSTRACT

Francisella tularensis is a zoonotic microorganism that can infect different species of animals and sometimes humans. The aim of this study was to determine the seroprevalence of *F. tularensis* in people from two areas in Turkey (Sivas and Tokat). This is a retrospective study of the serum samples collected in 2006 from people living in rural areas (n:360) and from people living in urban areas (n:90). *F. tularensis*-IgG antibodies were investigated by ELISA method. Antibody positivity against *F. tularensis* was found in 7.5% of 360 serum collected from rural areas and 1.1% of 90 serum collected from urban areas (OR 7.216, 95% CI 0.967-53.836, p=0.025). While there was no difference in *F. tularensis* seropositivity between different genders (p=0.424), tick contacts (p=0.303) and occupational groups (p=0.807), *F. tularensis* seropositivity was found to be higher in the Tokat region than in the Sivas region (p=0.047). Moreover, risk factors were observed in people over 40 years of age (p=0.045) and in those who consume fresh cheese (p=0.036). Our findings revealed that tularemia cases can be seen in these regions even though tick bite cases in the Sivas and Tokat regions were not an important influence on the transmission of *F. tularensis* to humans on the dates of our research.

Keywords: *Francisella tularensis*, tick bite, tularemia, Turkey

INTRODUCTION

Francisella tularensis is a non-motile, non-spore and highly infectious microorganism in coccobacillus form. Tularemia, a disease caused by *F. tularensis*, is a zoonotic disease that is usually located between 30°-71° latitude north, particularly in rural enviroments. However, cases of tularemia in many European countries including Turkey have also been reported (Ellis et al. 2002; Hestvik et al. 2015).

F. tularensis has 4 subspecies in nature and the most common and clinically important subtypes are subtype *tularensis* (A) and subtype *holarctica* or *palaearctica* (B) (Ellis et al. 2002; Zellner and Huntley 2019; Kılıç 2010). Type-A with higher virulence is transmitted by vectors such as ticks and infected animals. Type-B causes mild to moderate infection in the northern hemisphere and is transmitted from water sources. *F. tularensis* (A), which has higher virulence, is quite common in nature. The natural reservoirs of *F. tularensis* are mostly small mammalian species. Several arthropods, such as ticks, lice, fleas and flies, have been reported to be infected with *F. tularensis* (Socolovschi et al. 2009). In Europe, *F. tularensis* was isolated from *Ixodes ricinus*, *Dermacentor reticulates* and *D. marginatus* species (Milutinović et al. 2008; Genchi et al. 2015). Since blood-sucking insects such as ticks are vectors of tularemia for transmission to mammals, people living in rural areas are at greater risk (Gürcan 2014).

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F. tularensis is transmitted to humans by infection-bearing animals, contact with infected tissue and body fluids, arthropod bites, consumption of contaminated waters and inhalation of aerosols capable of infection. The transmission of *F. tularensis* through tick bites is usually sporadic (Ellis et al. 2002). In Turkey, after the first tularemia case was seen in 1936, several outbreaks occurred and the number of cases gradually increased. Between 1936 and 2011, 1441 cases diagnosed as tularemia were reported (Gürcan 2014). According to recent studies, *F. tularensis* is mostly transmitted by consumption of contaminated water in Turkey (Kilic et al. 2015; Duzlu et al. 2016).

Symptomatic manifestation of infection may vary depending on the type of disease. The ulceroglandular clinical form of the disease is the most common form of infection with 45-80% worldwide (Maurin and Gyuranecz 2016). In Turkey, epidemics mostly occur in northwest regions and Central Anatolia and the most prevalent clinical form is oropharyngeal form (Ulu-Kilic and Doganay 2014). IgG antibodies developed to *F. tularensis*, whether symptomatic or asymptomatic, may be detectable up to 10 years in the patient's serum even at low titers (Koskela and Salminen 1985). In epidemiological studies, IgG antibodies can easily be determined by methods such as microagglutination and ELISA. Since *F. tularensis* and *B. abortus* have common antigens, serological cross-reactions might occur between both, especially in the agglutination test. The sensitivity and specificity of the ELISA test is much higher than is the case with the agglutination tests (Ellis et al. 2002; Porsch-Ozcürümez et al. 2004).

The Tokat and Sivas regions in Turkey are geographic areas where tick-borne zoonoses are especially common (Gunes et al. 2012). This study aimed to determine whether there is a difference in the positivity of *F. tularensis* antibody between different factors including living area, tick contact, age, gender, occupation, city, and consumption of fresh cheese. In addition, *B. abortus*-IgG antibodies were studied to detect cross- reacting antibodies.

MATERIAL AND METHODS

Study area

Sivas is located in the Central Anatolia Region of Turkey with a 28,458 km^2 surface area and it has a population of around 650 thousand people. Tokat, which is a city located in the Black Sea region of Anatolia, has a population of around 600 thousand people and its surface area is approximately 10,000 km^2 (Figure 1). Sivas and Tokat districts have a fauna that hosts many animal species and a large flora and whose economy is based mainly on agriculture and livestock farming.

Collection of blood samples

In June-September 2006, 56 villages from 14 districts in Tokat and Sivas where tick-borne infections were endemic were selected as the study area (Figure 1). Blood samples were taken from 1093 people who were engaged in livestock farming and lived in rural areas of the Sivas and Tokat regions, and from 90 people who were not related to rural areas and animal husbandry and who lived in the city center. A questionnaire was formed and the participants were questioned about their names, gender, age, contact with animals (especially ticks) and consumption of fresh cheese (Table 1). Serums of blood samples were obtained. To minimize the drawbacks of the freeze-thaw, each serum was divided into 5 separate tubes and stored at -80°C prior to analysis. A total of 450 serum samples were included in this study and 360 serum samples from 1093 serums stored at -80°C were selected with the random sampling method. Of the 360 subjects included in the study group, 180 were male (mean age: 40.79±19.62), and 180 were female (mean age: 40.750±16.26 years). In total, 125 people from the Sivas region and 235 from the Tokat region were included in the study (Table 1). The study was approved by the Non-interventional Clinical Research Ethics Board (Decision No: 2017-11 / 12).

Serological tests

F. tularensis IgG antibodies were investigated by ELISA method. For this purpose, Serion ELISA classic *F. tularensis* IgG kits produced by Virion/Serion company and classic *B. abortus* IgG kits produced by Nova-Tec Company were used. The *F. tularensis* ELISA-IgG kit used in this study was able to screen antibodies produced against both the *F. tularensis* subtype tularensis (type-A) and *F. tularensis* holarctica (type-B) subtype. The experiments were conducted in accordance with the user manual contained in the kits. In the final stage, the microplates were read on the ELISA microplate reader (EL 312, Bio-Tek Instruments, Inc., Winooski, Vermont, USA) at a wavelength of 405 nm and the absorbance values of serum samples and standard serums were determined. The absorbance values of serums were examined and the cut off value was calculated with the formula specified in the kit prospectus. The absorbance values determined for each group were compared with the calculated cut off value and the serums having a higher value than the cut off value were evaluated as positive. Furthermore, the antibodies to *B. abortus* were examined by ELISA (NovaLisa Brucella IgG) to determine whether there was a serological relationship between *F. tularensis* and *B. abortus* in terms of cross reactions (in 56 seronegative serums and 27 seropositive serums for *F. tularensis).*

Statistical analysis

The data were transferred to the computer and statistically analyzed by the licensed The Statistical Package for the Social

Figure 1. Map of the study area, 656x472mm (96 x 96 DPI).

Table 1. *F. tularensis* antibody findings in risk categories

Categories of risk factors	n	Positive (%)	p	Odds (95% CI)#
Total	450	28 (6.222)		
Village. City				
City residents*	90	1 (1.111)	0.025	7.216 (0.967-53.836)
Village residents	360	27 (7.555)		
Tick Contact				
Bitten by tick*	90	4 (4.444)	0.303	1.265 (0.328-4.871)
Cleaning ticks	90	5 (5.555)		2.687 (0.810-8.912)
Bitten by tick and cleaning ticks	90	10 (11.111)		2.098 (0.608-7.232)
No tick contact	90	8 (8.888)		
Residential Areas				
Sivas*	125	5 (4.000)	0.047	2.479 (0.915-6.715)
Tokat	235	22 (9.361)		
Gender				
Male*	180	11 (6.111)	0.424	1.499 (0.675-3.326)
Female	180	16 (8.888)		
Occupational Groups				
Livestock (only) *	23	1 (4.348)	0.807	2.870 (0.277-29.713)
Farming (only)	26	3 (11.539)		1.732 (0.222-13.524)
Livestock and farming	274	20 (7.299)		1.941(0.190-19.869)
Other	37	3 (8.108)		
Consumption of fresh cheese				
No*	86	2 (2.326)	0.036	4.217 (0.978-18.182)
Yes	274	25 (9.124)		
Age. 40				
Age ≤ 40 y*	189	9 (4.762)	0.045	2.353 (1.027-5.389)
Age > 40 y	171	18 (10.526)		

CI: Confidence interval, *: Reference category in Odds ratio

Sciences (SPSS) for Windows 14' program (SPSS, Inc., Chicago, IL, USA). Chi-square test method was used to evaluate risk categories such as tick bites, tick contacts, sex, age and occupational groups in terms of "p value". The chi-square test was used to calculate the odds value in two categorical variables (2x2), and a binary logistic regression analysis was used to calculate the odds value in more than one categorical variable (3x2, 4x2). Confidence interval was accepted as 95% and p value <0.05 for statistical significance.

RESULTS

According to the results, IgG antibodies were detected against *F. tularensis* in 27 (7.56%) of the 360 people living in rural areas and 1 (1.11%) of 90 people living in urban areas, and seroprevalence was found to be higher in people living in rural areas (p=0.025, odds ratio (OR)= 7.216, 95% confidence interval (CI)= 0.967-53.836) (Table 1).

When the presence of *F. tularensis* antibody was evaluated, antibody positivity was found in in 4 (4.4%) of those bitten by ticks, in 5 (5.6%) of those who were cleaning ticks from animals, in 10 (11.1%) of those who were both bitten by ticks and were cleaning ticks from animals, and in 8 (%8.9) of the subjects who lived in the village but had no contact with ticks. The results showed that seroreactivity against *F. tularensis* was not statistically significant between the groups in terms of contact with the ticks (p=0.303).

The rate of *F. tularensis* antibody positivity was 5 (4%) in serum samples of 125 from Sivas, and there were 22 (9.4%) positive results in 235 samples taken from Tokat (p=0.047, OR=2.479, 95% CI=0.915-6.715). When the results were evaluated in terms of sex, in 11 (6.1%) of 180 men, in 16 (8.9%) of 180 women, reactive antibodies against *F. tularensis* were found (p=0.424, OR=1.499, 95% CI=0.675-3.326).

Evaluation of the serums in terms of occupational groups showed that seropositivity against *F. tularensis* was found in 1 (4.4%) out of 23 people who fed only animals, in 3 (%11.5) out of 26 people engaged in farming, in 20 (7.3%) out of 274 people engaged in both livestock and farming, and in 3 (8.1%) out of 37 of the other occupational group employees. No significant difference was found in the frequency of tularemia infection among different occupational groups (p=0.807).

While *F. tularensis* seropositivity was observed in 25 (9.1%) out of 274 people who consumed fresh cheese, it was also found in 2 (2.3%) out of 86 people who did not consume it. The seroprevalence of *F. tularensis* in people consuming fresh cheese

was found to be high compared to those not consuming fresh cheese (p=0.036, OR=4.217, 95% CI=0.978-18.182).

In 18 (10.5%) of 171 people over 40 years of age and in 9 (4.8%) of 189 people under 40 years of age antibody positivity was found and the difference was statistically significant (p=0.045, OR=2.353, 95% CI=1.027-5.389).

IgG seropositivity against *B. abortus* was determined in 5 (18.5 %) of 27 serums that were positive for *F. tularensis* and in 14 (25 %) of the 56 seronegative samples for *F. tularensis*. The seroprevalence of *B. abortus* was not statistically different in *F. tularensis* negative and positive sera (p=0.587).

DISCUSSION

Tularemia is endemic in Europe, Finland and Sweden and cases have also been reported in Austria, Germany, Spain, Hungary, Bulgaria and Turkey (Ellis et al. 2002; Leblebicioglu et al. 2008; Gürcan 2014). This seroepidemiological study suggests that asymptomatic or symptomatic tularemia infections may be seen in the Tokat and Sivas regions.

13 % of 18,343 tularemia cases in Europe between 1992 and 2012 were reported by Turkey (Hestvik et al. 2015). In epidemiological studies conducted by different researchers after the tularemia outbreak in Turkey, *F. tularensis* seroprevalence was found between 2,6%-20.9% depending on the region. (Dedeoğlu Kilinç et al. 2007; Gürcan 2014). In this study, the prevalence of tularemia in people living in rural areas was approximately 7 times higher than in people living in urban areas (OR= 7.216). *F. tularensis* seroprevalence, which we found in 7.6% of the rural population, indicates that tularemia may pose a risk to the health of people living in rural areas in the Tokat and Sivas regions.

Since *F. tularensis* is a zoonotic bacterium, people engaged in livestock farming, farmers and especially hunters have higher seropositivity (Jenzora et al. 2008; Esmaeili et al. 2014). In our results, no statistically significant difference was observed in *F. tularensis* seroprevalence between different occupational groups (p=0.807). Of the 360 participants included in this study, 272 (76%) were engaged both in animal husbandry and farming. It is not typical for people living in rural areas to have only one occupation type. Considering that the transmission of *F. tularensis* subsp. holarctica in Turkey is generally waterborne, it can be assumed that people from different occupational groups living in the villages usually use similar water resources.

In Europe , *F. tularensis* has been detected in *Ixodes ricinus*, *Dermacentor reticulatus* and *D. marginatus* ticks. However, the prevalence of *F. tularensis* (0-3.8%) determined in ticks is quite low compared to other tick-borne agents (Milutinović et al. 2008; Reye et al. 2010; Karasartova et al. 2018). According to the scientific data, tick bites do not have a significant importance in the transmission of *F. tularensis* to humans (Clark et al. 2012). In our study, in terms of their contact to ticks, there was no difference in the seroprevalence of *F. tularensis* among 4 groups in rural areas (p=0.303). These results confirm that tick bites in humans is not very important in the transmission of *F. tularensis*. However, infected ticks may be of importance in the transfer of *F. tularensis* between wild reservoirs.

The Tokat region, compared to the Sivas region, is more suitable for the survival of many rodent species that can be a reservoir for *F. tularensis*. This is because the climate in the Tokat region is similar to that in the Black Sea region. According to this study, people living in the villages of Tokat compared to the villagers of Sivas, have a 2.5 times greater risk of contact with *F. tularensis* (OR:2.479). In terms of survival of small mammals such as rabbits, mice and squirrels, which may be reservoirs for tick-borne infectious agents, we think that the Tokat region is a more suitable geography than Sivas.

According to the scientific data, there is generally no difference in the seroprevalence of *F. tularensis* among women and men living in rural areas (Gutiérrez et al. 2003; Clark et al. 2012; Esmaeili et al. 2014). Similar results were obtained in our study, too (p: 0.424). The fact that men and women living in rural areas are dealing with similar jobs can be one of the reasons for this result. Considering that the *F. tularensis* infections seen in our country are generally waterborne, another reason for this is that the risk of using contaminated water is similar for both sexes.

In our study, a significant difference of the *F. tularensis* seroprevalence was found between the groups who consumed fresh cheese and those who did not (p=0.036; OR: 4.217). In general, non-pasteurized raw milk consumption in Turkey is not common, but in rural areas, in the production of dairy products such as cheese, the use of non-boiled milk can occur.. Consumption of the products produced with non-boiled milk causes many infections, especially brucellosis. We predict that the possibility of coming into contact with *F. tularensis* infection is high in individuals who consume fresh cheese due to the lack of awareness of hygiene and protection against infections.

With increasing age, there is also an increase in *F. tularensis'* seroprevalence (Clark et al. 2012; Esmaeili et al. 2014). According to our findings, *F. tularensis* seropositivity (10.5%) was statistically higher in individuals over 40 years of age than in those aged 40 and below (4.8%). The fact that people over the age of 40 are more interested in animal feeding and agricultural activities, and also the fact that IgG antibodies remain at a detectable level in serum for years after infection, may provide some reasons for this difference.

In serological tests between *B. abortus* and *F. tularensis*, false positive results may be observed due to cross-reactions. The possibility of cross-reaction is higher in microagglutination tests (Behan and Klein 1982). In this study, the seroprevalence of *B. abortus* was not found to be higher in *F. tularensis* positive serums than *F. tularensis* negative serums. We assume that the possibility of cross-reaction due to *B. abortus* in ELISA test is too low to have importance attached to it. In this study, *F. tularensis* and *B. abortus* co-seroprevalence were detected in 1.39% of 360 serum samples of individuals, and we believe that the active co-infection rate is probably even lower.

CONCLUSION

F. tularensis infection is an important zoonosis which can be seen in almost every region, especially in rural areas. In the presence of symptoms such as sudden high fever muscle pain, sore throat and swelling of lymph nodes in people dealing with agriculture and livestock farming, it is very important to make differential diagnosis by diagnostic tests for the treatment and control of the disease, given that there is a possibility of contracting tularemia.

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Original Article

Synthesis, characterization, antibacterial and antifungal evaluation of novel cyclohexanone benzoylhydrazones

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ABSTRACT

A novel series of benzoyl hydrazones (2a-j) were synthesized and evaluated, *in vitro*, for antimicrobial activity against selected bacteria and fungi. The structures of the compounds were established by IR, ^{1}H-NMR, ^{13}C-NMR (APT), electrospray ionization mass spectrometry (ESI-MS) and microanalysis (C, H, N). All of the tested compounds, except for compound 2h, displayed weak antibacterial properties against *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus aureus* ATCC 29213. Compounds 2a, 2b, 2e, 2f and 2i further exhibited marginal antifungal activity against *Candida parapsilosis*.

Keywords: Hydrazone, cyclohexanone, antibacterial activity, antifungal activity

INTRODUCTION

The spread of antibiotic-resistant bacteria is one of the biggest threats to global health. Mortality, morbidity and medical costs due to antibiotic resistance are increasing worldwide. A growing list of infections such as tuberculosis, pneumonia, gonorrhoe, blood poisoning and foodborne diseases, are becoming harder to treat as the antimicrobial agents become less effective (WHO 2018). Every year, around 214,000 deaths in newborns are attributable to drug-resistant pathogens, especially in low- and middle-income countries (Laxminarayan et al. 2016). Despite a growing clinical need, the development of new antibacterial agents to deal with the threat is insufficient. Only two novel antibiotic classes have been discovered in the last 20 years (oxazolidinones and lipopeptides) both of which provide coverage against Gram-positive bacteria (Luepke et al. 2017; Tacconelli et al. 2018). The approval rate of U.S. Food and Drug Administration (FDA) for new antibiotics has fallen to dismally low levels during the past 30 years (Shlaes et al. 2013). There is an urgent need for new antibiotics with activity against resistant microorganisms.

Hydrazide-hydrazones, R_1R_2C=N-NR_3COR_4 (R_{1-4}=alkyl, aryl or H), are well known as compounds with a wide range of antimicrobial properties (Popiolek 2017). Several *N*-aroylhydrazones derived from aryl- and heteroaryl hydrazides are emerging in the literature as potential antibacterial agents with wide spectra of activity against both Gram-(+) and Gram-(-) bacteria (Vicini et al. 2002; Gürkök et al. 2009; Moldovan et al. 2011; Xavier et al. 2012; Pieckzonka et al. 2013; Qing Ge et al. 2014; Kaplancıklı et al. 2014; Morjan et al. 2014; Nastasa et al. 2015; Tatar et al. 2016; Sridhar et al. 2016). Some of these derivatives have also been reported to have an inhibitory effect on fungi, especially on Candida species (Vicini et al. 2002; Gürkök et al. 2009; Xavier et al. 2012; Kaplancıklı et al. 2014; Nastasa et al. 2015). In an early report, Backes et al. identified a series of N'-(2-hydroxybenzylidene) benzohydrazides with potent antifungal activity against two human pathogenic species, *Candida albicans* and *Candida glabrata*, at low µM concentrations (Backes et al. 2014).

In this study we report the synthesis and structural characterization of novel *N*-benzoylhydrazones which were obtained by the condensation of 2-hydroxy-4-methoxybenzohydrazide and appropriate cyclohexanone derivatives. These new compounds

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were evaluated for *in vitro* antibacterial and antifungal activity against a variety of pathogenic bacteria and fungi species.

MATERIALS AND METHODS

Chemistry

Melting points were determined in open capillary tubes with a Buchi B-540 melting point apparatus and were uncorrected. Microanalyses were performed on a Thermo Finnigan Flash EA 1112 elemental analyzer. IR spectra were recorded in KBr discs (υ_{max} in cm^{-1}) on a Shimadzu IRAffinity-1 FTIR spectrophotometer. ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (APT) (DMSO-d_6) spectra were run on VarianUNITYINOVA (500 MHz) instrument. Chemical shifts were reported as δ (ppm) relative to TMS as internal standard and coupling constants (*J*) were given in hertz (Hz). MS (ESI+/-) were determined on a Finnigan LCQ Advantage Max mass spectrometer (br.: broad/distorted, cyc.: cyclohexylidene, ar.: aromatic).

General procedure for the synthesis of 2-hydroxy-4-methoxy-N'-[(non)substituted cyclohexylidene]benzohydrazides (**2a**-**j**)

A mixture of 2-hydroxy-4-methoxybenzohydrazide (0.003 mol) and an appropriate cyclohexanone (0.003 mol) in absolute ethanol (20 mL) was refluxed on a water bath for 5-6 h. After cooling, the product was precipitated by adding distilled water. The solid thus obtained was filtered off and recrystallized from ethanol.

N'-cyclohexylidene-2-hydroxy-4-methoxybenzohydrazide (**2a**)

Light brown crystals (81%); mp 200-201°C; IR(KBr): υ_{max} 3311 (N-H), 1633 (C=O), 1612 (C=N), 1587, 1562, 1544, 1519 (C=C); ^{1}H-NMR (DMSO-d_6/500MHz): δ 1.59-1.68 (6H, m, CH_2-cyc.), 2.31-2.35 (4H, m, CH_2-cyc.), 3.77 (3H, s, 4-OCH_3-ar.), 6.46 (1H, d, *J=2.5*, H3-ar.), 6.51 (1H, dd, *J=8.5, 2.5*, H5-ar.), 7.86 (1H, d, *J=8.5*, H6-ar.), 10.87 (1H, s, NH/OH), 12.26 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 25.48, 26.06, 27.21, 28.13, 35.36 (CH_2-cyc.), 55.79 (4-OCH_3-ar.), 101.69, 106.61 (C3,C5-ar.), 109.62 (C1-ar.), 131.11 (C6-ar.), 160.50, 163.71, 164.42 (C2,C4-ar.,C=N,C=O). MS (ESI+) m/z (%): 263.2 ($[M+H]^+$, 55.0), 285.2 ($[M+Na]^+$, 100). Anal. Calcd for $C_{14}H_{18}N_2O_3$ (262.30): C, 64.10; H, 6.92; N, 10.68. Found: C, 63.74; H, 7.10; N, 10.32.

2-hydroxy-4-methoxy-N'-(4-methylcyclohexylidene)benzohydrazide (**2b**)

Beige crystals (78%); mp 204-205°C; IR(KBr): υ_{max} 3280 (N-H), 1651 (C=O), 1616 (C=N), 1604, 1535, 1504 (C=C); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 0.92 (3H, d, *J=6.8*, 4-CH_3-cyc.), 1.04-1.20 (2H, m, CH_2-cyc.), 1.67-1.71 (1H, m, CH-cyc.), 1.80-1.87 (2H, m, CH_2-cyc.), 2.01 (1H, td, *J=13.7, 5.4*, CH_2-cyc.), 2.26 (1H, td, *J=13.2, 4.9*, CH_2-cyc.), 2.42 (1H, br. d, *J=13.7*, CH_2-cyc.), 2.73 (1H, br. d, *J=14.1*, CH_2-cyc.), 3.77 (3H, s, 4-OCH_3-ar.), 6.46 (1H, d, *J=2.4*, H3-ar.), 6.51 (1H, dd, *J=8.8*, 2.4, H5-ar.), 7.86 (1H, d, *J=8.8*, H6-ar.), 10.86 (1H, s, NH/OH), 12.27 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 21.73 (4-CH_3-cyc.), 27.27 (CH_2-cyc.), 31.49 (CH-cyc.), 33.97, 34.57, 35.16 (CH_2-cyc.), 55.80 (4-OCH_3-ar.), 101.69, 106.58 (C3,C5-ar.), 109.60 (C1-ar.), 131.09 (C6-ar.), 160.58, 163.72, 164.02, 164.42 (C2,C4-ar.,C=N,C=O). MS (ESI+) m/z (%): 277.1 ($[M+H]^+$, 100). Anal. Calcd for $C_{15}H_{20}N_2O_3$ (276.33): C, 65.20; H, 7.30; N, 10.14. Found: C, 65.03; H, 7.50; N, 9.88.

N'-(4-ethylcyclohexylidene)-2-hydroxy-4-methoxybenzohydrazide (**2c**)

White crystals (86%); mp 166-168°C; IR(KBr): υ_{max} 3305 (N-H), 1633 (C=O), 1610 (C=N), 1580, 1550, 1516 (C=C); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 0.89 (3H, t, *J=7.3*, 4-$CH_2\underline{C}H_3$-cyc.), 1.06-1.18 (2H, m, CH_2-cyc.), 1.25 (2H, quin., *J=7.3*, 4-$C\underline{H}_2CH_3$-cyc.), 1.44-1.48 (1H, m, CH-cyc.), 1.86-2.03 (3H, m, CH_2-cyc.), 2.25 (1H, td, *J=13.5, 4.9*, CH_2-cyc.), 2.43 (1H, br. d, *J=13.7*, CH_2-cyc.), 2.73 (1H, br. d, *J=14.2*, CH_2-cyc.), 3.77 (3H, s, 4-OCH_3-ar.), 6.46 (1H, d, *J=2.5*, H3-ar.), 6.51 (1H, dd, *J=9.0, 2.5*, H5-ar.), 7.86 (1H, d, *J=8.5*, H6-ar.), 10.86 (1H, s, NH/OH), 12.26 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 11.95 (4-$CH_2\underline{C}H_3$-cyc.), 27.25, 28.63, 31.64, 32.72, 34.52 (CH_2-cyc., 4-$\underline{C}H_2CH_3$-cyc.), 38.06 (CH-cyc.), 55.79 (4-OCH_3-ar.), 101.69, 106.60 (C3,C5-ar.), 109.60 (C1-ar.), 131.10 (C6-ar.), 160.53, 163.71, 164.03, 164.72 (C2,C4-ar.,C=N,C=O). Anal. Calcd for $C_{16}H_{22}N_2O_3$ (290.36): C, 66.18; H, 7.64; N, 9.65. Found: C, 66.40; H, 7.80; N, 9.52.

2-hydroxy-4-methoxy-N'-(4-propylcyclohexylidene)benzohydrazide (**2d**)

White crystals (87%); mp 158-160°C; IR(KBr): υ_{max} 3304 (N-H), 1637 (C=O), 1612 (C=N), 1558, 1519 (C=C); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 0.87 (3H, t, *J=7.3*, 4-$CH_2CH_2\underline{C}H_3$-cyc.), 1.07-1.22 (4H, m, 4-$CH_2\underline{C}H_2CH_3$, CH_2-cyc.), 1.28-1.34 (2H, m, 4-$C\underline{H}_2CH_2CH_3$-cyc.), 1.54-1.58 (1H, m, CH-cyc.), 1.85-2.02 (3H, m, CH_2-cyc.), 2.25 (1H, td, *J=13.8, 4.9*, CH_2-cyc.), 2.43 (1H, br. d, *J=14.2*, CH_2-cyc.), 2.73 (1H, br. d, *J=13.7*, CH_2-cyc.), 3.77 (3H, s, 4-OCH_3-ar.), 6.46 (1H, d, *J=2.5*, H3-ar.), 6.51 (1H, dd, *J=8.8, 2.5*, H5-ar.), 7.86 (1H, d, *J=8.5*, H6-ar.), 10.86 (1H, s, NH/OH), 12.26 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 14.65 (4- $CH_2CH_2\underline{C}H_3$-cyc.), 20.12, 27.28, 32.02, 33.10, 34.54, 38.25 (CH_2-cyc., 4-$\underline{C}H_2CH_2CH_3$-cyc.), 36.04 (CH-cyc.), 55.78 (4-OCH_3-ar.), 101.69, 106.59 (C3,C5-ar.), 109.60 (C1-ar.), 131.09 (C6-ar.), 160.55, 163.71, 163.99, 164.71 (C2,C4-ar.,C=N,C=O). Anal. Calcd for $C_{17}H_{24}N_2O_3$ (304.38): C, 67.08; H, 7.95; N, 9.20. Found: C, 67.33; H, 8.11; N, 9.13.

N'-(4-*tert*-butylcyclohexylidene)-2-hydroxy-4-methoxybenzohydrazide (**2e**)

White crystals (91%); mp 203-205°C; IR(KBr): υ_{max} 3302 (N-H), 1635 (C=O), 1609 (C=N), 1558, 1516, 1480 (C=C); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 0.86 (9H, s, 4-$C(CH_3)_3$-cyc.), 1.13-1.24 (2H, m, CH_2-cyc.), 1.31-1.36 (1H, m, CH-cyc.), 1.88-1.97 (3H, m, CH_2-cyc.), 2.25 (1H, td, *J=13.2, 4.9*, CH_2-cyc.), 2.46 (1H, br. d, *J=13.7*, CH_2-cyc.), 2.81 (1H, br. d, *J=14.2*, CH_2-cyc.), 3.77 (3H, s, 4-OCH_3-ar.), 6.46 (1H, d, *J=2.5*, H3-ar.), 6.51 (1H, dd, *J=9.0, 2.5*, H5-ar.), 7.86 (1H, d, *J=8.5*, H6-ar.), 10.85 (1H, s, NH/OH), 12.27 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 26.75, 27.25 (CH_2-cyc.), 27.82 (4-$C(\underline{C}H_3)_3$-cyc.), 32.62 (4-$\underline{C}(CH_3)_3$-cyc.), 34.98 (CH_2-cyc.), 46.80 (CH-cyc.), 55.78 (4-OCH_3-ar.), 101.69, 106.60 (C3,C5-ar.), 109.58 (C1-ar.), 131.07 (C6-ar.), 160.51, 163.71, 164.05, 164.67 (C2,C4-ar.,C=N,C=O). MS (ESI+) m/z (%): 319.2 ($[M+H]^+$, 100). Anal. Calcd for $C_{18}H_{26}N_2O_3$ (318.41): C, 67.90; H, 8.23; N, 8.80. Found: C, 67.53; H, 8.25; N, 8.83.

2-hydroxy-4-methoxy-N'-(3-methylcyclohexylidene)benzohydrazide (**2f**)

White crystals (95%); mp 167-169°C; IR(KBr): υ_{max} 3311 (N-H), 1622 (C=O), 1613 (C=N), 1581, 1545, 1508 (C=C); ^{1}H-NMR (DM-

SO-d_6/500 MHz): δ 0.94, 0.95 (3H, 2d, *J=6.3*, 3-CH_3-cyc.), 1.12-1.20 (1H, m, CH/CH_2-cyc.), 1.37-1.49 (1H, m, CH/CH_2-cyc.), 1.60-1.94 (4H, m, CH/CH_2-cyc.), 2.17 (1H, td, *J=13.5, 5.4*, CH_2-cyc.), 2.35-2.44 (1H, m, CH_2-cyc.), 2.65-2.71 (1H, m, CH_2-cyc.), 3.77 (3H, s, 4-OCH_3-ar.), 6.46, 6.47 (1H, 2d, *J=2.5*, H3-ar.), 6.50-6.52 (1H, m, H5-ar.), 7.87, 7.89 (1H, 2d, *J=8.8*, H6-ar.), 10.89 (1H, s, NH/OH), 12.27 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 22.17, 22.35 (3-CH_3-cyc.), 24.81, 25.91, 27.51 (CH_2-cyc.), 32.77, 33.70 (CH-cyc.), 33.79, 34.83, 36.00, 43.31 (CH_2-cyc.), 55.78 (4-OCH_3-ar.), 101.69, 106.58 (C3,C5-ar.), 109.58, 109.68 (C1-ar.), 131.09, 131.18 (C6-ar.), 160.50, 160.60, 163.72, 163.91, 164.33 (C2,C4-ar.,C=N,C=O).). MS (ESI-) m/z (%): 275.5 ([M-H]$^-$, 100). Anal. Calcd for $C_{15}H_{20}N_2O_3$ (276.33): C, 65.20; H, 7.30; N, 10.14. Found: C, 65.30; H, 7.52; N, 10.06.

2-hydroxy-4-methoxy-N'-(3,3,5-trimethylcyclohexylidene)benzohydrazide **(2g)**

White crystals (94%); mp 203-206°C; IR(KBr): υ_{max} 3304 (N-H), 1658 (C=O), 1627 (C=N), 1604, 1543, 1504 (C=C); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 0.78, 0.83 (3H, 2s, 3-CH_3-cyc.), 0.93-0.95 (3H, m, 5-CH_3-cyc.), 0.99, 1.02 (3H, 2s, 3-CH_3-cyc.), 1.09-1.15 (1H, m, CH/CH_2-cyc.), 1.44, 1.48 (1H, br. 2d, *J=13.1*, CH/CH_2-cyc.), 1.72-1.81 (2H, m, CH/CH_2-cyc.), 2.03-2.12 (1H, m, CH/CH_2-cyc.), 2.38 (1H, br. d, *J=9.8*, CH/CH_2-cyc.), 2.74 (1H, br. d, *J=13.2*, CH_2-cyc.), 3.76, 3.77 (3H, 2s, 4-OCH_3-ar.), 6.46, 6.47 (1H, 2d, *J=2.5*, H3-ar.), 6.50-6.54 (1H, m, H5-ar.), 7.87, 7.89 (1H, 2d, *J=9.0*, H6-ar.), 10.92 (1H, s, NH/OH), 12.11, 12.32 (1H, 2s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 22.57, 22.74, 25.62, 25.65, 28.44 (3-$(CH_3)_2$, 5-CH_3-cyc.), 30.00, 32.18 (CH-cyc.), 34.00, 34.61, 35.65 (CH_2-cyc.), 43.20, 47.57, 47.66 (CH_2-cyc., C3-cyc.), 55.80 (4-OCH_3-ar.), 101.71, 101.77, 106.57, 106.64 (C3,C5-ar.), 109.48, 110.32 (C1-ar.), 131.00, 131.68 (C6-ar.), 159.61, 160.74, 161.95, 163.62, 163.74, 164.10, 164.31 (C2,C4-ar.,C=N,C=O). MS (ESI-) m/z (%): 303.6 ([M-H]$^-$, 100). Anal. Calcd for $C_{17}H_{24}N_2O_3$ (304.38): C, 67.08; H, 7.95; N, 9.20. Found: C, 67.36; H, 8.12; N, 9.08.

N'-[4-(acetylamino)cyclohexylidene]-2-hydroxy-4-methoxybenzohydrazide **(2h)**

White flakes (90%); mp 242-245°C; IR(KBr): υ_{max} 3342, 3298 (N-H), 1631 (C=O), 1609 (C=N), 1537, 1514, 1497 (C=C); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 1.36-1.50 (2H, m, CH_2-cyc.), 1.81 (3H, s, 4-NHCO<u>C</u>H_3-cyc.), 1.87-1.94 (2H, m, CH_2-cyc.), 2.13-2.19 (1H, m, CH_2-cyc.), 2.35 (1H, td, *J=13.3, 4.9*, CH_2-cyc.), 2.44-2.49 (1H, m, CH_2-cyc.), 2.68 (1H, br. d, *J=15.1*, CH_2-cyc.), 3.77 (3H, s, 4-OCH_3-ar.), 3.82-3.90 (1H, m, CH-cyc.), 6.46 (1H, d, *J=2.5*, H3-ar.), 6.51 (1H, dd, *J=8.8, 2.5*, H5-ar.), 7.86 (1H, d, *J=7.5*, H6-ar.), 10.88 (1H, s, NH/OH), 12.26 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 23.20 (4-NHCO<u>C</u>H_3-cyc.), 25.71, 31.18, 32.35, 32.96 (CH_2-cyc.), 46.40 (CH-cyc.), 55.80 (4-OCH_3-ar.), 101.69, 106.64 (C3,C5-ar.), 109.50 (C1-ar.), 131.10 (C6-ar.), 160.63, 163.24, 163.78, 164.11 (C2,C4-ar.,C=N,C=O), 168.92 (4-NH<u>C</u>OCH_3-cyc.). Anal. Calcd for $C_{16}H_{21}N_3O_4$ (319.36): C, 60.17; H, 6.63; N, 13.16. Found: C, 59.73; H, 7.05; N, 12.93.

N'-[2-(2-cyanoethyl)cyclohexylidene]-2-hydroxy-4-methoxybenzohydrazide **(2i)**

White crystals (69%); mp 147-149°C; IR(KBr): υ_{max} 3304 (N-H), 1649 (C=O), 1614 (C=N), 1543, 1508 (C=C); ^{1}H-NMR (DMSO-d_6/500 MHz): δ 1.32-1.40 (1H, m, CH/CH_2-sp.), 1.49-1.74 (5H, m, 2-C<u>H</u>$_2$$CH_2$CN-cyc. and/or CH/$CH_2$-sp.), 1.88-1.94 (1H, m, CH/CH_2-sp.), 2.04-2.11 (2H, m, 2-C<u>H</u>$_2$$CH_2$CN-cyc. or CH/$CH_2$-sp.), 2.37-2.41 (1H, m, CH/CH_2-sp.), 2.60-2.65 (3H, m, 2-CH_2C<u>H</u>$_2$CN-cyc., CH/CH_2-sp.), 3.77 (3H, s, 4-OCH_3-ar.), 6.47 (1H, d, *J=2.5*, H3-ar.), 6.52 (1H, dd, *J=8.5, 2.5*, H5-ar.), 7.87 (1H, d, *J=8.5*, H6-ar.), 10.94 (1H, s, NH/OH), 12.22 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 14.78 (2-CH_2<u>C</u>H_2CN-cyc.), 24.04, 26.51, 27.45 (CH_2-cyc., 2-<u>C</u>$H_2$$CH_2$CN-cyc), 33.30, 41.88 ($CH_2$-cyc.), 43.20 (CH-cyc.), 55.80 (4-OCH_3-ar.), 101.71, 106.66 (C3,C5-ar.), 109.58 (C1-ar.), 121.31 (2-$CH_2$$CH_2$<u>C</u>N-cyc), 131.12 (C6-ar.), 160.57, 163.77, 164.10, 165.46 (C2,C4-ar.,C=N,C=O). Anal. Calcd for $C_{17}H_{21}N_3O_3$ (315.37): C, 64.74; H, 6.71; N, 13.32. Found: C, 64.29; H, 6.86; N, 13.46.

2-hydroxy-4-methoxy-N'-(4-phenylcyclohexylidene)benzohydrazide **(2j)**

White crystals (84%); mp 221-222°C; IR(KBr): υ_{max} 3263 (N-H), 1638 (C=O) 1607 (C=N), 1543, 1502 (C=C); ^{1}H-NMR (DMSO-d_6/500MHz): δ 1.60-1.75 (2H, m, CH_2-cyc.), 1.97-2.04 (2H, m, CH_2-cyc.), 2.16 (1H, td, *J=14.1, 5.4*, CH_2-cyc.), 2.46 (1H, td, *J=13.7, 4.9*, CH_2-cyc.), 2.56 (1H, br. d, *J=13.7*, CH_2-cyc.), 2.85-2.91 (2H, m, CH-cyc., CH_2-cyc.), 3.77 (3H, s, 4-OCH_3-ar.), 6.47 (1H, d, *J=2.5*, H3-ar.), 6.52 (1H, dd, *J=8.5, 2.5*, H5-ar.), 7.18-7.31 (5H, m, 4-C_6H_5-cyc.), 7.88 (1H, d, *J=9.0*, H6-ar.), 10.94 (1H, s, NH/OH), 12.27 (1H, s, NH/OH); ^{13}C-NMR (APT) (DMSO-d_6/125 MHz): δ 27.81, 33.24, 34.27, 35.13 (CH_2-cyc.), 42.93 (CH-cyc.), 55.80 (4-OCH_3-ar.), 101.71, 106.65 (C3,C5-ar.), 109.62 (C1-ar.), 126.61, 127.16, 128.84 (4-C_6H_5(C2-6)-cyc.), 131.17 (C6-ar.), 146.19 (4-C_6H_5(C1)-cyc.), 160.55, 163.46, 163.75, 164.04 (C2,C4-ar.,C=N,C=O). Anal. Calcd for $C_{20}H_{22}N_2O_3$ (338.40): C, 70.99; H, 6.55; N, 8.28. Found: C, 70.57; H, 6.67; N, 8.36.

Antibacterial and Antifungal Activity Assays

The *in vitro* antimicrobial activity of compounds **2a**-**j** was evaluated against four Gram negatif bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153), three Gram positive bacteria (*Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212) and three fungi (*Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 750) using the microbroth dilution method according to the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI 1997; CLSI 2006) and compared with the standard drugs.

Serial twofold dilutions ranging from 2500 µg/mL to 1.22 µg/mL were prepared in the test medium, i.e. Mueller-Hinton broth for bacteria and RPMI-1640 medium for yeast strains. The inoculum was prepared using a 4-6 h broth culture of each bacteria type and 24 h culture of yeast strains adjusted to a turbidity equivalent to 0.5 McFarland standard, diluted in broth media to give a final concentration in the test tray of 5×10^5 cfu/mL for bacteria and 5×10^3 cfu/mL for yeast. The trays were covered and placed into plastic bags to prevent evaporation. The bacteria trays were incubated at 35°C for 18-20 h while the yeast-containing trays were incubated at 35°C for 46-50 h. The MIC was defined as the lowest concentration of compound giving complete inhibition of visible growth. As a control, antimicrobial effects of the solvents against the tested microorganisms were also investigated.

RESULTS AND DISCUSSION

Chemistry and Structural Characterization

The synthetic pathway for the preparation of the target hydrazones (**2a**-**j**) are illustrated in Scheme 1. Reactions occured readily under mild temperatures. The structures of the obtained compounds were established using IR, ^{1}H-NMR, ^{13}C-NMR (APT), electrospray ionization mass spectrometry (ESI-MS) and microanalytical data.

Scheme 1

IR spectra of the new hydrazone derivatives **2a**-**j** showed single N-H band in the 3263-3311 cm^{-1} region, while the IR spectrum of the starting hydrazide **(1)** exhibited three seperate N-H strechings at 3149, 3275 and 3319 cm^{-1}. The C=O groups of compounds **1** and **2a**-**j** absorbed in the 1635 and 1622-1658 cm^{-1} regions, respectively. No phenolic O-H streching vibrations were observed in the IR spectra of the hydrazide (**1**) or hydrazones (**2a**-**j**). Absence of the O-H bands in the expected regions is presumably due to the strong intramolecular hydrogen bonding between the phenolic O-H and C=O groups (Silverstein et al. 2005).

^{1}H-NMR spectra displayed the N-H and O-H resonances in the δ 10.85-10.94 ppm and δ 12.11-12.32 ppm regions as singlets. The resonances of the $-OCH_3$ group and aromatic hydrogens were observed in the δ 3.76-3.77 ppm and δ 6.46-7.89 ppm, respectively. The splitting patterns of the aromatic H3, H5, H6 hydrogens were in accordance with the 1,2,4-trisubstituted aromatic ring system. The aliphatic protons of the cyclohexane residue resonated at about δ 1.04-3.79 ppm region depending on the substituents on the ring system.

Carbon assignments were made on the basis of APT experiments which provided information about the number of protons attached to a ^{13}C atom. The quaternary C=N and C=O carbon resonances appeared as positive signals in downfield region together with the aromatic quaternary C2 and C4 carbon resonances (δ 159.61-165.46 ppm). Observation of upfield resonances (δ 11.95-47.66 ppm) assigned to the CH/CH_2 carbons of the cyclohexane residue further proved the intended conversion.

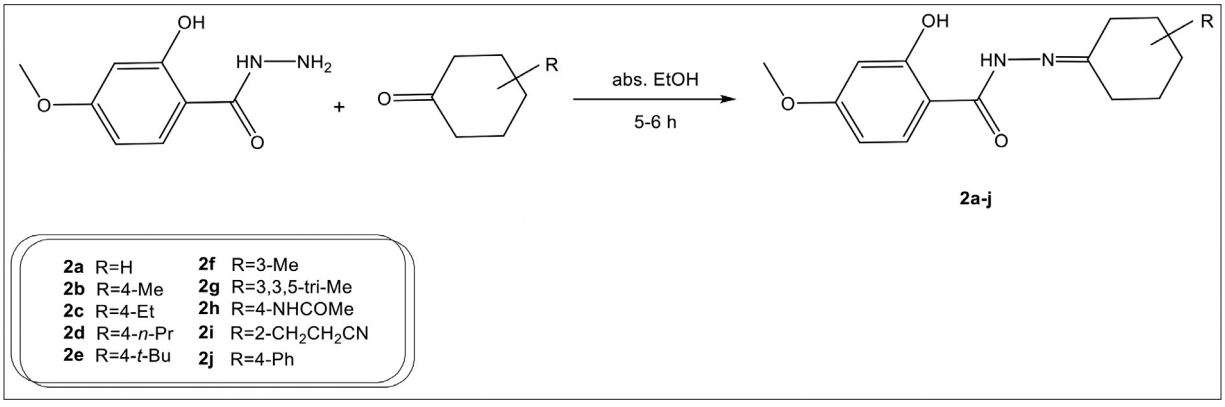
The proton spectra of compounds **2f** and **2g** displayed two sets of signals for some protons. Aromatic protons of **2f** and **2g** absorbed as two doublets (H3 and H6) and distorted multiplets (H5). Methyl substituents on the cyclohexane ring appeared as duplicated doublets for both compounds. The OH/CONH resonance about 12.00 ppm and the $-OCH_3$ resonance of compound **2g** were also detected as two separate singlets. Similarly, two signal sets appeared for most of the carbon atoms in the APT spectra of compounds **2f** and **2g**. The multiplicity in the signals pointed to the presence of two isomers due to the restricted rotation about the N=C double bond. It is assumed that the methyl substituents at 3- or 5-positions interrupt the symmetry of the molecules and give rise to the formation of *E* and *Z* isomers for compounds **2f** and **2g**.

ESI-MS was used to verify the molecular weights of compounds **2a**, **2b**, **2e**, **2f** and **2g**. Compounds **2a**, **2b** and **2e** were analyzed under negative-ion ESI conditions while compounds **2f** and **2g** were analyzed under positive-ion ESI conditions. Deprotonated $[M-H]^-$ or protonated $[M+H]^+$ molecular ions observed in the ESI-MS confirmed the molecular weights of the compounds.

Antibacterial and Antifungal Activity

The antibacterial and antifungal activity of compounds **2a-j** was evaluated *in vitro* against the following strains: *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Enterococcus faecalis* ATCC 29212, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 29213, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 750. The compounds were prepared using twofold dilutions starting at 2500 µg/mL. The lowest concentration of compound giving complete inhibition of visible growth was referred as the MIC (minimum inhibitory concentration).

As shown in Table 1, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 29213 and *Candida parapsilosis* ATCC 22019 were the most sensitive strains to the tested hydrazone derivatives. All of the tested compounds, except for compound **2h**, showed weak antibacterial activity against *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus aureus* ATCC 29213 with MIC values of 312.5-1250 µg/mL. Compounds **2a**, **2b**, **2e**, **2f** and **2i** further exhibited antifungal activity against *Candida parapsilosis*, showing complete inhibition at MIC val-



Scheme 1. Synthesis of **2a-j.**

Table 1. Antimicrobial properties of compounds 2a-j against selected bacteria and fungi

	MIC (µg/mL)[a]										
Microorganism	2a	2b	2c	2d	2e	2f	2g	2h	2i	2j	Reference antimicrobials
P. aeruginosa ATCC 27853	-[b]	-	-	-	625	-	-	-	-	-	2.4 (Ceftazidime)
E. coli ATCC 25922	-	-	-	-	-	-	-	-	-	--	4.9 (Cefuroxime-Na)
K. pneumoniae ATCC 4352	-	-	-	-	-	-	-	-	-	-	4.9 (Cefuroxime-Na)
P. mirabilis ATCC 14153	-	-	-	-	-	-	-	-	-	-	2.4 (Cefuroxime-Na)
E. faecalis ATCC 29212	-	-	-	-	-	-	-	-	-	-	128 (Amikacin)
S. epidermidis ATCC 12228	625	1250	1250	1250	625	1250	1250	-	1250	625	9.8 (Cefuroxime)
S. aureus ATCC 29213	625	1250	1250	1250	625	312.5	625	-	312.5	312.5	1.2 (Cefuroxime-Na)
C. albicans ATCC 10231	-	-	-	-	-	-	-	-	-	-	4.9 (Clotrimazole)
C. parapsilosis ATCC 22019	312.5	312.5	-	-	625	625	-	-	312.5	-	0.5 (Amphotericin B)
C. tropicalis ATCC 750	-	-	-	-	-	-	-	-	-	-	1 (Amphotericin B)

[a] Minimum inhibitory concentration: the lowest concentration of compound giving complete inhibition of visible growth.
[b] No activity at the highest concentration tested.

ues of 312.5 and 625 µg/mL. Neither of the test compounds displayed antimicrobial activity below 312.5 µg/mL.

Benzaldehyde phenylhydrazones incorporating an aromatic system linked to the hydrazide moiety have been extensively studied by different research groups (Kumar et al. 2010; Niazi et al. 2010; Tajudeen et al. 2013; Sapra et al. 2014; Backes et al. 2014). This type of compounds was found to be highly active against different bacteria and fungi species. Replacing the unsaturated aromatic ring with a saturated cyclohexane system seemed to have a negative effect on antimicrobial activity since compounds **2a**-**j** were found to be slightly active against the tested bacteria and fungi.

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Original Article

The cytotoxicity and insecticidal activity of extracts from *Delphinium formosum* Boiss. & Huet

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ABSTRACT

Delphinium species are well-known toxic plants with diterpenoid alkaloid contents. There has been no previous investigation on the cytotoxicity of *Delphinium formosum*. The extracts of the different parts of *D. formosum*, an endemic species in Turkey, were investigated for their cytotoxic activity against the human liver carcinoma cell line (HepG2) and primary human umbilical vein endothelial cells (HUVEC). The cytotoxic effects of twelve extracts and subfractions were determined against HepG2 cells using the MTT assay. The only active extract was applied to the HUVEC as a model for healthy cells. Only one of the alkaloid-containing extracts from the aerial parts was toxic (IC50=244,9 µg/mL against HepG2 and 144,4 µg/mL against HUVEC), while the root extracts were inactive. The results were improbable although it is predicted secondary metabolites, such as diterpene alkaloids (methyllycaconitine, browniine, lycoctonine, avardharidine, antranoyllycoctonine, delsemine A/B and lycoctonine). Based on previous studies in the literature, the cytotoxic plants were also expected to exhibit insecticidal activity. Therefore, the cytotoxic extract of *D. formosum* was examined for its adulticidal and larvicidal activity against the yellow fever, dengue fever and the Zika virus vector *Aedes aegypti* L.

Keywords: *Delphinium formosum*, cytotoxic activity, HepG2, HUVEC, natural insecticidal, *Aedes aegypti*

INTRODUCTION

The *Delphinium* L. genus (Ranunculaceae), with almost 300 species worldwide, consists of 29 species in Turkey (17 endemic to Anatolia). Their usages have a long history of medicinal use. Dioscorides mentioned their use against lice and scorpions. The powders of *D. staphisagria* L. and *D. peregrinum* L. were used against lice by the British army in the battle of Waterloo. The dried and mature seeds of *D. staphisagria*, known as "kokarot, kokarotu, müzüdek, mevezek and mevzek", (Baytop 1999) were used as emetics, purgatives and sedatives (in the treatment of rabies, tetanus, and epilepsy) in traditional Turkish medicine. The usage was left aside due to their high toxicity. Despite their toxicity, their external applications (as ointment, powder form or infusion (1-

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3%), to the body or on the head) were observed also, especially for their insect repellent activity. The seeds of *D. ajacis* L (Syn: *Consolida orientalis* (Gay) Schröd.) and *D. consolida* L. (Syn: *Consolida regalis* S.F. Gray subsp. *regalis*) are antiparasitic (Ulubelen et al. 2001; Hiller and Melzig 2006).

Vector-borne diseases cause epidemics, leading to serious human health problems. *Aedes aegypti* (Culicidae), one of these vectors, transmits viruses like Dengue, Yellow fever, Zika, and Chikungunya. According to WHO, yellow fever is an acute viral haemorrhagic disease transmitted by infected mosquitoes, and Dengue is a mosquito-borne viral infection, which causes a flu-like illness, and evolves on occasion into lethal complications. WHO also emphasized that, the symptoms of the infection of Zika virus, transmitted primarily by *Aedes* mosquitoes, are skin rash, mild fever, muscle and joint pain, headache, conjunctivitis or malaise, and is also a cause of Guillain-Barré syndrome and microcephaly (Masi et al. 2017; WHO February 2018; WHO May 2018; WHO April 2019). Many interventions are conducted to prevent these diseases, and one of them is integrated mosquito control, including the implementation of personal protection, destruction and reduction of its habitats, and insecticidal treatment regimens against adult and larval mosquitoes for the reduction of the virus spread. Reducing the amount of synthetic insecticides or pesticides applied, is preferred because of their undesirable and toxic effects. Frequent chemical interventions can also cause the development of insecticide resistance (Tabanca et al. 2013a; Masi et al. 2016). Therefore, an increasing number of plant-based extracts, essential oils or phytochemicals are being investigated with the aim of determining an effective agent against *A. Aegypti* (Kamaraj et al. 2010; Pitarokili et al. 2011; Maheswaran and Ignacimuthu 2012; Liu et al. 2012; Kumar et al. 2012; Tabanca et al. 2013a, b; Reegan et al. 2015; Cantrell et al. 2016; Masi et al. 2016; Dias et al. 2017; Carroll et al. 2017; Chantawee and Soonwera 2018; Stappen et al. 2018; Tabanca et al. 2018, 2016a, 2016b).

The alkaloids, one of the most remarkable groups of natural products in these plants, have a wide variety of biological activities. The structures of over a hundred alkaloids have been identified; many of them have elicited an expected anticancer activity (Lu et al. 2012). In addition to the studies on cytotoxic and anticancer effects (Liu et al. 2017; Nugroho et al. 2015; Chanakul et al. 2011), several investigations into insecticidal activities have been published (Bandara et al. 2000; Garcez et al. 2009; Liu et al. 2012; Masi et al. 2017). The diterpene and norditerpene alkaloids have also shown insecticidal activity (Ulubelen et al. 2001; Kukel and Jennings 1994). Also based on previous studies in literature, the cytotoxicity and insecticidal activities are related to each other, and diterpene alkaloids can be protective agents for parasite control strategies (Gonzalez-Coloma et al. 2004; Reina and Gonzalez-Coloma 2007).

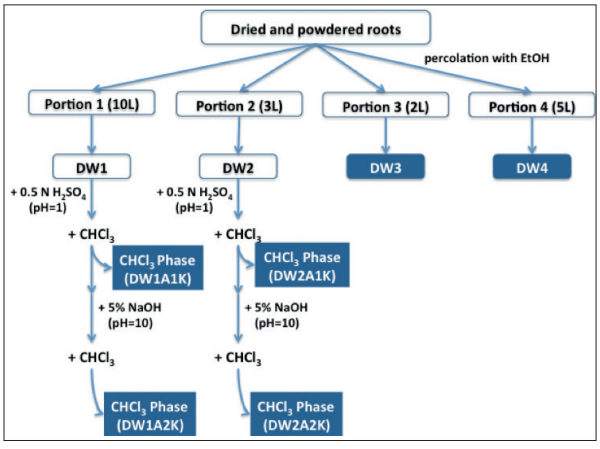

Figure 1. Representative scheme of extraction procedure of the roots, collected in the first year.

This preliminary study was presented to assess the cytotoxic and insecticidal potentials of extracts, obtained from *Delphinium formosum* an endemic species in Trabzon (Turkey), which has a role in traditional medicine and causes serious poisoning. The different extracts of its aerial parts, roots, and flowers were investigated for their cytotoxic activity against the human liver carcinoma cell line (HepG2) and primary human umbilical vein endothelial cells (HUVEC). The mosquitocidal activity of the cytotoxic extract against *Ae. aegypti* was also determined for the possibility to use its lethal potential as an insect repellent.

MATERIALS AND METHODS

Plant material

Delphinium formosum was collected from Köprübaşı-Trabzon and Macka-Trabzon, in August 2011 and July 2012. The voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE 102747; 98086).

Extraction procedure

Dried and powdered roots, collected in the first year, were percolated with EtOH in four portions (10 L, 3 L, 2 L, 5 L) and each portion was evaporated to dryness at 40°C *in vacuo* (DW1, DW2, DW3 and DW4). The residues of the portions (DW1 and DW2) were acidified to pH1 by 200-300 mL 0.5 N H_2SO_4 and extracted with $CHCl_3$. These $CHCl_3$ extracts were evaporated to dryness (DW1A1K and DW2A1K). The acid solutions were basidified with 5% NaOH to pH10 and extracted a second time with $CHCl_3$. The extracts were evaporated to dryness, and named as DW1A2K and DW2A2K (Figure 1).

Dried and powdered aerial parts, flowers and roots, collected next year, were percolated with EtOH in one portion and evaporated to dryness at 40°C *in vacuo*. The same process was applied. Two different chloroform extracts of each part (HA1K, HA2K, FA1K, FA2K, 2DWA1K, and 2DWA2K) were acquired (Figure 2).

Biological Assays

Cytotoxicity Assays

Cytotoxicity test, cell lines, culture conditions and treatments

Roswell Park Memorial Institute Medium 1640 (without glutamine; RPMI-1640) with glutamine, 10% FCS and penicillin / streptomycin mixture was used for the human liver carcinoma cells (HepG2). Primary human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by digestion with 0.01% collagenase A solution (Roche) and grown in Endothe-

lial Cell Growth Medium with a supplement mix (Promocell; C-39215) containing 10% FCS, penicillin (100 U/mL) / streptomycin (100 mg/mL) mixture, and kanamycin (50 mg/mL). Umbilical cords were obtained with the consent of patients (permission by the local ethics committee). All cells were grown at 37°C and 5% CO_2 in a humidified cell incubator. The culture medium was changed every 2 days. Monolayer cells grown to 75-85% confluence were detached with trypsin-ethylenediamine tetraacetic acid to make single cell suspensions, and the viable cells were determined using the trypan blue exclusion test and diluted with medium to give a final density of 10^5 cells/mL. The passage number range for HepG2 cell lines was maintained between 21 and 26. The passage number for primary HUVEC cells was maintained between 3 and 5. The extracts were initially dissolved in dimethyl sulfoxide (DMSO) and the stock solution of each extract was prepared at a concentration of 20 mg/mL. Next, the stock solutions were diluted in the medium to obtain final concentrations of 10-400 µg / mL. 100 µL of cell suspension per well were seeded into 96-well plates at a plating density of 10^4 cells/well for the HepG2 cells and $2x10^4$ cells/well for HUVEC, and incubated to allow for cell attachment at 37°C and 5% CO_2 for 24 h. After 24 h, the medium was aspirated, and the cells were treated with 100 µL serial concentrations of all extracts. The plates were incubated at 37°C and 5% CO_2 for 24 h. The medium without samples (negative control) and with DMSO as solvent served as controls. For each extract, all concentrations were tested n=2 or n=3 in quadruplicate. The cells in each well were quantified by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) test (Kessler et al. 2013; Diesel et al. 2011; Kiemer et al. 2002; Mosmann 1983).

Cytotoxicity studies

For MTT tests, the cells were then incubated with 150 µL MTT (0.5 mg/mL in medium) solution for 3 h. After the removal of the MTT solutions, the formed formazan crystals were solubilized in 80 µL of DMSO and then, the absorbance was measured at 550 nm and at 690 nm (as control wavelength) using a microplate reader.

The cell viability in treated cells compared to that of negative control cells was calculated. Then, the half maximal inhibitory concentration (IC_{50}) was expressed as the sample concentration that caused an inhibition of 50% in cytotoxicity in the cells calculated by extrapolation. The percentage of cell viability was calculated with respect to solvent control as follows:

$$\% \text{ Cell viability} = \text{Abs}_{\text{Compounds}} / \text{Abs}_{\text{Solvent Control}} \times 100$$

The results were expressed as cell death (%) compared to the negative control.

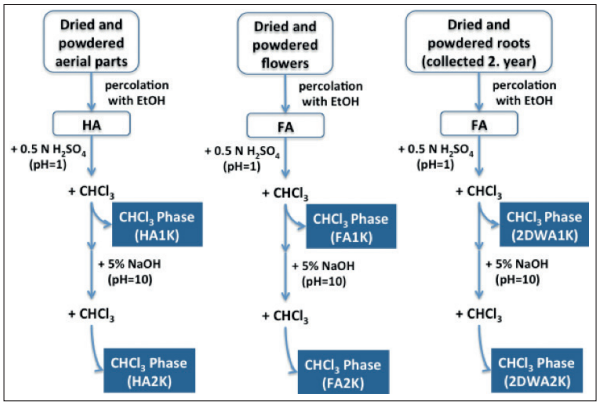

Figure 2. Representative scheme of extraction procedure of the aerial parts and flowers, collected in the first year, and the roots, collected in the second year.

Insecticidal Activity

Mosquito Colony

Aedes aegypti used for testing were pesticide susceptible, and provided by the CMAVE insectary. The "Orlando1952" strain was collected near Orlando, Florida, USA in 1952, and has been in continuous laboratory colony for 64 years. Rearing procedures are standardized and have been described previously (Tabanca et al. 2016b).

Larvicidal Activity

Larvicidal activity testing was performed essentially as described previously (Pridgeon et al. 2008), but the assay was modified for 96-well plates to conserve limited amount of test samples by the use of smaller volumes (Masi et al. 2017). The HA1K extract was diluted in dimethyl sulfoxide (DMSO) to make 100 mg/mL. Mortality was determined in the larval assays at four different concentrations (1.0, 0.5, 0.25, and 0.1 ¼g/¼L) in a final volume of 200 ¼L of larval rearing media. For each assay, a positive control of permethrin stock and a negative control of ethanol or DMSO was included. Assays were repeated at least three times on separate days using different hatches of eggs.

Adulticidal Activity

The toxicity of HA1K extract was tested in assays against adult *Aedes aegypti* using cohorts of 3-6-day post-emergence females as described previously (Pridgeon et al. 2008). Mosquitoes were cold anesthetized on ice, and groups of 10 females sorted into individual plastic cups. An application of 0.5 µL of the appropriate dilution of the test chemical was made by repeater pipettor (Hamilton PB600) with a 25 µL blunt tip glass syringe (Hamilton 7100 series) to at least twenty females at each dose. Permethrin mixture of 46.1% cis and 53.2 trans isomers (Chemservice, West Chester, PA) was used as a positive control, and acetone was used as negative control. After treatment, the mosquitoes were kept in plastic cups at 24-26°C and 80% humidity, and supplied with 10% sucrose in water for 24 h prior to recording mortality.

RESULT AND DISCUSSION

Due to the diterpenoid alkaloid content of *Delphinium* species, we expected distinct cytotoxic actions when testing their effect on HepG2 cells. Only one extract (HA1K) was active among them, in the studied concentration ranges. The same extract was also examined against the HUVEC cells. The IC_{50} values of HA1K against two cell types (244.9 µg/mL for HepG2 and 144.4 µg/mL for HUVEC) were similar.

The MTT assay demonstrated the effect of the extracts on the % cell viability of HepG2 and HUVEC cell lines as shown in Figure 3.

The HA1K extract, found cytotoxic against both cell lines, was further investigated for its insecticidal activity (Table 1). The extract was evaluated at the dose of 5 mg/mosquito and had 97% mortality against adult *Ae. aegypti*. The positive control permethrin at 6.33 pg/mL resulted in 53±11% mortality, while the negative control acetone resulted in 0% mortality. In larval activity, the HA1K extract showed 80% mortality at the highest dose of 1 mg/mL against 1st instar *Ae. aegypti*; however, the mortality tittered off quickly at the lower doses. Negative control mortality in larvicidal assays was 0% for DMSO, and positive control permethrin resulted in 100% at the 47.4 pg/mL. Since HA1K extract produced 97% mortality against adult *Ae. aegypti*, isolation of the active compound/-s through bioassay-guided fractionation and its/their characterization may be promising.

This is the first investigation on the cytotoxicity and insecticidal activity of *D. formosum*. On the other hand, several studies on the cytotoxic effect of the isolated compounds from other species exist in the literature. De Inés et al. evaluated 43 norditerpenoid alkaloids from *Aconitum*, *Delphinium* and *Consolida* species for their cytotoxic effects on the tumor cell lines SkMel28 (human malignant melanoma), HeLa (human cervical adenocarcinoma), SkMel25 (human melanoma), CT26 (murine colon adenocarcinoma), and SW480 (human colon adenocarcinoma) and the non-tumor cell line CHO (Chinese hamster ovary cells). Browniine, ajadelphinine, 8-*O*-methylcolumbianine, dehydrotakaosamine, lycoctonine, 14-deacetylajadine, pubescenine, 14-deacetylpubescenine, 1,14-diacetylcardiopetaline, 18-*O*-demethylpubescenine, delphatine and neoline showed selective cytotoxicity. The most active compound was a gadesine-type norditerpene alkaloid,

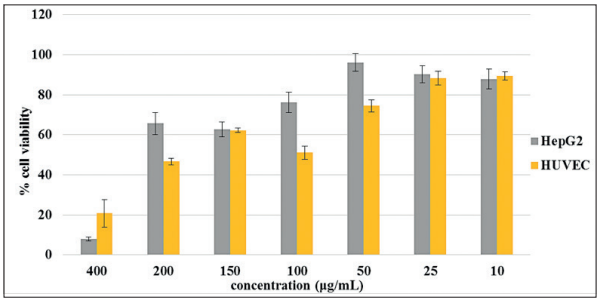

Figure 3. The % cell viability values of the HA1K against HepG2 and HUVEC cell lines.

Table 1. The mortalities of the HA1K against adult female mosquito *Ae. aegypti* and 1st instar *Ae. aegypti* larvae

Adult female mosquito *Aedes aegypti*	1st instar *Aedes aegypti* larvae (% mortality)			
5 µg/mosquito (% mortality)	1 µg/µL	0.5 µg/µL	0.25 µg/µL	0.1 µg/µL
96.7±5.8	80	53.3±23.1	13.3±11.5	0

dehydrotakaosamine. Its IC_{50} values were detected 0.40 µg/mL against HeLa cells and 6.25 µg/mL against CT26, SW480 and SkMe125 cells. Lycoctonine was found active against CT26 and SW480 (IC_{50}=50 µg/mL), while methyllycaconitine showed an activity against CHO, CT26, SW480 and HeLa (IC_{50}=12.50 µg/mL, 12.50 µg/mL, 50 µg/mL, 50 µg/mL; respectively). All IC_{50} values of browniine were more than 100 µg/mL against all cell lines, but an effect was seen by browniine against CT26 and SW480 cells (De Ines et al. 2006). In another study, from *Aconitum vulparia* isolated compounds (vulparine, finetiadine, anthranoyllycoctonine) exhibited a cell growth inhibitory activity against cervix adenocarcinoma (HeLa) and breast adenocarcinoma (MCF-7) cell lines. Finetiadine was the most cytotoxic compound at 30 µg/mL with the 39.48±3.42% on MCF-7 cell line and 25.59±1.87 % inhibition on HeLa cell line, while septentriodine and anthranoyllycoctonine also showed tumour cell inhibitory activity. Septentriodine showed an activity (34.50±3.72 % inhibition) against MCF-7 cells only at the concentration of 30 µg/mL (Csupor et al. 2007).

In a study on *Delphinium* alkaloids to determine the inhibition of α-bungarotoxin binding to insect and rat neural membranes, 17 *Delphinium* alkaloids were tested. Among them, glaudelsine showed the highest inhibition on the insect binding site even 10x more potent than methyllycaconitine, which was known as the most potent alkaloid until that date (Kukel and Jennings 1994). In another study, 29 diterpene and norditerpene alkaloids from *Delphinium*, *Consolida* and *Aconitum* species were investigated against *Tribolium casteneum* (Herbst.) to evaluate the repellent effects. Twenty-one alkaloids exhibited promising insect repellent activity (Ulubelen et al. 2001). Hetisine, a diterpen alkaloid, was found to have the most active repellency (59.12%). The compounds, delsemin-B, lycoctonine and browniine, showed also an activity against *Tribolium casteneum* (Ulubelen et al. 2001).

In a previous study from the Black Sea area (Trabzon) in Turkey, *D. formosum* was collected and, delsemine A/B, 14-demethylajacine (*N*-acetyldelectine), lycoctonine, anthranoyllycoctonine, delcosine and delectine were identified from the aerial parts (Mericli et al. 1996). Additionally, lycoctonine and delsemine were isolated from *D. formosum* roots, collected in Trabzon, in a previous study (Tanker and Ozden 1975). Durust et al. (1999) also isolated some phenolic acids such as p-coumaric, p-hydroxy benzoic, caffeic, protocatechic and vanillic acids from the flowers of *D. formosum*, collected in same region, with four different methods. In our recent study, we reinvestigated norditerpenoid alkaloids from the *D. formosum* roots, and methyllycaconitine, browniine, lycoctonine, avardharidine, antranoyllycoctonine and delsemine A/B were isolated (Sen-Utsukarci et al. 2018).

All of the aforementioned studies on the cytotoxic effects of diterpenoids and norditerpenoid alkaloids have a contribution on the cytotoxicity. The difference of the cytotoxicity of *D. formosum* (aerial parts and roots) can be explained with the diversity of secondary metabolites in the root-extracts. In light of it all, it gives the impression that the extracts may contain different percentages of the active substances.

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Informed Consent: N/A.

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

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Original Article

Investigation of antioxidant and cytotoxic activities of *Opuntia ficus-indica* (L.) Mill. fruit extract

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ABSTRACT

Opuntia ficus-indica fruits are claimed to possess several health-beneficial properties. This study was carried out to determine the phytochemicals as well as to compare antioxidant activity and cell viability of the methanol extract of OFI-fruits. Antioxidant activity was evaluated by various *in vitro* tests such as DPPH and ABTS. LC-MS and HPLC analyses were then performed. The activity of the OFI-fruit extract on viability of U87-MG (glioblastoma multiform), HT-29 (colon cancer) and human primary fibroblast cells was investigated by determining viable cells on a luciferase based system (Cell TiterGlo; Promega; USA). The results showed that OFI-fruits were a source of antioxidant compounds. Moreover, the extract was found to have rich phenolic-flavonoid contents. Quercetin was found in the extract according to LC-MS and HPLC studies. A remarkable increased cell death in the HT-29 and U87 cell lines was detected in a concentration-dependent manner (1-10mg/mL). Among these cell lines, HT-29 responded to OFI-fruit treatment more sensitively to even the lowest dose (1mg/mL). The results of this study showed that the OFI-fruit has significant antioxidant capacity and leads to strong cytotoxic activity on various cancer cells. Therefore, it can be suggested that OFI-fruits may have chemo-preventive potential and can be studied further to clarify cytotoxic abilities.

Keywords: Extract, *Opuntia ficus-indica*, brain cancer cell line, colon cancer cell line, antioxidant

INTRODUCTION

Opuntia ficus-indica (L.) Miller (Prickly pear cactus) is located in the Mediterranean and Aegean Region and belongs to the Cactaceae family (Kabas et al. 2006). This species is known as "kaynana dili, firenk inciri, hint inciri" in Turkey (Güner et al. 2012) and as "prickly pear" elsewhere in the world. The nutritional properties of this species have long been known. In Mediterranean countries, cladodes are not typically consumed as nutrients, but the fruits are largely consumed due to natural antioxidants which are may be protective against oxidative damage (Lee et al. 2002). The fruits are used also in traditional medicine for their hypolipidemic and hypoglycemic actions (Butera et al. 2002). The phytochemical compounds of prickly pear fruits and cladodes are vitamins, carotenoids, betalains, and polyphenolic compounds which have proven biological activities and health benefits (anti-cancer, anti-diabetic, anti-inflammatory, neuroprotective effect) (Ammar et al. 2018; Mena et al. 2018). OFI is especially rich in flavonoids and phenolic compounds. Due to the high amount of these substances the prickly pear fruit has a potent antioxidant activity (Butera et al. 2002; Lee et al. 2002;

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Dok-Go et al. 2003). *O. ficus-indica* (OFI) has become a popular research subject in recent years because of these therapeutical and biological properties (Bouaouine et al. 2018).

Cancer, in its various types, is the uncontrolled growth of abnormal cells. . Normal cells frequently fix DNA mutations but once the repair system fails, the cells become cancerous (Abdel-Hady et al. 2018). Although many drugs have been developed for the treatment of cancers, there are concerns about the therapeutic effects and safety of these drugs. The major problem of chemotherapeutic drugs used as a standard treatment in various types of cancers is their toxicity (Carroll et al. 2012; Lee et al. 2014; Livshits et al. 2014). However, products from plants have been proven to be effective and safe in the treatment of cancers. Therefore, cancer drug discoveries are also directed to plant derived products obtained from natural plants (Demain and Vaishnav 2011; Bishayee and Sethi 2016). These products act as anti-cancer agents by interfering with the initiation, development and progression of cancer through the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis (Sreekanth et al. 2007; Rajesh et al. 2015).

The herbal products are used worldwide in the prevention and treatment of various chronic diseases, and their potential anticancer and antimutagenic effects are under current investigation (Ahmad et al. 2014). The present study aims to determine and evaluate total phenolic and total flavonoid compounds, in vitro antioxidant properties, and in vitro cytotoxic activities against U87-MG (glioblastoma multiform; brain cancer), and HT-29 (colon cancer) cell lines.

MATERIALS AND METHODS

Materials

The standard chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and the HPLC-grade solvents were purchased from Merck. DPPH (1,1diphenyl-2-picryl hydrazyl radical), ABTS (2-2'azinobis(3-ethylbenzthiazoline-6-sulphonic acid), Folin-Ciocalteu reagent and methanol were purchased from Sigma-Aldrich (Germany). All other reagents and solvents used were of analytical grade.

Preparation of extract samples

OFI-fruit samples were collected from Turunç, Marmaris (Date: 04.08.17). Voucher samples were deposited in the Herbarium of the Faculty of Pharmacy at Ankara University (No: AEF 28753). The pinkish fruits were thinly cut and air-dried. Samples were powdered and extracted with methanol on a magnetic stirrer (Heidolph MR3001, Germany) (200 g sample, 200 ml x 3) followed by filtration. The extract was distilled by the rotary evaporator (Heidolph WB2000, Germany). The yield of the extraction procedure was calculated as 8,98% (w/w).

Total phenolic and flavonoid contents of the extract

TPC (Total phenolic content) of OFI-fruit extract was determined by Folin-Ciocalteu method. 5 ml of extract sample was mixed with 0.2 ml of 7.5% Na_2CO_3 and 0.25 ml of Folin-Ciocalteu's reagent. The mixtures were incubated for 15 min at 45ºC and the absorbance was calculated at 765 nm. TPC was determined by the calibration curve (R^2=0.9811) (Okur et al. 2018a) and the result was expressed in terms of mg gallic acid equivalents (GAE) per 100 g (Spanos and Wrolstad 1990).

The colorimetric aluminum chloride method was utilized in order to determine TFC (Total flavonoid content) of the OFI-fruit extract. Briefly, 50 µL OFI-fruit extract was diluted to 1 mL volume with methanol. After being added to 5% $NaNO_2$ (0.3 mL) solution and water (4 mL), the mixture was left for incubation (5 min) and then a solution of 10% $AlCl_3$ *(0.3 m*L) was added and the mix was kept for 6 minutes. Next, a solution of 1 mol/L NaOH (2 mL) was added and the mix was made up to 10 mL with water. The mixture was kept for 15 minutes, and absorbance was measured spectrophotometrically at 510 nm. TFC was measured by the calibration curve (R^2=0.9978) (Okur et al. 2018b) and the result was expressed in terms of mg quercetin equivalents (QE) per 100 g. For analysis of flavonoid and phenolic compounds studies of extract sample, three replications were performed.

In vitro antioxidant assays

DPPH scavenging assay

The antioxidant capacity of the OFI-fruit extract was determined in terms of hydrogen donating or radical scavenging ability using DPPH by its capability to bleach the stable radical (Blois 1958). The reaction mix contained 100 µM DPPH in methanol and several concentrations of the crude extract. After 30 minutes, absorbances were measured at 517 nm using an UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 25±2°C and the radical scavenging activity (RSA) was determined as the percentage of radical reduction. The study was performed on Ascorbic acid as the reference (Okur et al. 2018a). The outcomes were declared as IC50 as follows:

DPPH RSA % = [(Absorbance control – Absorbance test sample)/Absorbance control)] x 100

Each experiment was performed in triplicate. Ascorbic acid was used as the reference (Okur et al. 2018).

The DPPH test is based upon the reduction of methanolic DPPH solution in the presence of hydrogen do-nating antioxidants through the formation of non-radical form (DPPH-H) (Lee et al. 2012).

ABTS radical scavenging assay

The antioxidant capacity of OFI-fruit extract was evaluated by the ABTS radical cation decolorization test (Re et al. 1999). ABTS solution was prepared by mixing aqueous ABTS (7 mM) and potassium persulfate (2.45 mM). The mixture was stored for 12-16 h in the dark at 25±2ºC. To regulate its absorbance at 734 nm, this final solution was diluted with ethanol and the percent inhibition was calculated. The test was carried out in triplicate. To determine absorbance of the extract, 990 µL ethanol was used instead of ABTS in the control. The study was performed on Trolox as the reference (Okur et al. 2018b). The results were expressed as IC_{50} as follows:

ABTS RSA % = [(Absorbance $_{control}$ – Absorbance $_{test\ sample}$)/Absorbance $_{control}$)] x 100

Extraction for LC-MS and HPLC analysis

For Liquid Chromatography Mass Spectroscopy (LC-MS) analysis 5 mg methanol extract of OFI-fruit was dissolved in 5 mL

ethanol and filtered by 0.22 µm membrane filters. Also, the same sample was used for High Pressure Liquid Chromatography (HPLC) analysis.

Qualitative and quantitative chromatographic analysis with LC-MS and HPLC systems

Methanol extract of OFI-fruit was analysed using LC-MS on a single quadrupole mass spectrometer (1200 LC, Agilent). For the chromatropic separation, LC-MS was run on an Agilent 4.6x250 mm, 5 µm particle size, octadecyl silica gel C18 analytical column and its temperature was maintained at 40°C. The elution gradient which consisted of mobile phases were A (Acetonitrile: Water: Formic acid (10:89:1, v/v/v)) and B (Acetonitrile: Water: Formic acid (89:10:1, v/v/v)). The gradient elution was established in the time frame 0-40 min, B% 15-100. The solvent flow rate was maintained at 0.7 ml/min. The injection volume was 20 µL (Gulsoy-Toplan et al. 2018).

Quercetin concentration in OFI-fruit methanol extract was determined using HPLC technique. The HPLC analysis was performed on an Agilent 1200 series instrument. The analytical HPLC column was Agilent C18 column of 5 µm particle size and 4.6 mm x 250 mm dimensions. LC-MS mobil phase was used. A reference standard of quercetin was prepared from the USP. The flow rate was set to 0.7 mL/min; injection volume and column temperature were adjusted to 20 µL and 40°C, respectively. The detection was performed at 330 nm (Gulsoy-Toplan et al. 2018). The HPLC method was validated based on the experimental results; linearity, selectivity, LOD, LOQ, accuracy, precision, RSD, recovery and robustness (Üstündağ-Okur et al. 2015; Okur et al. 2018b). The quercetin concentration in each extract was calculated by the following regression equation y= 59671x-18,47 with good linearity (r^2=0.9996). All standard and sample solutions were injected in triplicate.

Table 1. TPC and TFC contents of OFI-fruit extract

	TPC mg GAE/ 100g Extract	TFC mg QE/ 100g Extract
OFI Fruit Extract	363.20±0.02	1490.34±0.05

TPC: Total phenolic; TFC: total flavonoid

Table 2. DPPH and ABTS scavenging activities of OFI-fruit methanol extract

	OFI Fruit Extract	References
	IC50 ±SD (mg/mL)	
DPPH	4.12±0.03	4.67±0.04 (Ascorbic acid)
ABTS	2.46±0.04	3.84±0.04 (Trolox)

Table 3. Determined flavonoids of OFI-fruit methanol extract by LC-MS

Compound	RT	Area	Base Peak (m/z)
Myrcetin	11.436	6268	316.93
Quercetin	15.250	6926446	301.02
Luteolin	19.153	39288	285.03

Cell culture

U87-MG (glioblastoma multiform; brain cancer), HT-29 (colon cancer) and human primary fibroblast ((HDFa) ATCC® PCS-201-012) were purchased from ATCC (U.S.). The cells were cultured in growth medium either DMEM (U87-MG, Human primary fibroblast) or RPMI (HT-29,Gibco) medium each with 10% fetal bovine serum (Gibco), 1% antibiotic (penicillin/streptomycin) at 37°C in 5% CO_2 incubator. Confluent cells were then removed from the flask with trypsin/EDTA (Gibco) and seeded into 96 well plates for viability assays.

Cell viability assay

Cells were plated in 96 well plates at a density of $5x10^3$ cells/well for cell viability assays (luciferase based Cell Titer Glo Assay: Promega) and incubated for 24 h at 37°C in 5% CO_2 incubator. The extract was dissolved in dimethylsulfoxide (DMSO) and filtered to prepare stock solutions, and then serial dilutions were made using growth mediums considering 0.5% DMSO threshold for cellular toxicity. Then the medium was aspirated from all wells and treated with the OFI-fruit extract at a concentration range between 1-10 mg/mL. Accordingly, to normalize the measurements, control cells were treated with DMSO for each dose accordingly. All treatments were performed as triplicates. After 48 h, the medium was removed and Cell Titer Glo reagent (Promega) was applied to the wells and measurements of metabolically active cells were determined using SpectraMax i3x Multi-Mode Detection Platform (Sittampalam et al. 2004, Rodenhizer et al. 2018, Zhang et al. 2018).

Statistical analysis

For cell viability assays, statistical comparisons were performed by unpaired Student's t-test assuming equal variance. Differences were considered as statistically significant at $0.001<p^*<0.05$ and $p^{**}<0.005$. Data are the mean±standard error of the mean (S.E.M.).

RESULTS

Total phenolic and flavonoid contents of the extract

TPC and TFC of the OFI-fruit extract were determined. The Folin-Ciocalteu method is a widespread assay which is used for quantitative determination of phenolic compounds. Table 1 shows the total phenolic and flavonoid contents. OFI-fruit extract contained a significantly high antioxidants content (363.20±0.02 GAE/100g extract).

In vitro antioxidant assays

Detection of antioxidant capacity of the OFI-fruit extract is given in Table 2. According to the DPPH test results, antioxidant activity of OFI fruit extract was found as 4.12±0.03mg/mL and according to other (ABTS) test results, OFI fruit extract as 2.46±0.04 mg/mL.

Qualitative–quantitative chromatographic analysis by LC-MS and HPLC

The flavonoid contents of the methanol extract were detected by HPLC and LC/MS analyses**.** Standards were detected in accordance with quantitative and mass analyses using reversed-phase chromatography. Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one) was identified in the methanol

extract as major flavonoid component. Additionally, myrcetin and luteolin were detected in the extract as flavonoids. The LC-MS chromatogram of the methanol extract is shown in Table 3.

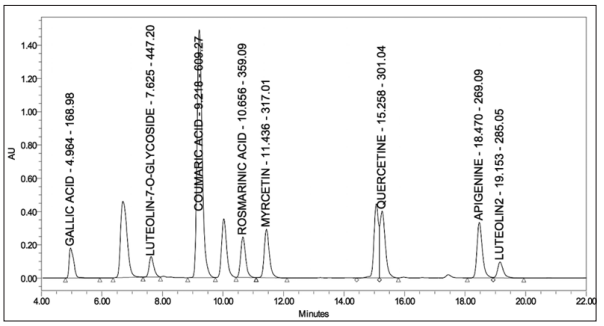

Figure 1. LC-MS standard chromatogram. Standards: 1, Gallic acid (R.T. 4,96); 2, Luteolin-7-o-glycoside (R.T. 7,62); 3, Coumaric acid (R.T. 9,21); 4, Rosmarinic acid (R.T. 10,65); 5, Myrcetin (R.T. 11,43); 6, Quercetin (R.T. 15,25); 7, Apigenine (R.T. 18,47); 8 Luteolin (R.T. 19,15) (R.T.=Retantion time).

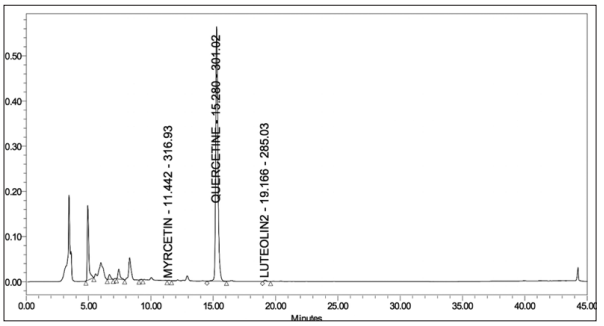

Figure 2. OFI-fruit metanol extract LC-MS chromatogram.

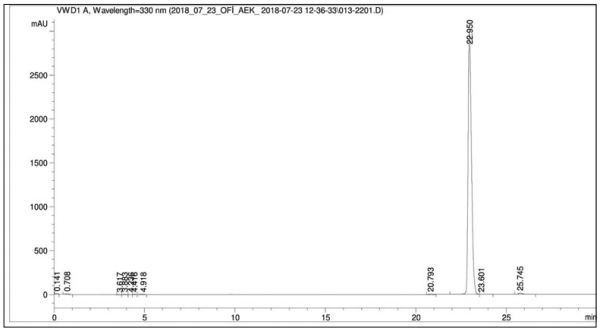

Figure 3. Quercetin Standard chromatogram. Quercetin (R.T. 22,86).

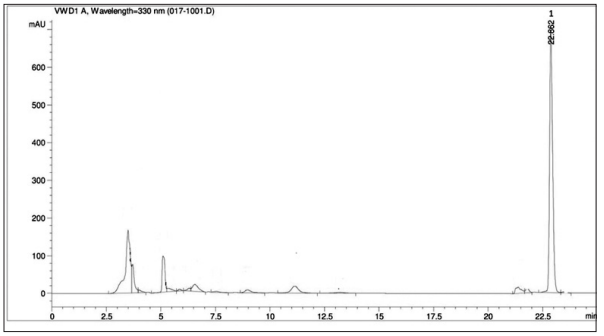

Figure 4. OFI-fruit methanol extract HPLC Chromatogram. 1, Quercetin (R.T. 22,86).22,86).

Determined flavonoids of OFI-fruit methanol extract by LC-MS were given in Figure 1. Figure 2 shows OFI-fruit extract LC-MS chromatogram.

Quercetin amounts in the methanol extract was examined by HPLC (Figures 3 and 4). Additionally, the HPLC method was validated according to the experimental results of quercetin: linear range (0.02-0.0015 mg/ml); recovery (99.82±3.97%); LOD (0.0006 mg/mL); LOQ (0.0015 mg/mL) and RSD (6.16%). The extract has 0.401±0.012% amount of quercetin.

Cell viability

The OFI-fruit extract (1-10mg/mL) showed significant cytotoxic effects on HT-29 (colon) and U87 (brain) cancer cell lines while the extract was not cytotoxic for human primary fibroblast at the same concentrations (Figure 5. a-c.). Cytotoxicity of the OFI-fruit extract on U87, HT-29 cancer cell lines and human primary fibroblast (as healthy control) was analyzed by measuring cell viability based on their metabolically active state. According to the obtained results, the OFI-fruit extract resulted in a significantly increased cell death in the HT-29 (colon cancer) and U87 (brain cancer) between the concentration range of 1-10 mg/mL in a concentration dependent manner. Among these cell lines, HT-29 responded to OFI-fruit extract treatment more sensitively even at the lowest dose (1 mg/mL). On the other hand, 5mg/ml treatment with OFI-fruit extract was sig-

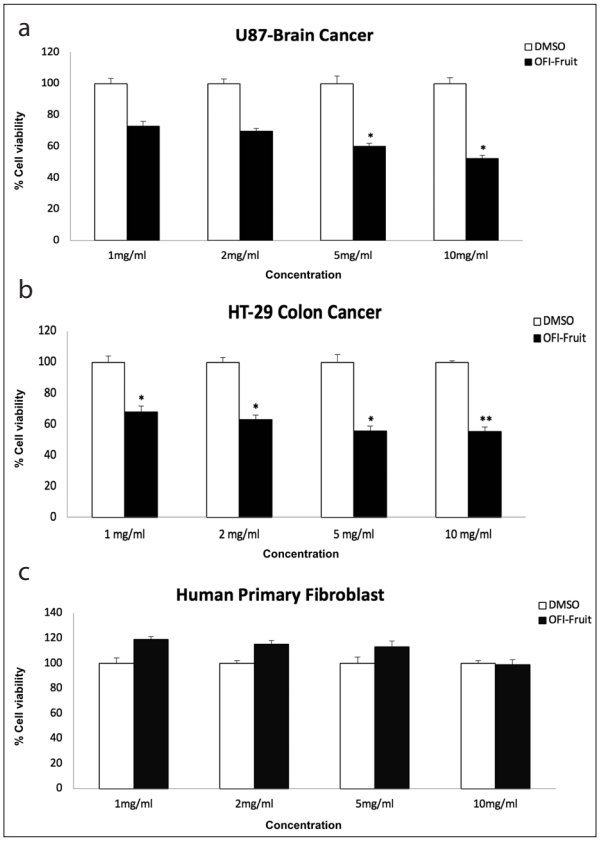

Figure 5. a-c. Cell viability of a) U87 (brain cancer) cell line, b) HT-29 (colon cancer) cell line, c) human primary fibroblast (normal cell) cell line upon 48 hours treatment with 1-10 mg/mL OFI-fruit extracts. Data are expressed as the mean±SEM (0.001<p*<0.05 and p**<0.005).

nificantly toxic to U87 cell line and cell death increased when treated with 10 mg/mL extract.

DISCUSSION

Cancer is a major public health problem in the world. In recent years, the use of herbal medicines in cancer treatment has received increasing attention due to their varied phytometabolic contents with multiple biological activities (Mann 2002). In our study, TPC of OFI-fruit extract was determined by Folin-Ciocalteu method. TPC of OFI-fruit extract was determined as 363.20±0.02 GAE/100g extract. The phenolic and flavonoid compounds are the major ingredients of various plants. It has been stated that these compounds possess protective and therapeutic activities to heal various diseases due to their hydroxyls groups which are responsible for the free radical scavenging activity (Abdel-Hady et al. 2018). OFI is rich in flavonoid and phenolic compounds and therefore it has high antioxidant activity (Lee et al. 2002). Flavonoids belong to secondary plant metabolites with a polyphenolic structure, which is commonly found in fruits and vegetables. They have various significant biochemical effects such as antioxidative, anti-inflammatory, antimicrobial, antithrombogenic, antimutagenic and anticarcinogenic activities (Panche et al. 2016). More than 5000 naturally occurring flavonoids have already been identified. The outcomes displayed in Table I show that the OFI-fruit extract exhibit high flavonoid and phenolic contents.

Antioxidants play an important role in neutralizing free radical species which are produced as end or by-products of normal biochemical reactions in a normal system (Jiménez-Estrada et al. 2013). Oxidative stress causes many diseases (cardiovascular diseases, diabetes, cancer, alzheimer etc.). Current studies about free radicals have supported that antioxidant rich-foods display a leading role in the prevention of cancers. Therefore, much interest has been focused on the benefit of natural antioxidants to keep protection against free radicals which cause damage (Lee et al. 2012). Many methods of analysis have been developed for *in vitro* and *in vivo* antioxidant activity. However, there are only a few reliable and fast methods for the evaluating of the antioxidant capacity of plant extracts. Total antioxidant activity tests, such as DPPH and ABTS, is most common for large-scale examination (Cai et al. 2004). In the present work the antioxidant capacity of OFI-fruit extract was evaluated by the DPPH and ABTS techniques. The antioxidant capacity of OFI-fruit extract was found as 2.46±0.04 mg/mL while trolox was determined as 3.84±0.04 mg/mL with ABTS method. Accoring to the DPPH method, antioxidant capacity of OFI-fruit extract was found as 4.12±0.03 mg/mL. Many studies have shown that plant components can be effective and protective against oxidative damage (Butera et al. 2002; Lee et al. 2002). Several phenolics such as phenolic acids, flavonoids, coumarins, curcuminoids, lignans, quinones, stilbenes, etc. possess potent antioxidant capacity as well as potential anti-cancer activity, (Sun et al. 2002; Cai et al. 2004).

Prior research has reported the relationship between antioxidant activity and TPC (Sun et al. 2002; Cai et al. 2004). Quercetin is one of the most prominent antioxidants, which is a member of the flavonoids family. It has favourable biochemical activity. Most of the anti-cancer agents have been shown to possess antioxidant potential that can play an important role in the protection of some forms of cancer (Khanapur et al. 2014). Quercetin has perfect antioxidant activity. It is the most effective scavenger of ROS (Boots et al. 2008) and also previous studies have shown that quercetin, myricetin and luteolin have anti-cancer activity (Lu et al. 2006; Chahar et al. 2011; Majumdar et al. 2014; Rauf et al. 2018; Sun et al. 2018). The total phenolic compounds and flavonoids which are responsible for antioxidant activity were also rich in fruits. By LC-MS analysis, 3 flavonoids were determined (Myrcetin, Quercetin and Luteolin) (Table III) and quercetin compound was also found to be rich in methanol extract of OFI-fruits.

The evaluation of the anti-cancer activity of plant extracts is essential for safe treatment. Various anti-cancer activity studies with cell lines used in this study are available on plant extracts (Okur et al. 2019). OFI fruits are also in the safe range in terms of toxicity because they are consumed as food among the public. OFI-fruit extract was analyzed for potential *in vitro* cytotoxic effects by using cancer cell lines from different tissue origins including colon and brain cancers. DMSO was used as the control group, and the IC_{50} value from this group was set as the background. In this present study, OFI fruit extract was shown to have selectivity against the HT-29 and U87 cancer cell lines with 4.386 mg/mL, 5.297 mg/mL IC_{50} values, respectively. It can be said that our study is the first which evaluates the cytotoxic effects of *O. ficus-indica* plant extracts in the specific cell lines, since previous conducted studies evaluated cytotoxicity in different cancer lines. More specifically, OFI plant has shown positive results, according to previous studies which have been conducted against ovarian and cervical cancer cell lines (Kaur et al. 2012). El-Beltagi et al. studied the anticancer activities of OFI peel and pulp extracts on Liver (HepG2), colorectal adenocarcinoma (Caco-2) and Breast (MCF-7) cell lines. OFI peel and pulp extracts were tested at 3 mg/mL and decreasing doses and a decrease in growth of cancer cells was observed at high doses (El-Beltagi et al. 2019). In another previous study prickly pear cactus seed oil was studied for its anti-cancer activity against colon cancer cell lines. As a result, cell viability has been observed significantly in some colon cancer cell lines (Becer et al. 2018). In addition, the anti-cancer activity of indicaxanthin, which is known to be highly present in the fruits of *O. ficus-indica*, on melanoma cancer was investigated by in vivo and in vitro methods and very successful results were obtained (Allegra et al. 2018). In this study, an increasing activity was observed in increasing doses as well as selectivity. In conclusion, OFI-fruit extracts were significantly toxic to HT-29 (colon cancer) cells even at the lowest dose 1mg/mL while U87 (glioblastoma brain cancer) cells showed significantly increased cell death upon treatment with 5mg/mL extract and viability decreased on both lines in a dose dependent manner. It can be said that 10 mg/mL of OFI-fruit extract was cytotoxic to all the cancer cell lines tested. Different cytotoxic efficacies to OFI-fruit extract might be linked to diverse mutagenic profiles of the cancer cell lines which facilitate their escape from cell death mechanisms. To sum up, this study demonstrates that OFI-fruit extract has significant cytotoxic effects on cancer cell lines from different origins such as colon (HT-29) and

brain (U87) cancers and is found to be promising to extensively study cytotoxic activities of OFI extracts that can shed light on underlying mechanisms.

CONCLUSION

In this study, total phenolic compounds and total flavonoid contents of OFI fruits were determined and they were studied in respect to their antioxidant and cytotoxic activities. The flavonoid contents of the extracts revealed HPLC and LC-MS. Cytotoxicity of OFI-fruit extract was measured by determining *in vitro* cell viability of different cancer cell lines composed of brain (U87-MG), colon (HT-29) and human primary fibroblast upon treatment with OFI-fruit extract. According to cell viability assays, the OFI-fruit extract showed a significant cytotoxic effect (more potently at the 10 mg/mL dose) on both U87-MG, HT-29 and human primary fibroblast. Further studies are required to isolate cytotoxic compounds.

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Original Article

Development of paclitaxel and flurbiprofen co-loaded PLGA nanoparticles: understanding critical formulation and process parameters using Plackett–Burman design

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ABSTRACT

Nano drug co-delivery system is a popular strategy for combined application of two or more anticancer and/or synergistic drugs. Synergistic effects of nonsteroidal anti-inflammatory drugs and anti-cancer drugs in cancer treatment are shown in the literature. This study aimed to screen and understand the critical formulation and process parameters in the preparation of flurbiprofen and paclitaxel co-loaded nanoparticles to develop an anti-cancer nano co-delivery system. With this aim, critical parameters were determined using the Plackett–Burman experimental design (DoE). Flurbiprofen and paclitaxel drug loading amounts were considered as critical quality attributes to control the effective drug loading ratio. Furthermore, average particle size and zeta potential were also defined as critical quality attributes in order to optimize passive drug targeting and colloidal stability. Surfactant type was determined as the most significant factor for the average particle size and zeta potential. For flurbiprofen and paclitaxel drug loading into the nanoparticles, amounts of both flurbiprofen and paclitaxel were determined as critical factors. Consequently, paclitaxel and flurbiprofen were efficiently loaded into nanoparticles, and the impact of the formulation variables was successfully screened by a DoE. By controlling the determined parameters, the therapeutic efficacy of co-loaded drug nanoparticles could be maximized in further studies.

Keywords: Nanoparticles, paclitaxel, flurbiprofen, PLGA, design of experiments, Plackett–Burman

INTRODUCTION

Previous studies showed that nonsteroidal anti-inflammatory drugs (NSAIDs) are promising anticancer drugs, the effects of which were well confirmed in clinical trials (Thun et al. 2002). Anticancer effects of R-flurbiprofen, a NSAID, have been shown *in vitro* and *in vivo* models of prostate and colon cancer (Liu et al. 2012). It was demonstrated that R-flurbiprofen increased levels of the tumor suppressor neurotrophin receptor in gastric cancer cells and reversed multidrug resistance (Jin et al. 2010).

Paclitaxel is one of the most important anticancer drugs, approved by the United States Food and Drug Administration (FDA) for clinical use in chemotherapy. It is a *Permeability-glycoprotein* (P-gp) substrate (Yerlikaya et al. 2013). For increasing pharmaco-

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kinetic profiles, reducing toxicity, overcoming multidrug resistance and increasing efficacy of paclitaxel, many nano-delivery systems were developed and evaluated. To date, paclitaxel albumin-bound nanoparticles (Abraxane®) have been approved by the FDA (Ma and Mumper, 2013).

Nanotechnology offers some advantages such as improved drug release, intracellular drug delivery and tumor accumulation by active and passive targeting properties (Hillaireau and Couvreur, 2009; Wicki et al. 2015). Poly (lactic-co-glycolic acid) (PLGA) is the most frequently used polymer to prepare nanoparticles because of its biodegradable and biocompatible nature (Dinarvand et al. 2011; Danhier et al. 2012). Co-delivery of two or more anticancer drugs with PLGA nanoparticles became an attention grabbing strategy to provide a synergistic effect. These nano drug co-delivery systems provide a unique opportunity for targeting and simultaneous drug delivery of drug combinations (Qi et al. 2017; Kozlu et al. 2018). NSAIDs, that could overcome *multiple drug resistance* by inhibiting P-gp, show synergistic effects while used concurrently with anticancer drugs (Thun et al. 2002; Jin et al. 2010).

To enhance the pharmaceutical development through design efforts, the FDA encourages risk-based approaches and the adoption of Quality-by-design (QbD) principles in drug product development. To identify and control critical source of variability in the process, and understand the impact of formulation components and process parameters on the critical quality attributes are defined in the objectives of the QbD approach. The pharmaceutical characteristics of the nanoparticles could be influenced by many factors in the manufacturing process, including the formulation materials. To evaluate the effects of these factors, many statistical designs of experiment (DoE) are used. The most commonly used (DoE) is Plackett–Burman, which is a very efficient screening design used when only the main effects are of interest to be investigated. (Rahman et al. 2010; Yerlikaya et al. 2013; Yu et al. 2014; Kozlu et al. 2018)

In this study, we aimed to screen and understand the critical formulation and process parameters in the preparation flurbiprofen and paclitaxel co-loaded nanoparticles to develop an anti-cancer nano co-delivery system. With this aim, critical parameters were determined using the Plackett–Burman experimental design. Flurbiprofen and paclitaxel drug loading amounts were considered as critical quality attributes to control effective drug concentration ratios. Average particle size and zeta potential were also defined as critical quality attributes in order to optimize passive drug targeting and colloidal stability.

MATERIALS AND METHODS

Materials

Paclitaxel was donated by DEVA Pharmaceuticals (Istanbul, Turkey).Flurbiprofen (R/S enantiomer) was donated by ILKO Pharmaceuticals (Istanbul, Turkey). R-Flurbiprofen, PLGA polymers, poly(vinyl alcohol) (PVA), D-α-tocopherol polyethylene glycol 1000 succinate (TPGS), dimethyl sulfoxide (*DMSO*) and acetone were purchased from Sigma-Aldrich (Saint Louis, USA). All other reagents used were either analytical or reagent grade.

Methods

Nanoparticles were prepared using nanoprecipitation *technique.* Briefly, paclitaxel, R/S-flurbiprofen (or R-flurbiprofen) and PLGA were dissolved in 5 mL of acetone. This organic phase was transferred into the aqueous phase comprising either 10 mL, 1% (w/v) of PVA or 0.2% (w/v) of TPGS by dropping while homogenizing (IKA RET Basic, Germany). Following the acetone's evaporation overnight on a magnetic stirrer, the obtained suspension was centrifuged at 13.500rpm for 60 min (Z 383 K,Hermle; Germany). The resulting nanoparticles were washed with pure water and collected. For the screening of process parameters and formulation variables, Plackett–Burman DoE was used. Nine factors were tested at 12 runs and statistical evaluation, including the design matrix and randomization, were conducted by using Minitab software (Minitab Ltd., UK). The selected factors and their levels are given in Table 1, and the experimental design matrix is given in Table 2. The selection of the parameters and their levels were based on preliminary studies and on literature data. The average particle size (Y_1), zeta potential (Y_2), flurbiprofen loading (Y_3) and paclitaxel loading (Y_4) were determined as response variables. Smaller average particle size was targeted in order to provide enhanced permeability and retention (EPR) effect (Acharya and Sahoo, 2011) and high negative or positive zeta potential was targeted to provide colloidal stability (Malvern; Ostolska and Wiśniewska, 2014). The selection reason for flurbiprofen/paclitaxel loading values is to specify critical parameters that can affect each drug loading because of optimum flurbiprofen/paclitaxel concentration ratio and will be evaluated in further cell culture studies to find to determine maximum synergistic effect. The statistical significance value (p) was set at 0.05. *Ethical approval* was *not required for this study.*

Particle size distribution and surface charge

To measure particle size distribution and zeta potential of nanoparticles, a particle size analyzer (Malvern Nano ZS, Malvern Instruments, UK) was used. All samples were dispersed in ultrapure water and examined for the mean particle diameter, polydispersity index and surface charge.

Table 1. The Factors and Their Levels Used in Plackett–Burman Design

	Levels	
Factors	Low	High
X_1:FLUR Amount (mg)	2	5
X_2: PTX Amount (mg)	2	5
X_3:FLUR Enantiomers	R	R/S
X_4:PLGA Amount (mg)	50	100
X_5:PLGA Terminal Group	Acid	Ester
X_6:PLGA Molecular Weight (kDa)	7-17	24-38
X_7:Surfactant Type	TPGS	PVA
X_8:Homogenization Rate (rpm)	500	1100
X_9:Dropping Rate (Drop per Second)	0.5	1

FLUR: Flurbiprofen; PTX: Paclitaxel

Determination of drug loading

Drug loading was determined as paclitaxel or R/S flurbiprofen or R-flurbiprofen amounts in final nanoparticle formulations. For calculation, a certain amount of nanoparticles were dissolved in DMSO, and analyzed using a high-pressure liquid chromatography (HPLC) system equipped (Agilent 1200 Series, USA) with a reversed-phase column (Inertsil® ODS-3, Particle size 5 µm, 4,6x250 mm, GL Sciences, China). For the quantification of paclitaxel, the mobile phase was composed of water:acetonitrile (48:52, v:v). The flow rate of the mobile phase was set to 1 mL/min, and the injection volume was 20 µL. The detector was set to 227 nm. For the quantification of RS or R-flurbiprofen, the mobile phase was composed of acetonitrile:0.1M acetate buffer (40:60). The flow rate of the mobile phase was set to 1 mL/min and the injection volume was 25µL. The detector was set to 247 nm.

Below equations were used to determine the drug loading values: Drug loading (*µg*/mg)= (Amount of drug in nanoparticles)/(Amount of nanoparticles)

RESULTS AND DISCUSSION

The average particle size of the nanoparticle formulations varied between 143.9 nm and 270.5 nm. Formulations showed uniform particle size distributions. The polydispersity indices (PDI) of the nanoparticles were lower than 0.3. The response values are shown in Table 3. The R^2 values indicate that a good correlation was obtained between predicted and actual values (R^2= 0.9798). However, despite the fact that the p value of main effects obtained from ANOVA was 0.088 and therefore was not statistically significant, the most significant factors and effects of other factors were evaluated. For the average particle size (Y_1), the surfactant type (p=0.021) was determined as the most significant factor (Table 4 and Figure 1A). Nanoparticles prepared with 0.2% TPGS showed smaller particle size than the formulations that were prepared with 1% PVA. This could be explained by the stronger emulsification effect of TPGS over PVA (Zhang et al. 2012). It was demonstrated that the emulsification efficiency of TPGS is 66.7 times higher than PVA and TPGS

Table 2. Plackett–Burman Randomized Design Matrix

Formulation	X_1 FLUR Amount (mg)	X_2 PTX Amount (mg)	X_3 FLUR Enantiomers	X_4 PLGA Amount (mg)	X_5 PLGA Terminal Group	X_6 PLGA Molecular Weight (kDa)	X_7 Surfactant Type	X_8 Homogenization Rate (rpm)	X_9 Dropping Rate (Drop per Second)
A1	2	5	R	50	Acid	24-38	PVA	1100	0.5
A2	2	2	R	100	Ester	24-38	TPGS	1100	1
A3	5	5	R	100	Ester	7-17	PVA	500	0.5
A4	5	2	R/S	50	Acid	7-17	PVA	1100	1
A5	5	5	R	100	Acid	7-17	TPGS	1100	1
A6	2	2	R/S	100	Ester	7-17	PVA	1100	0.5
A7	2	5	R/S	50	Ester	7-17	TPGS	500	1
A8	5	2	R/S	100	Acid	24-38	TPGS	500	0.5
A9	5	5	R/S	50	Ester	24-38	TPGS	1100	0.5
A10	2	2	R	50	Acid	7-17	TPGS	500	0.5
A11	5	2	R	50	Ester	24-38	PVA	500	1
A12	2	5	R/S	100	Acid	24-38	PVA	500	1

Table 3. The Results of Dependent Variables Obtained Through Plackett–Burman Design

Formulation	Y_1 Average Size (nm)	Y_2 Zeta Potential (mV)	Y_3 Flurbiprofen Loading (µg/mg)	Y_4 Paclitaxel Loading (µg/mg)
A1	173.8	-3.5	31.4	68.8
A2	154.8	-22.8	25.7	22.1
A3	270.5	-4.7	49.8	36.2
A4	167.6	-4.4	60.4	36.7
A5	153.7	-21.4	54.4	32.2
A6	206.1	-6.6	16.8	12.4
A7	173.9	-19.0	21.7	56.5
A8	165.4	-22.5	38.7	19.9
A9	146.6	-18.1	53.5	59.6
A10	143.9	-23.7	33.1	14.6
A11	203.8	-8.0	96.4	28.6
A12	194.1	-5.7	13.3	42.4

Table 4. Statistical analysis of Average particle size (Y_1), zeta potential (Y_2), flurbiprofen loading (Y_3) and paclitaxel loading (Y_4) results

	Average particle size (Y_1)		Zeta potential (Y_2)		Flurbiprofen loading (Y_3)		Paclitaxel loading (Y_4)	
	Coefficient	p	Coefficient	p	Coefficient	p	Coefficient	p
Constant	179.51	<0.001*	-13.34	0.001*	41.278	0.004*	35.804	0.007*
X1	5.10	0.274	0.19	0.687	17.601	0.023*	-0.304	0.928
X2	5.93	0.224	1.30	0.083	-3.912	0.286	13.463	0.046*
X3	-3.89	0.372	0.64	0.248	-7.209	0.117	2.087	0.558
X4	11.25	0.081	-0.59	0.277	-8.164	0.095	-8.313	0.109
X5	13.11	0.061	0.18	0.695	2.717	0.422	0.071	0.983
X6	-6.42	0.200	-0.08	0.861	1.888	0.558	4.712	0.278
X7	23.11	0.021*	7.87	0.003*	3.409	0.336	1.688	0.630
X8	-12.43	0.068	0.57	0.286	-0.891	0.774	2.796	0.449
X9	-4.86	0.290	-0.18	0.690	4.043	0.275	0.588	0.863

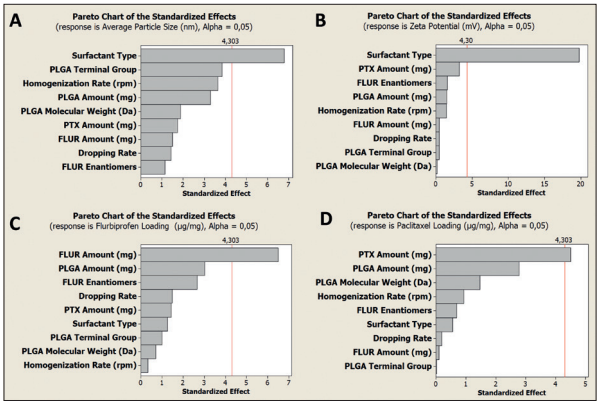

Figure 1. a-d. The Pareto charts showing statistically significant formulation and process variables influencing average particle size (a), zeta potential (b), flurbiprofen loading (c) and paclitaxel loading (d).

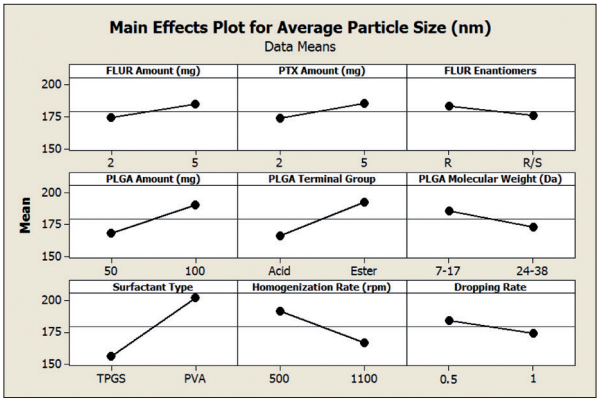

Figure 2. Main effects plot for average particle size.

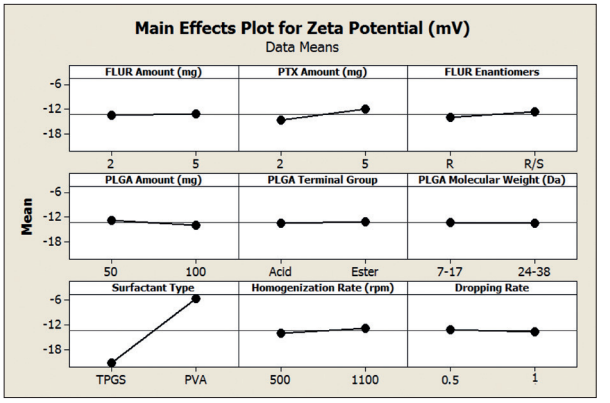

Figure 3. Main effects plot for zeta potential.

emulsified nanoparticles are much more uniform and smaller than the PVA-emulsified nanoparticles (Win and Feng, 2006; Saadati and Dadashzadeh, 2014). Briefly, the results showed that the average particle size was decreased with increasing homogenization rates (Figure 2), since large droplets were mixed more efficiently with higher shear rates (Rahman et al. 2010; Yerlikaya et al. 2013). Also, using higher PLGA amounts and ester-terminated PLGA increased the average particle size (Figure 2). Increasing viscosity of the dispersed phase might enhance the resistance against shear forces and cause agglomeration (Warsi et al. 2014; Sahin et al. 2017). Usage of different enantiomers of flurbiprofen, paclitaxel amount, flurbiprofen amount, dropping rate and PLGA molecular weight slightly affect the average particle size (Figure 1a and Figure 2). These results indicate that the targeted average particle size of nanoparticles could be obtained by controlling the critical parameters.

The zeta potential values of the nanoparticle formulations were found to be negative, and ranged between -3.5 and -23.7 mV. The response values are shown in Table 3. The R^2 values indicate that a good correlation was obtained between predicted and actual values (R^2= 0.9951). The p value of main effects obtained from ANOVA was 0.022 and was considered as significant. Surfactant type was determined as the most significant factor for zeta potential (Y_2) (p=0.003) (Table 4 and Figure 1B). It was observed that the zeta potentials of nanoparticles were strongly influenced by the emulsifier used in the preparation process. The nanoparticles that were prepared with TPGS showed more negative surface charges (Figure 3). It is known that increased zeta potential could enhance the colloidal stability. If all the particles in suspension have a high negative or

positive zeta potential, they tend to repel each other, and there will be no tendency for the particles to come together (Malvern 2017; Ostolska and Wiśniewska, 2014). Recently, garcinol loaded vitamin E TPGS emulsified PLGA nanoparticles were prepared with nanoprecipitation method by Gaonkar et al., and a similar satisfactory zeta potential (-28.10±2.1) was obtained (Gaonkar et al. 2017). On the other hand, slightly negative zeta potentials were found in previous studies which were used PVA as emulsifier (Sahin et al. 2017a; Sahin et al. 2017b). Additionally, TPGS possess potential to be a preferable surfactant for preparing nanoparticular systems due to its anticancer activity and P-gp inhibition (Collnot et al. 2010; Yang et al. 2018). Because of these properties, TPGS could be more effective than PVA for the preparation of a P-gp substrate drug containing PLGA nanoparticles.

Nano drug co-delivery system is a feasible and popular strategy for the combined application of two or more anticancer and/or synergistic drugs (Qi et al. 2017). NSAIDs, that could overcome *multiple drug resistance* by inhibiting P-gp, show synergistic effects while used concurrently with anticancer drugs (Thun et al. 2002; Jin et al. 2010). In this study, critical parameters for flurbiprofen and paclitaxel loading amounts were investigated to provide the targeted optimum drug loading amount and ratio in further studies. Drug loading values ranged between 13.3 and 96.4 µg/mg flurbiprofen nanoparticles and between 12.4 and 68.8 µg/mg paclitaxel nanoparticles. The response values are shown in Table 3. The R^2 values indicate that a good correlation was obtained between predicted and actual values (R^2= 0.9705 and R^2 =0.9409 for Y_3 and Y_4, respectively). Although the *p* values of main effects obtained from ANOVA were 0.126 and 0.240 for Y_3 and Y_4, respectively, significant factors and effects of other factors were evaluated. The flurbiprofen amount was determined as the most significant factor for flurbiprofen loading (Y_3) (p=0.023)(Figure 1c and Table 4). Similarly, the paclitaxel amount was determined as the most significant factor for paclitaxel loading (Y_4) (p=0.046) (Figure 1d and Table 4). Experimental designs showed that an increased flurbiprofen or paclitaxel amount in the organic phase resulted in increased drug loading. Drug amounts in nanoparticles were controlled by drug amounts in used levels (Figures 4 and 5). Additionally, increased PLGA amount decreased flurbiprofen and paclitaxel concentration in nanoparticles, but this factor did not reach a statistically significant level (Table 4, Figures 4 and 5). Additionally, it was observed that drug loading values were not significantly influenced by the emulsifier used in the preparation process. Zu et al. showed that increased encapsulation efficacy was obtained by using TPGS (Zhu et al. 2014). On the other hand, Saadati et al. found that encapsulation efficacy was decreased when TPGS was used as emulsifier in the nanoprecipitation method (Saadati and Dadashzadeh, 2014). These results clearly indicated that targeted drug amounts and ratio of paclitaxel and flurbiprofen for anticancer activity could be loaded together in PLGA nanoparticles.

CONCLUSION

In this study, several process parameters and formulation variables were screened by using a DoE approach to understand

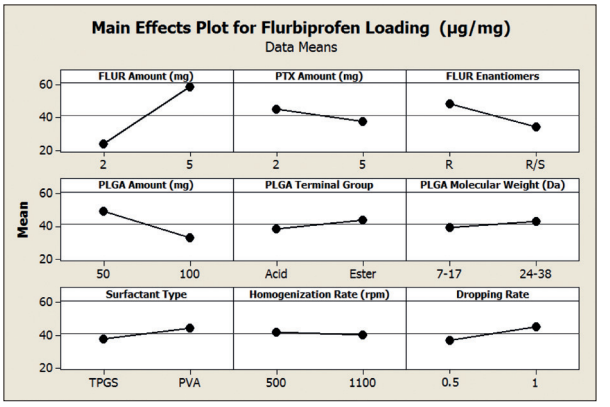

Figure 4. Main effects plot for flurbiprofen loading.

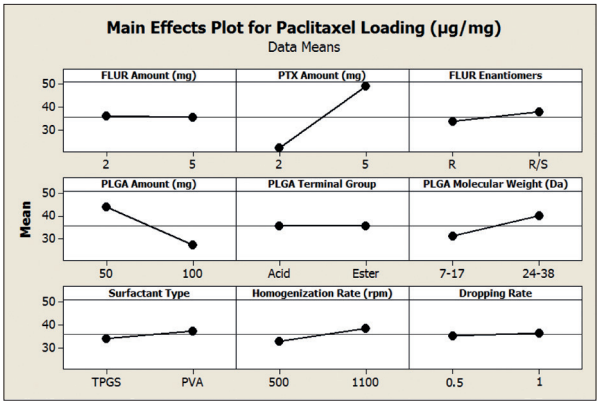

Figure 5. Main effects plot for paclitaxel loading.

the most significant factors influencing the characteristics of the nanoparticles. It was found that the surfactant type was determined as the most significant factor for the average particle size and zeta potential. For flurbiprofen and paclitaxel drug loading into the nanoparticles, the amounts of both flurbiprofen and paclitaxel were determined as critical factors. Consequently, paclitaxel and flurbiprofen were efficiently loaded into nanoparticles and the impact of the formulation variables were successfully screened by a DoE.

Further studies to provide maximum efficacy of co-loaded nanoparticles, firstly the optimum synergistic concentration of flurbiprofen and paclitaxel will be evaluated on cancer cells to achieve superior therapeutic efficacy, and determined formulation parameters will be optimized. By controlling the determined parameters, the therapeutic efficacy of co-loaded drug nanoparticles could be maximized in further studies and prepared formulations could be promising tools for the treatment of various cancer types.

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Original Article

The subcronic effects of acetamipride on the global DNA methylation levels in Sprague-Dawley rat brain and liver

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ABSTRACT

Acetamiprid, which is a neonicotinoid insecticide, is used to control leafy vegetables, fruiting vegetables, fir seeds, citrus fruits, pome fruits, grapes, cotton and ornamental plants and absorbent insects on flowers. The present study aim to evaluate global DNA methylation and gene expression of DNA methylation related enzymes in liver and brain tissues of male Sprague-Dawley rats after a 90-day subchronic exposure to acetamiprid at low doses of 12.5, 25 and 35 mg/kg body weight (b.w.). Global DNA methylation resulted in a significant decrease in the levels of 5-methylcytosine (5-mC%) at the doses of 25 and 35 mg/kg b.w. in the liver and 35 mg/kg b.w. in the brain compared to the vehicle control group. Consistently, expression of DNA methyltransferase enzymes decreased at doses of 12.5, 25 and 35 mg/kg b.w. in liver and 35 mg/kg b.w. in brain. It has been suggested that non-genotoxic (epigenetic) mechanisms may be involved in the toxicity of acetamiprid and further investigations are needed to elucidate the epigenetic effects of neonicotinoid insecticides.

Keywords: Acetamiprid, DNA methylation, Sprague-Dawley rats, liver, brain

INTRODUCTION

Pesticides are defined as substances or mixtures of substances used to remove, reduce, suppress or degrade harmful organisms in order to increase productivity in agriculture. Pesticides are claimed to be quite useful as a result of their use in appropriate doses and conditions for their intended purpose. However, when they are used incorrectly and in higher than recommended doses, they harm human health as well as increase environmental pollution and affect other living things. Neonicotinoids are a relatively new type of insecticide used to control a variety of pests in agriculture and livestock (Honda et al. 2006). These pesticides are currently preferred to organophosphates and carbamates throughout the world owing to their ability of resistance against day light and higher toxicity to insects than mammals and aquatic organisms because of their affinity to the nicotinic acetylcholine receptors in insects (Kiriyama et al. 2003; Tomizawa and Casida, 2003; Casida and Quistad, 2004; Whitacre and Ware, 2004; Ford 2008; Yu 2008). Neonicotinoids are systemic acting insecticides and affect the central nervous system of insects, resulting in paralysis and death. They can also be persistent in the environment. Some neonicotinoids are suspected to be carcinogenic and mutagenic in mice (Dich et al. 1997; Office of Prevention, Pesticides and Toxic Substances 2003; Green et al. 2005). Acetamiprid (N-[(6-chloropyridin-3-yl)methyl]-N′-cyano-N-methylethanimidamide), a neonicotinoid pesticide, was the second insecticide to be manufactured in this group after the launch of imidacloprid and was first marketed in Japan under the brand name Mospilan (Yamamoto and Casida 1999; Ünver and Uysal 2014). It has been reported that acetamiprid is widely used in agriculture in Turkey (Kocaman and Topaktaş 2007). Acetamiprid is a selective agonist of nicotinic acetylcholine receptor in postsynaptic membrane. LD_{50} value of acetamiprid is 140-417 mg/kg body weight (b.w.) in different rat strains (Kanungo and Solecki 2011).

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Acetamiprid may lead to oxidative damage by producing reactive oxygen species in target tissues (Yao et al. 2006; Ford et al. 2011; Zhang et al. 2011). Additionally, it has been reported that chronic exposure to acetamiprid caused disturbance of matrix oxidative status, and a loss of mitochondrial membranes integrity in rat brain via generating reactive oxygen species (Gasmi et al. 2016 and 2017). Acetamiprid accumulates in the brain of murine and rats (Devan et al. 2015; Terayama et al. 2016). Besides, it can cause changes in brain functions -such as the break-down of learning ability (Mondal et al. 2014; Mandal et al. 2015). In a few studies, the genotoxic and cytotoxic effects of acetamiprid have been investigated. It has been reported that acetamiprid induced frequency of sister chromatid exchange, chromosomal aberrations, and micronucleus formation in human peripheral lymphocytes (Kocaman and Topaktas 2007), micronucleus formation and DNA damage in Caco-2 cells (Cavas et al. 2012) and micronucleus formation and DNA damage in human lung fibroblast cells (Cavas et al. 2014), while acetamiprid did not increase micronucleus formation in peripheral blood lymphocytes (Muranli et al. 2015). According to these studies results on the mechanisms of action in acetamprid toxicity have been controversial. It has become crucial for evaluation of possible genotoxic and cytotoxic effects of acetamiprid on living organisms to take place, due to it becoming increasingly widespread in the world.

DNA methylation, one of the most studied non-genotoxic (epigenetic) modifications, plays an important role in cell proliferation and various diseases such as cancer and diatebetes (Baylin 1997; Moggs et al. 2004; Jones and Baylin 2007; Kulis and Esteller 2010; Anderson et al. 2012; Bansal and Pinney 2017). DNA methylation contributes to alterations in gene expression of key molecular pathways including global DNA hypomethylation and hypermethylation of CpG islands of tumor suppressor genes (Baylin et al. 1986; Watson and Goodman 2002). There has only been one study that shows alterations of global and gene-spesific DNA methylation after acetamiprid exposure in mouse embryonic stem cells (Wang et al. 2019). Therefore, we aimed to investigate the role of DNA methylation changes in acetamiprid toxicity. For this purpose, we determined the global levels DNA methylation and gene expression of related enzymes in liver and brain tissues after subcronic acetamiprid exposure to Sprague-Dawley albino adult rats. This is the first study which analyzed the effects of acetamiprid on global DNA methylation as a key molecular mechanism of epigenetic modulation in rat tissues. Because acetamiprid is a neuro-active insecticide and acts as neurotoxic agent and because the liver is the main target organ for acetamiprid metabolism and elimination, we selected brain and liver tissues for the evaluation of DNA methylation analysis.

MATERIALS AND METHODS

Chemicals

Acetamiprid, technical purity 97%, was obtained from a national company (Hektaş Ticaret T.A.Ş. Istanbul, Turkey) and weekly suspended in an aqueous solution of 0.5% methylcellulose (Merck, Darmstadt, Germany) before use. All other supplements were purchased from Wisent Bioproducts (Saint-JeanBaptiste, QC, Canada) and sterile plastic materials were purchased from Nest Biotechnology (Jiangsu, China). DNA, RNA isolation kits and cDNA synthesis kits were obtained from Roche Life Sciences (Penzberg, Germany). 5-methylcytosine (5-mC) DNA ELISA kit was purchased from Epigentek Research (Farmingdale, NY, USA). Syber green master mix was obtained from Bioline (London, UK) and primers for gene expressions were obtained from Sentromer DNA Technologies (Istanbul, Turkey).

Animal treatments

In this study male Sprague-Dawley albino adult rats, aged 8-12 weeks and weighing 250-375 g were obtained from Aziz Sancar Institute of Experimental Medicine, Istanbul University. The animals were housed throughout the experiment in polypropylene standard cages in which 4-5 animals were placed. Animals were maintained under controlled conditions of temperature at 22-24°C, normal photoperiod (12-12 h light–dark cycle) and relative humidity of (50±10%). The animals were allowed free access to standard dry pellet diet and tap water ad libitum. The experiments reported here complied with the current laws and regulations of the Turkish Republic on the care and handling of experimental animals and the local ethics committee of experimental animals of Istanbul University (IUHADYEK; 2016/35 and 2016/42).

Experimental design

The animals were randomly divided into four experimental groups. The substances were administered in the morning (between 09.00 and 11.00 a.m.) to rats who had not fasted.-

Group I (Vehicle control Group): Control rats received intragastrically (i.g.) a vehicle (0.5% methylcellulose) (n=11).

Group II: Acetamiprid at the dose of 12.5 mg/kg body weight (b.w). acetamiprid (NOAEL) in a vehicle (0.5% methylcellulose) was applied to rats i.g. once a day for 90 days (n=12).

Group III: Acetamiprid at the dose of 25 mg/kg b.w. acetamiprid in a vehicle (0.5% methylcellulose) was applied to rats i.g. once a day for 90 days (n=12).

Group IV: Acetamiprid at the dose of 35 mg/kg b.w. acetamiprid in a vehicle (0.5% methylcellulose) was applied to rats i.g. once a day for 90 days (n=13).

Selection of treatment concentrations in the present study were based on no observable adverse effect level (NOAEL) of acetamiprid (12.4 mg/kg b.w.), and on increased liver weight and centrilobular hepatocyte hypertrophy for the 90 days rat study (EFSA 2016). At the end of the treatments, the rats were sacrificed on the 90th day by removing a large volume of blood from the orbital veins under inhalation anesthesia induced by diethyl ether. Brain and kidney samples were dissected, placed in a sufficient amount of phosphate buffered saline (PBS) (1x) and immediately stored at -80°C until analysis. The tissues were homogenized in 0.9% NaCl using a tissue homogenizer (Ultra-Turrax T-18, IKA Werke GmbH&Co., Staufen, Germany) to make up the 10% homogenate (w/v). After that DNA and RNA isola-

tion was carried out from these homogenates (10%) of liver and brain tissues.

Global DNA methylation analysis

Genomic DNA was isolated from liver and brain tissue homogenates (10%) using the High Pure PCR Template Preparation kit (Roche Life Sciences, Penzberg, Germany) according to the manufacturer's instructions. To measure global levels of 5-mC%, 100 ng of DNA samples were applied to MethylFlashTM Methylated DNA Quantification kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions as previously described (Karaman and Ozden 2019).

Gene expression analysis of DNA methyltransferases

Total RNA was isolated from liver and brain tissues using a High Pure RNA Tissue kit (Roche Life Sciences, Penzberg, Germany). Reverse transcription was performed by Transcriptor First Strand cDNA Synthesis kit (Roche Life Sciences, Penzberg, Germany) 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 DNA methyltransferases such as ***DNMT1***, ***DNMT3a***, ***DNMT3b***, were measured using Bio-Line 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 and Ozden 2019).

Statistical analysis

Results of 5-mC% levels and gene expression were represented as mean ± standard deviation (SD). Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test using The Statistical Package for the Social Sciences (SPSS) version 21.0 for Windows, statistical program (IBM Corp.; Armonk, NY, USA). P values of less than 0.05 and 0.001 were selected as the levels of significance.

RESULTS

Effects of acetamiprid on the global DNA methylation levels

Levels of 5-mC% were measured after 12.5, 25 and 35 mg/kg b.w. of acetamiprid treatments for 90 days by Elisa kit. 25 and 35 mg/kg b.w. of acetamiprid treatments resulted in a significant decrease in 5-mC% status (38.7%, p□0.05 and 77%, p□0.05, respectively) in liver comparison with the vehicle control group. 5-mC% levels reduced significantly (48.7%, p□0.05) after 35 mg/kg b.w. of acetamiprid treatments in brain comparison with the vehicle control group (Figure 1).

Effects of acetamiprid on DNA methyltransferases gene expression levels

We analysed the gene expressions of DNA methyltransferases (*DNMT1*, *DNMT3a*, *DNMT3b*) to support the global DNA methylation results of acetamiprid in liver and brain. In Figure 2a, our data showed that acetamiprid treatments (12.5, 25 and 35 mg/kg b.w.) significantly decreased expression levels of *DNMT1* (≥1.96 fold), *DNMT3a* (≥2.86 fold) and *DNMT3b* (≥1.95 fold) in comparison with the vehicle control group. In Figure 2b, 35 mg/kg b.w. of acetamiprid significantly decreased expression levels of *DNMT1* (2.44 fold), *DNMT3a* (2.63 fold) and *DNMT3b* (1.92 fold) in brain in comparison with the vehicle control group.

DISCUSSION

Due to their low cost and easy application, the use of pesticides have been preferred as a way of combating plant diseases, pests and weeds which cause a loss of significant amounts of the product or product quality at postharvest storage and agricultural production times. Because of unconscious, incorrect applications of pesticides by manufacturers at harvest and

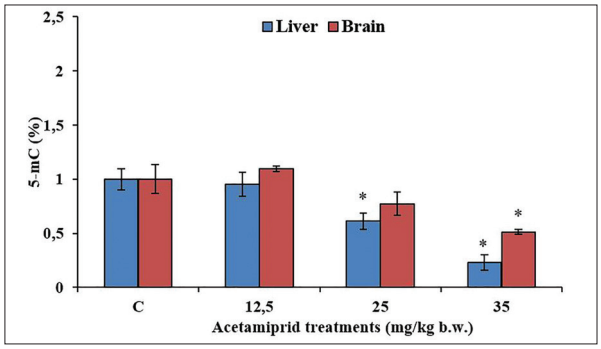

Figure 1. Effects of acetamiprid (12.5, 25 and 35 mg/kg b.w.) on levels of 5-mC% in liver and brain tissues of male Sprague-Dawley albino adult rats. Data are presented as mean ± SD. Statistically significant changes are indicated by *p<0.05. (one way ANOVA-Dunnett post hoc test.).

Table 1. Primers used real-time PCR analysis of DNA methyltransferases and the corresponding annealing temperatures

Gene	Primer sequence (5'-3')	Tm (°C)	Reference
DNMT1	F: GGTTCTGCGCGGGGACAGAC	66	Xu et al. 2015
	R: CCGGCAACATGGCCTCAGGG		
DNMT3a	F: GGTGTGTGTCGAGAAGCTCA	60	Xu et al. 2015
	R: CCAAGGGCCCACTCAATCAT		
DNMT3b	F: GGGCCGCTACCACGTTCAGG	64	Xu et al. 2015
	R: AGGGCCGTCCTGGCTCAAGT		
β-actin	F: GCGTCCACCCGCGAGTACAA	64	Xu et al. 2015
	R: ACATGCCGGAGCCGTTGTCG		

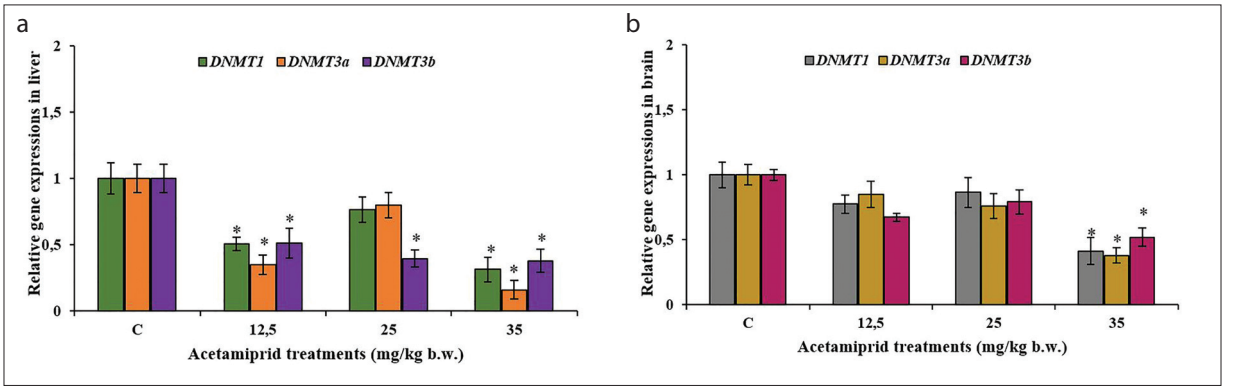

Figure 2. a, b. Effects of acetamiprid (12.5, 25 and 35 mg/kg b.w.) on relative gene expression levels *(DNMT1, DNMT3a, DNMT3b)* in liver (a) and brain (b) tissues of male Sprague-Dawley albino adult rats. Data are presented as mean±SD. Statistically significant changes are indicated by *p<0.05; **p<0.001. (one way ANOVA-Dunnett post hoc test.).

close to the harvest period, the consumption of pesticides above what is necessary to get higher quality products, high levels of drug residues are found on foodstuffs resulting in toxic effects on humans and the environment. Neonicotinoids which act as agonists on the nicotinic acetylcholine receptors (nAChRs) of insects and mammals, form a commercially important pesticide group used as insecticides with increasing use in recent years (Tomizawa and Casida 2003; Sanyal et al. 2008; Simon-Delso et al. 2015).

Acetamiprid is one of the most widely used neonicotinoids which is distributed throughout the body, especially in the liver, kidney, adrenal and thyroid glands, by reaching a high concentration (EFSA Panel 2013). Chakroun et al. (2016) evaluated the hematological, biochemical, and histopathologic effects of acetamiprid on Wistar rats during 60 days and have observed a significant decrease in t body weight gain, hematological parameters and an increase in the relative liver weight. They reported that acetamiprid induced liver toxicity through the increases in the activities of the enzymes including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase which are the indicators of hepatocellular damage (Chakroun et al. 2016). It has been reported that acetamipride inhalation in humans causes headache, dizziness, nausea, vomiting and other symptoms (Chen et al. 2007).

Investigating epigenetic alterations such as DNA methylation could be useful biomarkers for the toxicity assesments of environmental exposures (Baccarelli and Bollati 2009; Collotta et al. 2013; Greally and Jacobs 2013; Maqbool et al. 2016). It was shown that loss of 5-mC residues termed as global DNA hypomethylation is a common feature in the oncogenesis of many tumor tissues, leading to genomic instability (Gama-Sosa et al. 1983). Emerging evidence indicates that environmental chemical exposure such as dichlorodiphenyltrichloroethane (DDT), organochlorine pesticides, methylmercury chloride or polychlorinated biphenyls, causes epigenetic changes via DNA methylation machinery malfunctions with an increase of carcinogenic risk and developing neurodegeneration (Desaulniers et al. 2009; Shutoh et al. 2009; Kanthasamy et al. 2012; Collotta et al. 2013). It has been reported that blood levels of persistent organic pollutants which accumulate in adipose tissue were inversely related with global DNA methylation levels (Collotta et al. 2013). Kim et al. (2010) observed that exposure to organochlorine pesticides caused global DNA hypomethylation in healthy Koreans. As shown in previous studies it has been emphasized that potential role of epigenetic changes serve as markers for environmental chemical exposures and risk assesment. In the risk assessment process, the evaluation of epigenetic alterations in the toxicity of neonicotinoid insecticides is important. Only one study has been performed on the epigenetic alterations in acetamiprid exposure in cell culture (Wang et al. 2019). Therefore, we aimed to investigate global DNA methylation levels in response to acetamiprid exposure in rat liver and brain. We showed that acetamiprid decreased the global DNA methylation levels in liver and brain tissues of rats, and consistently expression levels of the genes regulating DNA methylation ***DNMT1***, ***DNMT3a***, ***DNMT3b*** have also decreased. Wang et al. (2019) showed that neonicotinoids induced global DNA methylation, and imidacloprid had greater effects than acetamiprid in embriyonic stem cells. Ivkovic et al. (2018) suggested that DNA methylation status was disrupted in acetamiprid treated-zebrafish embryos and they reported acetamiprid induced alterations in the methylation levels of certain genes such as ***CYP19A1***, ***p53***, ***p21*** during the early embryonic development of zebrafish.

In conclusion, we showed that global DNA methylation could be associated with acetamiprid toxicity in rat liver and brain tissues. Further studies are needed to better understand the role of epigenetic modifications in the mechanisms of toxicity for acetamiprid and also for other neonicotinoids in the risk assesment processes.

Ethics Committee Approval: The experiments reported here complied with the current laws and regulations of the Turkish Republic on the care and handling of experimental animals and the local ethics committee of experimental animals of Istanbul University (IUHADYEK; 2016/35 and 2016/42).

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Original Article

Bismuth oxide nanoparticles induced oxidative stress-related inflammation in SH-SY5Y cell line

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ABSTRACT

Bismuth (III) oxide nanoparticles' (Bi_2O_3-NPs) unique physicochemical properties have attracted attention in biological, industrial, technological and medical fields. Concurrently, increasing numbers of studies revealing their potential toxic effects and possible toxicity mechanisms are ongoing. In this study, we assessed the toxic potentials of Bi_2O_3-NPs in human SH-SY5Y neuroblastoma cell line. After Bi_2O_3-NPs characterization using TEM, the cytotoxic potentials were evaluated by MTT and LDH assays. The induction of reactive oxygen species production was evaluated by H_2DCFDA. In order to evaluate the oxidative damages, the changes in antioxidant catalase and superoxide dismutase and glutathione levels were determined. The cellular death pathway and the role of immune response were studied by measuring the mRNA expression levels of related genes. Our results showed that Bi_2O_3-NPs decreased the cell viability through disruption on mitochondrial activity (IC50:77.57 µg/mL) and membrane integrity (LDH%50:16.97 µg/mL). At 50 µg/mL Bi_2O_3-NPs, the production of reactive oxygen species (ROS) was induced significantly as well as the catalase and superoxide dismutase levels. In immune response, the mRNA expression levels of interleukin (IL)-6 increased more than 1.5-fold in all doses; whereas, TNF-α, NF-κB and MAPK8 expressions remained unchanged. Consequently, Bi_2O_3-NPs induced oxidative stress-related inflammation via activation of pro-inflammatory cytokine, IL-6.

Keywords: Bismuth (III) oxide nanoparticles, neurotoxicity, oxidative stress

INTRODUCTION

Nanoparticles, chemical structures between 1-100 nm in size, are one of the most promising elements of a new era in technology and science. NPs-based products are growing rapidly in a variety of fields such as health and fitness, electronics and computers, cosmetics, home and garden, food and beverage as well as biomedical applications (Mnyusiwalla et al. 2003; EPA, 2007). The Nanotechnology Consumer Products Inventory report, released in October 2013, listed 1814 nano-containing consumer products, in which the largest group of these products (37%) contains metals and metal oxides NPs (Vance et al. 2015). NPs potentially cause harm to both humans and the environment due to their unique physicochemical properties, size, shape and solubility (Maynard et al. 2006). NPs can easily penetrate the cell membrane due to their very small size and alter the crucial functions of cells. Growing evidence shows that various molecular mechanisms such as DNA damage, oxidative stress, mitochondrial disruption, apoptosis resulting in morphological changes and eventually cell death are responsible for the toxic effects of NPs (Ray et al. 2009; Dhawan and Sharma, 2010; Iavicoli et al. 2013).

Bi_2O_3 is one of the significant metal oxides which has rapidly attracted attention, perhaps because of its use in technology, industry and biomedical sciences (Hyodo et al. 2000; Rabin et al. 2006; Taufik et al. 2011). Despite Bi_2O_3-NPs widespread usages and the increasing intentional or unintentional exposure, there is limited knowledge about their toxicity (Thomas et al. 2012; Hernandez-Delgadillo et

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al. 2013; Liman 2013). Our previous studies show that the toxicity of Bi_2O_3-NPs varies according to the cells type (Abudayyak et al. 2017). Previous data indicate that different metal based NPs highly induce neurotoxicity (Abudayyak et al. 2017b; Song et al. 2014).There is no *in vivo* or *in vitro* study which evaluates the toxic effects of Bi_2O_3-NPs in neuronal cells or systems, and there seems to be an urgent need to gather data. Therefore, this study aims to assess the neurotoxicity of Bi_2O_3-NPs in human neuroblastoma SH-SY5Y cells which are widely used in neurotoxicity research and preferred in the evaluation of metal-based NP toxicity (Abudayyak et al. 2017b; Choi et al. 2007; Chen et al. 2008, Kim et al. 2010). For this purpose, the cytotoxicity was determined by MTT and LDH assays, the ROS production was evaluated by H_2DCFDA, the levels of glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) were measured by ELISA kits, and the mRNA expression levels of inflammation related genes were determined.

MATERIALS AND METHODS

Chemicals

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) and the other chemicals were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). The cell culture mediums and all other supplements were purchased from Multicell Wisent (Quebec, Canada), and sterile plastic materials from Corning (Amsterdam, The Netherlands).

Cell culture and exposure conditions

SH-SY5Y cells (CRL-2266) were purchased from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) F-12 and Eagle's Minimum Essential Medium (EMEM) in a 1:1 ratio. The medium was supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U-100 µg/mL). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Bi_2O_3-NPs were freshly prepared at 1 mg/mL by suspending them in the culture medium, and sonicated at room temperature for 15 minutes immediately before exposure to avoid the aggregation/agglomeration of NPs. The treatment concentrations of Bi_2O_3-NPs were 50, 25, 12.5 and 6.25 µg/mL and the exposure duration was 24 h. All assays were done in triplicate on independent days. Also, qPCR was performed in triplicate for each cDNA sample as a technical check.

Nanoparticles characterization

Bi_2O_3-NPs (Cat. No. 631930) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Transmission electron microscopy (TEM) (JEM-2100 HR, JEOL, USA) was used to images the nanoparticles. For that, Bi_2O_3-NPs were suspended in a cell culture medium or distilled water and dropped on a copper grid prior to analysis (Abudayyak et al. 2017).

Evaluation of cytotoxic potential

The cytotoxicity of Bi_2O_3-NPs was determined by MTT and lactate dehydrogenase (LDH) assays. MTT assay is based on colour changes from yellow to purple-blue during the reduction of tetrazolium salt to insoluble formazan crystals by the mitochondrial succinate dehydrogenase enzyme (Van Meerloo et al. 2011). The extracellular release of LDH is an indicator of cell membrane damage. LDH activity can be measured via absorbance change as the decrease rate of reduced nicotinamide adenine dinucleotide (NADH) during pyruvate-lactate cycle (Fotakis and Timbrell, 2006; Han et al. 2011).

The cells were seeded at 10^5 cells/mL in 96-well plates. After the culture medium was discarded, serial dilutions starting from 250 µg/mL of Bi_2O_3-NPs were added to the wells. The culture medium and 1% sodium dodecyl sulphate (SDS) were used as negative and positive controls, respectively, for MTT assay. Triton X-100 was used as a positive control for 100% release of LDH in LDH assay. MTT assay was done according to Abudayyak et al. (2017). The LDH release was measured by Cytotoxicity Detection LDH Kit (Roche, Indiana, USA) according to the manufacturer's instructions. The optical densities (ODs) were read at 590 nm and 490 nm for MTT and LDH assays, respectively, using an Epoch microplate spectrophotometer system (BioTek, Vermont, USA). The half-maximal inhibition of enzyme activity (IC_{50}) was calculated as compared to the negative control whereas the half percentage of LDH release ($LDH\%_{50}$) was calculated as compared to the Triton-X 100.

Evaluation of pro-oxidant level

The potential of ROS induction in SH-SY5Y cells due to exposure to Bi_2O_3-NPs was evaluated using H_2DCFDA fluorescent probe by flow cytometer (Eruslanov and Kusmartsev, 2010). For this, the cells were treated with 50, 25, 12.5 and 6.25 µg/mL of Bi_2O_3-NPs. After a 24 h exposure period, the assay was done according to Oztas et al. (2019). The ROS dependent fluorescence intensity of $5x10^4$ cells was measured in FITC channel (excitation at 488 nm; emission at 530 nm) on an ACEA NovoCyte flow cytometer (San Diego, California, USA) and the results were expressed as the median of fluorescence intensity (MFI).

Evaluation of oxidative damage potential

The oxidative damage potential of Bi_2O_3-NPs was evaluated with determination of ROS production and measurement of GSH, CAT and SOD levels (Abudayyak et al. 2017). The cells were seeded at $5x10^5$ density in 3 mL culture medium into each well of 6-well plates and incubated overnight for cell attachment. They were then exposed to 50, 25, 12.5, 6.25 µg/mL of Bi_2O_3-NPs for 24h and culture medium as control. The suspended exposed cells were used for measurement of protein amount, GSH content and antioxidant enzyme levels. The amount of protein was measured by Bradford (1976) method. The GSH, CAT and SOD levels were determined by ELISA kit (SunRed Biological Technology, Shanghai, PRC), based on biotin double antibody sandwich technology, according to the manufacturer's instructions. The levels were calculated using a standard calibration curve, and the results were expressed as µmol, µg and µg per g protein for GSH, CAT and SOD, respectively.

mRNA expression levels of cell death and inflammation genes

The mRNA expression levels of cell death and inflammation related genes (interleukin-6 [IL-6], mitogen-activated protein kinase 8 [MAPK8], nuclear factor kappa B [NF-κB] and tumor

necrosis factor-α [TNF-α]) were determined using LightCycler 480 Probes Master and Catalog Assays (Roche, Mannheim, Germany) on the Roche RealTime LightCycler 480 II platform according to the manufacturer's instructions and Oztas et al. (2019). Cells were seeded at $5x10^5$ density in 3 mL culture medium into each well of 6-well plate and incubated overnight, then, exposed to 50, 25, 12.5, 6.25 µg/mL of Bi_2O_3-NPs for 24h and culture medium as control. The RNA isolation was performed using High Pure RNA Isolation Kit (Roche, Mannheim, Germany) in a benchtop laminar flow hood. After RNA quantity and purity were checked, cDNA synthesis was carried out using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. β-actin [ACTB] was used as a housekeeping gene, and the results were expressed as the fold-change of the control.

Statistical analysis

Data was expressed as mean ±standard error (SE). The significance was calculated by one-way ANOVA Post Hoc Dunnett t-test using IBM SPSS v. 20.0 for Windows (IBM Corp.; Armonk, NY, USA). A two-tailed $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Nanoparticles characterization

The particle size and size distribution of Bi_2O_3-NPs in both distilled water and cell culture medium were analysed by TEM (Figure 1). The calculated average particle diameter in water was 149.7 nm. The agglomeration- aggregation could explain the increase in the average diameter (182.2 nm) of the particles after dissolution in cell culture medium (Abudayyak et al. 2017).

Evaluation of cytotoxic potential

Bi_2O_3-NPs decreased the cell viability through disruption on mitochondrial activity and membrane integrity which are determined by MTT and LDH assays, respectively. The IC_{50} values

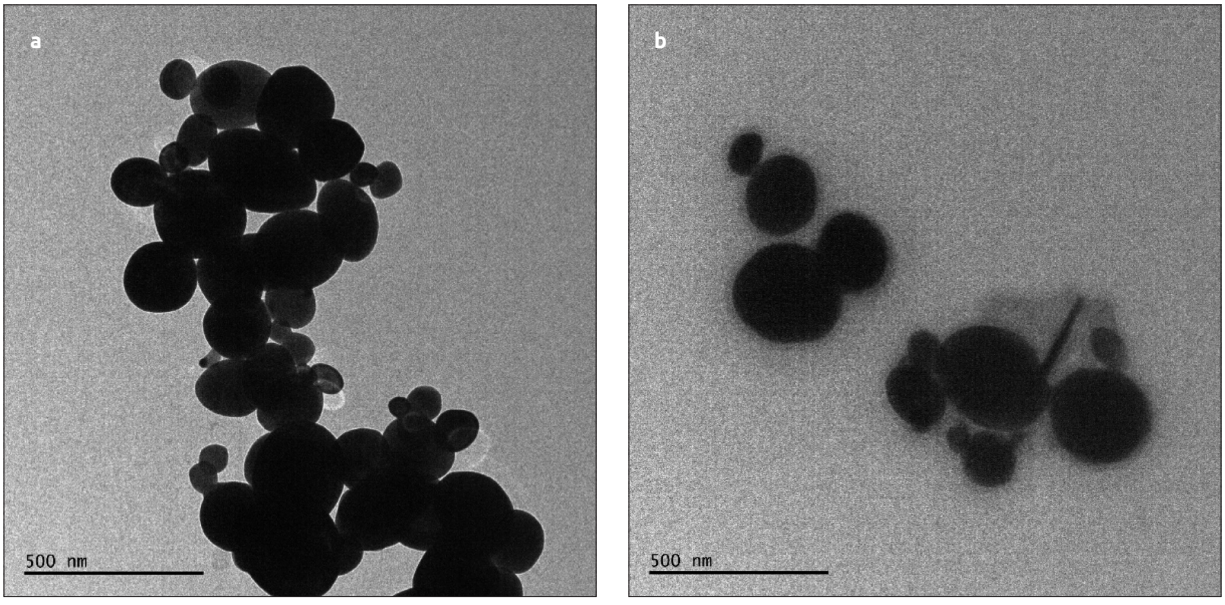

Figure 1. a, b. The Characterization of Bi_2O_3-NPs in (a) distilled water and (b) cell culture medium by TEM analysis.

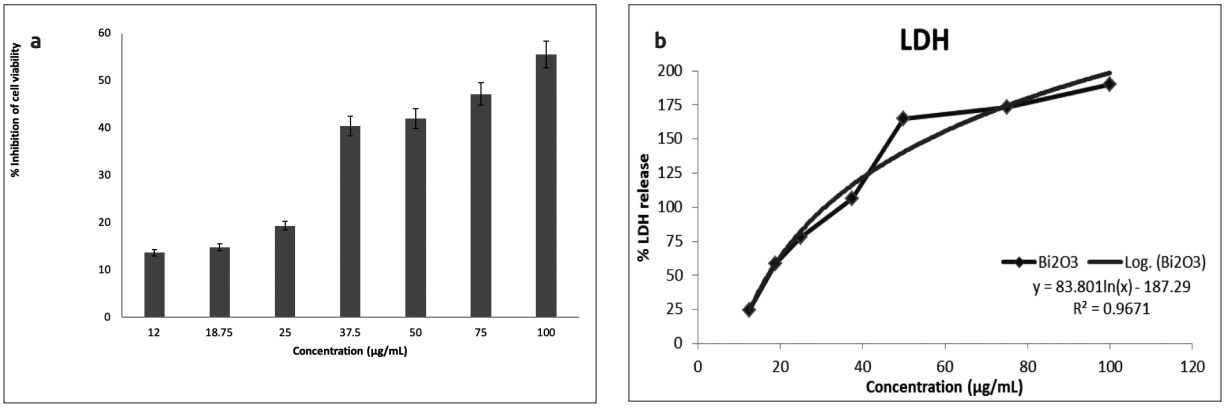

Figure 2. a, b. Inhibition of cell viability and extracellular release of LDH by MTT and LDH assays, respectively.

Inhibition of cell viability (a) and LDH% release (b, grey line) were measured in the cells exposed to Bi_2O_3-NPs (12.5-100 µg/mL). $LDH\%_{50}$ (b) was calculated according to the formula of the logarithmic (red line) curves. The IC_{50} values of Bi_2O_3-NPs were 77.57 µg/mL by MTT assay and $LDH\%_{50}$ was 16.97 µg/mL by LDH assay. Error bars represent ±standard deviation.

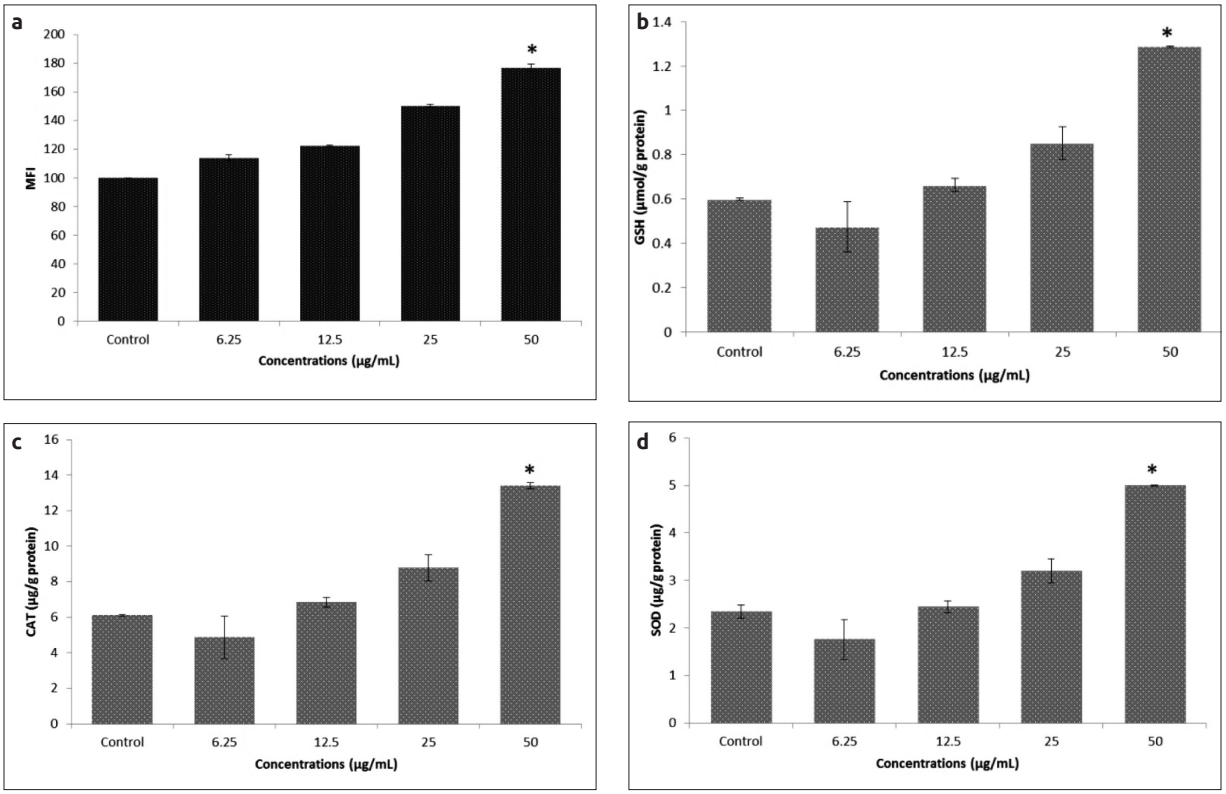

Figure 3. a-d. The pro-oxidant levels induction and oxidative damage potential of Bi_2O_3-NPs.

Pro- oxidant ROS production (a) and the levels of GSH (b), CAT (c) and SOD (d) were evaluated in cells exopsed to 6.25-50 µg/mL Bi_2O_3-NPs for 24 h. The ROS production was expressed as MFI and GSH, CAT and SOD levels were expressed as µmol, µg and µg per g protein, respectively. Cells exposed to culture medium used as control. The error bar represents ±standard error, one-way ANOVA Post Hoc Dunnett t-test was performed and *p<0.05.

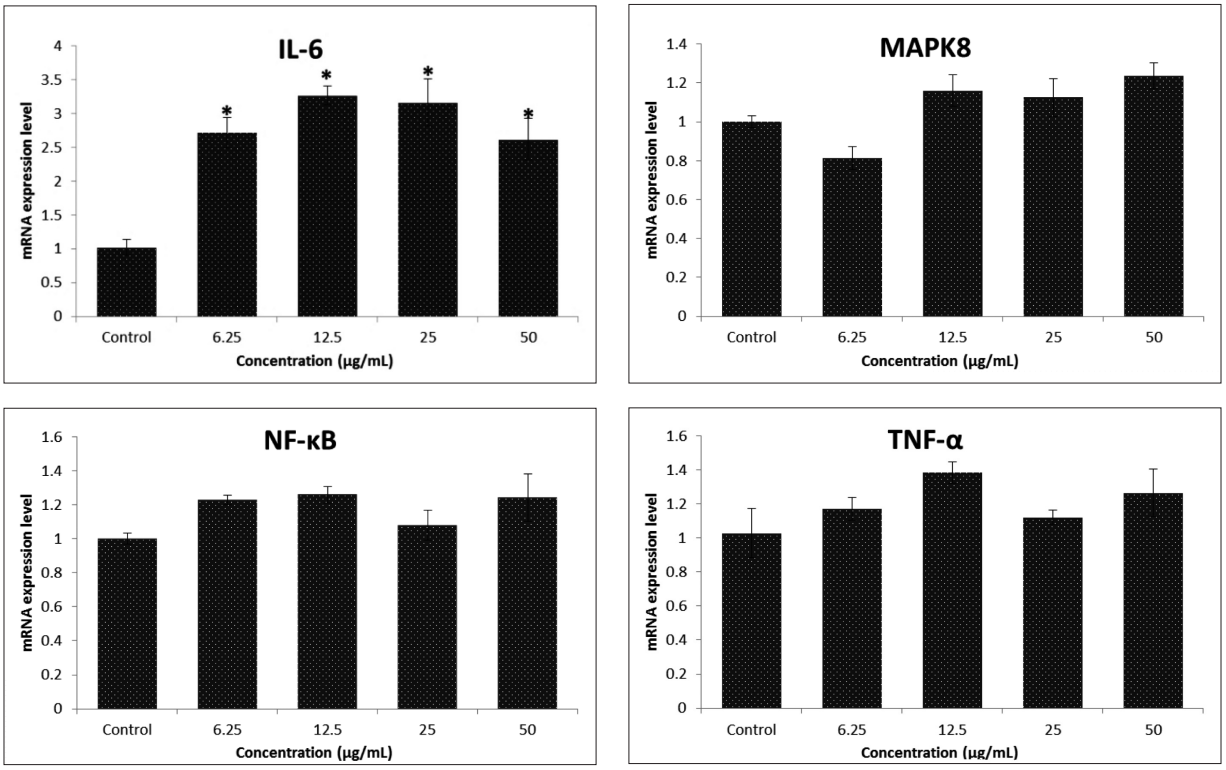

Figure 4. mRNA expression levels of cell death and inflammation related genes.

Cells were treated with various concentrations of Bi_2O_3-NPs (6.25-50 µg/mL). The results were expressed as the relative fold-change. Cells exposed to culture medium used as control, the error bar represents ±standard error one-way ANOVA Post Hoc Dunnett t-test was performed and *p<0.05.

of Bi_2O_3-NPs were 77.57 µg/mL by MTT assay (Figure 2a) and LDH%$_{50}$ was 16.97 µg/mL by LDH assay (Figure 2b).

Assay of pro-oxidant levels

The potential of Bi_2O_3-NPs to induce the production of ROS was evaluated by flow cytometer using H_2DCFDA fluorescent probe. The results showed an increase in ROS levels in a dose dependent manner (Figure 3a). The ROS production was significantly induced over 1.5-fold in the highest concentration (50 µg/mL) ($p<0.05$).

Oxidative damage potential

The oxidative damage potential of Bi_2O_3-NPs was evaluated by measuring the levels of GSH, CAT and SOD. Bi_2O_3-NPs induced oxidative damage in a dose-dependent manner. Additionally, GSH content, CAT and SOD activity were significantly higher with approximately 2-fold change at 50 µg/mL Bi_2O_3-NPs than control ($p<0.05$) (Figures 3b-d).

Cell death and inflammation related gene expressions

The regulations in cell death and inflammation related genes were determined in Bi_2O_3-NPs treated SH-SY5Y cells using specific hydrolysis probes on a qPCR platform (Figure 4). Bi_2O_3-NPs increased the mRNA expression levels of IL-6 more than 1.5-fold in all doses ($p<0.05$); whereas the changes in expression levels of MAPK8, NF-κB and TNF-α were insignificant.

DISCUSSION

Toxicity of metal-based NPs is still an emerging topic because they may have a quite different toxicity profile depending on their shape, size, coating, surface charge etc. than their macro-sized compounds or salts. Although, a few reports indicated that Bi_2O_3-NPs showed toxic effects in several cell lines, there is no data about the neurotoxicity potential and related molecular mechanism of Bi_2O_3-NPs. Differentiated or undifferentiated SH-SY5Y cells are widely used *in vitro* models in the neuroprotection or neurotoxicity studies due to their morphological, neurochemical and electrophysiological similarities to neurons (Xie et al., 2010). Therefore, the toxicity profile of Bi_2O_3-NPs in SH-SY5Y cells was evaluated and focused on oxidative stress related mechanisms.

In the present study, Bi_2O_3-NPs showed cytotoxicity in SH-SY5Y cells through disruption on mitochondrial activity and membrane integrity in a dose-dependent manner. Based on the IC_{50} value (77.57 µg/mL) and LDH%$_{50}$ value (16.97 µg/mL), it can be suggested that cell membrane is more vulnerable to Bi_2O_3-NPs in SH-SY5Y cells; since, the concentration caused the extracellular leakage of LDH to be lower. Similarly, Abudayyak et al. (2017) reported that Bi_2O_3-NPs showed cytotoxicity in several cell lines in which IC_{50} values ranged from 35.11-96.55 µg/mL and cell sensitivity to cytotoxic damage ranked at NRK-52E < Caco-2 ~ A549 < HepG2. Cornelio et al. (2011) reported that Bi_2O_3 caused slightly cytotoxic effect in murine periodontal ligament and rat osteosarcoma cells at 100 mg/mL. In a study by Song et al. (2014), they reported a cytotoxic potential of bismuth ferrite NPs in PC-12 rat neuronal cells. In a recent research by Akbarzadeh et al. (2018), it was noticed that Bi_2O_3-NPs and Folate-Conjugated Bi_2O_3-NPs induce cell death in KB nasopharyngeal carcinoma and A549 cell lines. Similar results were reported by Ahamed et al. (2019) and Bogusz et al. (2018) in MCF-7 human breast cancer and 9L malignant gliosarcoma cell lines. By contrast, Hernandez-Delgadillo et al. (2013) reported that aqueous colloidal Bi_2O_3-NPs did not induce cytotoxicity in Vero cells at ≤ 1 mg/mL. It should be considered that the controversial results could be associated with the selected cell types, with the cytotoxicity assays as well as with Bi_2O_3 size and formulation.

Toxicity of metallic NPs could be related with two main ways release of ions and oxidative stress (Seabra and Duran, 2015). NPs may interact with cellular proteins and enzymes resulting in increased ROS production which may cause DNA damage, lipid peroxidation, apoptosis and necrosis (Arora et al. 2012; Elsaesser and Howard, 2012). It is well known that oxidative stress plays an important role in many complications such as diabetes, atherosclerosis, cancer as well as aging. Since, NPs may induce excessive ROS production in the nervous system with their ability to cross blood brain barrier, neurodegenerative diseases such as Parkinson and Alzheimer could be a consequence of NP toxicity (Sorg 2004; Schrand et al. 2010; Afanas'ev 2011). Ahamed et al. (2019) reported oxidative stress mediates the cytotoxic effects of Bi_2O_3-NPs in MCF-7 cells. They also indicated the role of ROS in the oxidative stress. In the present study, Bi_2O_3-NPs induced oxidative damage in a dose-dependent manner through increasing in ROS production, GSH content, CAT and SOD activity. Similar to our results, both macro- and nano-structured Bi_2O_3 induced oxidative stress in cultured human blood cells and in HepG2, NRK-52E, Caco-2 and A549 cells (Abudayyak et al. 2017; Geyikoglu and Turkez, 2005), respectively.

It seems that NPs may induce ROS production by either innate immune response or autocatalysis (Love et al. 2012). Considering the bivious relationship between oxidative stress and inflammation, profound changes in mRNA expression levels of pro-inflammatory cytokines as key elements of immune response can be attributed as a consequence of oxidative stress (Marklová, 2007; Park and Park, 2009). Oxidative stress can trigger signalling pathways of MAPK8, and transcription factors such as NF-kB and TNF-α; and finally release of pro-inflammatory cytokines and some critical chemokines (Roach et al. 2002; Shishodia and Aggarwal, 2002). Ahamed et al. (2019) indicated the role of Bax/Bcl-2 pathway in cell death. Several reports indicated that NPs such as titanium dioxide (Monteiller et al. 2007), carbon black (Niwa et al. 2008) and crystalline silica (Rao et al. 2004) induced strong pro-inflammatory responses; however, in the present study Bi_2O_3-NPs induced pro-inflammatory response is evaluated for the first time. In the present study, Bi_2O_3-NPs increased the mRNA expression levels of IL-6, an important factor for the coordination of the innate and acquired immune response. MAPK8, NF-κB and TNF-α remained unchanged and it should be considered that these signalling pathways also play a crucial role in oxidative stress-mediated apoptosis and/or necrosis (Chandra et al., 2002). Considering the previous study (Abudayyak et al., 2017) reported that Bi_2O_3-NPs induce apoptosis in HepG2 and NRK-52E cells and necrosis in Caco-2 and A549 cells. This study is limited in that apoptotic/

necrotic cell status was not checked with any other methods such as Annexin V/Propidium iodide staining.

CONCLUSION

In the fields of drug delivery and toxicology, the application of NPs has been studied intensively in the past few decades. Potential toxic effects of metal oxide-based NPs have received growing attention and not been completely addressed. Preliminary data from *in vitro* experiments can potentially provide precautionary hazard identification about toxic effects on the people who come into contact with NPs. Obtained data will lead the way to safe usage of NPs in drug delivery and industrial applications.

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Original Article

The role of pharmacists in the management of autism spectrum disorder: A survey to determine the knowledge and perception of the parents

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ABSTRACT

Autism spectrum disorder (ASD) is one of the neuropsychiatric disorders of childhood and has many causes including genetic, neurobiological, environmental, as well as other risk factors that can trigger the disorder. The aim of this survey is to determine the knowledge of the families about ASD, the most prescribed medications, their side effects and the alternative therapies used besides to the pharmacological treatment. This study was survey-based and conducted among parents of children with ASD (N=50). Risperidone, aripiprazole, and valproate were the most prescripted drugs (64%) and weight gain (n=14), increased aggressive behavior (n=11) and drowsiness/sedation (n=11) were reported as the main side effects. Besides to pharmacological therapy, most of ASD children have used complementary and alternative medicine like gluten and casein-free diet (n =23) or the sugar diet (n=15), as well as omega-3 (n=43), multivitamins (n=40), heavy metal chelation agents (n=27), vitamin B12 (n=18), zinc (n=17) vitamin D (n=9), and probiotics (n= 7). It is revealed that pharmacists should be able to inform the parents about the general characteristics of ASD, and new treatment approaches, and provide a rational use of prescribed drugs in children with autism.

Keywords: Autism spectrum disorder, pharmacist role, pharmacological therapy, complemenatry and alternative medicine

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder, which is characterized by persistent deficits in social communication and social interaction, limitations and deviations in verbal and nonverbal skills, repetitive patterns of behavior, interests or activities. The worldwide prevalence of ASD has been steadily increasing in recent decades. In 2016, the USA National Health Center for Health Statistics reported that ASD could be found in 1 out of 36 children (Christensen et al. 2016; Sharma et al. 2018). Due to the large variation in deficiencies and the deviations in symptoms between patients, the diagnosis of ASD can be quite difficult. Therefore, it is necessary to distinguish ASD from other neuropsychiatric disorders, and use appropriate evaluation methods to diagnose correctly in order to improve the clinical management of the disease (National Institute of Mental Health 2018; Baumer and Spence 2018).

Recent studies have shown that multiple genetic factors play a role in the development of ASD (Lovato 2019). Siblings of ASD children also carry a higher risk of developing ASD (Bolton et al. 1994). It is reported that genetic and chromosomal abnormalities, such as Down's syndrome or fragile X syndrome, can be found in 10% of the children with ASD (DiGuiseppi et al. 2010; Hall et al. 2008). In addition, parental history of psychiatric disorders, premature birth and fetal exposure to psychotropic drugs, toxins, heavy metals and insecticides have been linked to the pathophysiology of ASD (Cattane et al. 2018; Sharma et al. 2018). With the emergence of possible etiologic causes, new therapy approaches may be introduced in the treatment of ASD (Emberti Gialloreti et al. 2019).

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The current treatment options of ASD include nonpharmacological (behavioral, phsycological, and educational therapy, food supplements, dietary interventions, etc.) and pharmacological therapies (Goel et al 2018; Baumer and Spence 2018). Pharmacological therapy includes different classes of drugs such as psychostimulants, antipsychotic drugs, antidepressants, NMDA receptor antagonists, alpha-2 adrenergic receptor agonists, cholinesterase inhibitors and antiepileptics (Aman et al 2008; Sharma et al. 2018). Although core symptoms of ASD may improve with appropriate pharmacological therapy, there is currently no appropriate cure. Many families of children with ASD use conventional as well as complementary and alternative (CAM) therapies (Goel et al 2018; DeFilippis 2018). Treatment should be individualized according to the patient's symptoms and family's needs, and also, should be closely monitored by the healthcare team, including the pharmacists. Pharmacists play an important role in the follow up of new treatment approaches, in providing the correct use of the prescribed drugs and over the counter medications (OTC), as well as the education of ASD families. Unfortunately, pharmacists are rarely included in the team of health professionals who routinely provide care to children with ASD (Shafiq and Pringsheim 2018; Breik et al. 2018; Wongpakaran et al. 2017; Luleci et al 2016).

The aim of this survey is to determine the knowledge of the families about ASD, the most prescribed medications, their side effects and the alternative therapies used besides to the pharmacological treatment. These findings may give a perspective to strengthen the role of pharmacist in the management of ASD therapy.

MATERIALS AND METHODS

This is a survey-based study which was developed by the authors and conducted among parents of children and adolescentse (2.5-18 years old) with ASD (N=50). Parents completed questionnaires during interviews scheduled at OÇEM (Autistic Children Education Centre) in Istanbul and Sakarya in Turkey. The first part of the survey consisted of demographic characteristics (i.e., age, sex, profession of parents, status of intermarriage, family history of ASD, Alzheimer's disease and dementia), knowledge and perception of parents regarding ASD (such as delayed reply to name, failure to show an interest to other children, repetitive, stereotypic movements, limited social interaction). In the second part of the survey, currently used therapies, such as pharmacological (stimulants, antipsychotics) and non-pharmacological (OTC medications, food supplements, dietary interventions etc.) therapies, presence of concomitant disorders, physicians' visit intervals and social care service support were assessed. The inclusion criterias of the study were: children and adolescents having a confirmed diagnosis of ASD; parents being able to read, understand and fill in the forms properly. All participants provided informed consent, and the study was approved by the Ethics Committees of Istanbul University Cerrahpasa Faculty of Medicine (Number:83045809/5563).

Table 1. Demographic variables of ASD patients and their families

	N	Percentage (%)
• Age		
<7 years	15	30
7-15 years	28	56
15-18 years	7	14
• Gender		
Female	10	20
Male	40	80
• Status of intermarriage		
Yes	10	20
No	40	80
• Family history of autism		
Yes	7	14
No	43	86
• Family history of neurodegenerative brain disorders (Alzheimer's disease and dementia)		
Yes	14	28
No	36	72

Statistical analysis

The statistical analysis was performed by GraphPad Prism software (version 6). Chi-square and Fisher's test were perfomed to show association between variables. Mean and standard deviation (SD) were calculated for demographic data and anthropometric measurements.

RESULTS AND DISCUSSION

In this study, a survey was conducted with the parents of children and adolescents with ASD aged between 2.5 and 18 years (N=50). The demographic data of the patients with ASD and their special family characteristics which may be associated with ASD are listed in Table 1. Studies have shown that the prevalence of ASD is four- to five-fold higher in boys than girls (Christensen et al. 2016). A similar male / female ratio was observed in the current study. Among the parents interviewed, the majority of the mothers (66%) were housewives and a special etiological link in terms of an occupational risk of toxicity was not determined.

ASD is known to have a genetic component (Lovato et al. 2018). Thus, we evaluated the genetic risk factors of the ASD patients. It was found that 20% of the parents enrolled in this study were consanguineous. In addition, 14% of the ASD parents have other relatives with ASD, and 28% of them had Alzheimer's and dementia in their families (Table 1). Indeed, due to the small sample size, these available data may not be sufficient for questioning the genetic origin of autism (Lovato et al. 2018; Gyawali and Patra 2019). Most of the ASD parents do not have an ASD association membership, however, 36% of them receive social care services called home care fees.

ASD symptoms in children are often first recognised by their parents and teachers (National Institute of Mental Health 2018; Baumer and Spence 2018). In this study, we determined that parents visit the physicians when they notice the symptoms,

such as delay in speaking and deficiency in eye contact (n=45), lack of facial expression and social communication (n=35), recurrent and stereotypic hand, finger, whole body movement (n=12) and lack of developing friendships (n=21). The age when families mostly recognise ASD symptoms in their children is generally around 3 years (Figure 1), which is consistent with the mean age for ASD diagnosis in children (4.3 years) in the USA (Baio et al 2018).

Children with ASD generally experience difficulties in developing social, speech, and behavioral skills. Therefore, pharmacological therapy is the mainstay in ASD therapy, in order to help patients to become functional in their daily activities, which additively needs to be supported by behavioral therapy as well

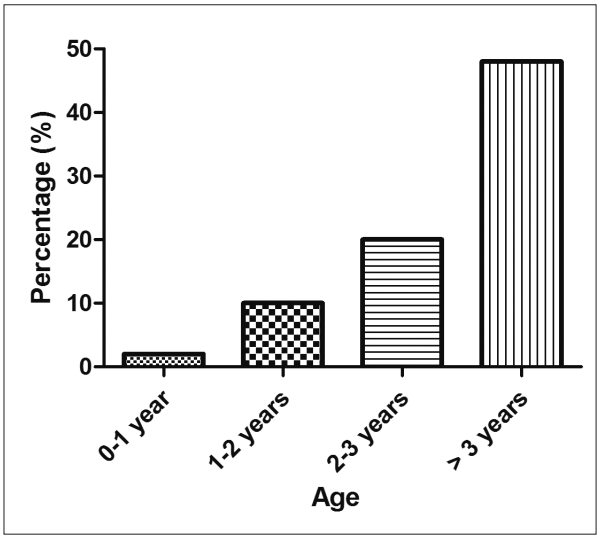

Figure 1. Rates of ASD Diagnosis by Age.

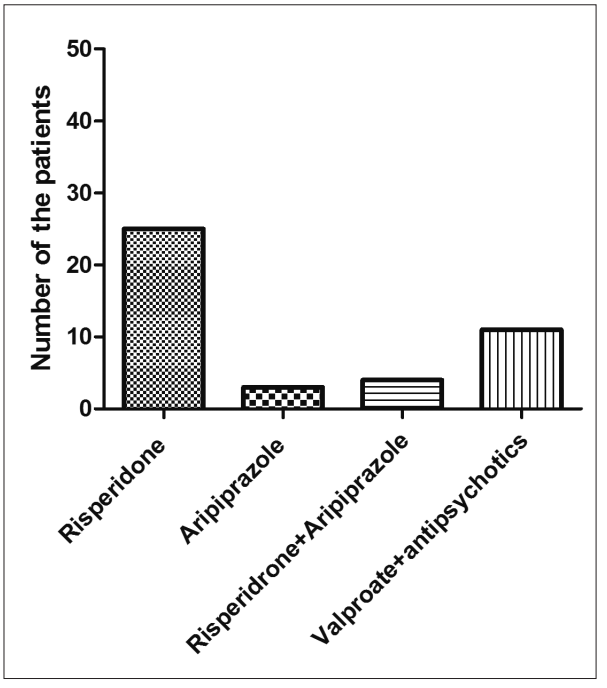

Figure 2. Rates of prescribed drugs for ASD treatment in this study.

(Goel et al. 2018; DeFilippis 2018). Risperidone and aripiprazole, the atypical antipsychotic agents, are the only medications approved by the USA Food and Drug Administration (FDA) to treat irritability and self-injurious and aggressive behaviors in children with ASD (Posey et al 2008, Oshikoya 2019, Wink 2017). In particular, Risperidone is the commonly prescribed antipsychotic with the most comprehensive data in ASD, and is used either alone or in combination with aripiprazole to treat children with ASD (Novaes et al. 2008). In this study, we observed that 36% (n = 18) of ASD children do not use any medication for ASD treatment. Among 50 ASD children, 32 (64%) were either on risperidone, aripiprazole or their combination therapy (Figure 2). 77% of the parents stated that drug treatment was effective. In addition, weight gain (n=14), increased aggressive behavior (n=11) and drowsiness/sedation (n=11) were reported as the main side effects by the parents. In line with our findings, weight gain and associated metabolic risks have been reported with antipsychotic treatment in other ASD patients. Indeed, it's well known that weight gain and drowsiness are the main side effects of risperidone, while sedation occurs especially with the use of aripiprazole (Wink 2017).

In this study, we documented that valproate was prescribed in 11 patients in combination with the antipsychotic agents, risperidone and aripiprazole. Indeed, valproate is an antiepileptic drug which can be added to ASD therapy in relation to the child's functioning and needs (Goel et al. 2018). In accordance with this data, we determined that among these 11 patients who were on combined drug regimen, 9 were also diagnosed with epilepsy along with ASD. Two clinical trials were noticed in the literature that investigated the effect of valproate in reducing the aggression and irritability symptoms of ASD patients. Therein, Hellings et al (2005) showed that there were no differences in the symptoms observed between the placebo and valproate groups following 8 weeks of treatment. However, Hollender at al. (2010) reported that valproate was superior to placebo in 12 weeks of treatment in controlling these symptoms in ASD children.

It is reported that, three-quarters of children with ASD may also have another medical, psychiatric, or neurological disorder, including attention-deficit hyperactivity disorder, anxiety, bipolar disorder, inflammatory bowel disease, epilepsy, fragile X syndrome, gender dysphoria, intellectual disability, neuroinflammation and immune disorders, non-verbal learning disorder, obsessive-compulsive disorder, schizophrenia, sensory problems, sleep disorders, tuberous sclerosis, Tourette syndrome and tic disorders (Sharma et al. 2018; Tye et al. 2019). In our study, half of the children with autism have intestinal problems, and almost all the children with this problem have also developed fungal infections. The surveyed children mostly (%76) have sleep disturbances. Other diseases determined to be associated with autism include epilepsy (n=9), intellectual disability (n=7) and hyperactivity (n=4) (Table 2). In this relation, children with ASD can use several medications at the same time for the treatment of different symptoms, as well as comorbid disorders (Wongpakaran et al. 2017). Therefore, it is important to consider the effectiveness, adverse effects and interactions of these medications used systemically during ASD therapy. In this regard, pharmacists can

develop systems to monitor these symptoms and provide data about responsiveness to drug therapy and thereby, can play an important role in the improvement of the prognosis and clinical outcomes in ASD patients.

We determined that most of children with autism in this survey study (74%) had undergone heavy metal assessment. As a result of this assessment, heavy metals, particularly mercury and lead, were detected in almost all the children in this study population, and in among 54% of them, the heavy metal chelation agents like 2,3-dimercaptosuccinic acid (DMSA) were preferably used (n=27). Although, there is no evidence of the efficacy and potential side effects of this chelation therapy in ASD, the heavy metal ratio is quite high in ASD patients and thus, it is suggested that there is a relationship between the heavy metals ratio and ASD (Pelch et al. 2019). However, reviewers of the chelation therapy concluded that this treatment is not recommended for individuals with ASD, and that risks associated with chelation therapy outweigh any potential benefits (Davis et al. 2012; James et al. 2015; DeFilippis 2019). Chelation agents can bind ions nonspecifically, and thus cause a decrement in the plasma levels of calcium, iron, and magnesium as well. This may associated with hypertension, hypotension, cardiac arrhythmias, and hypocalcemia, the latter of which can be fatal (James et al. 2015; DeFilippis 2019). In this context, pharmacists can determine and monitor the problems related to the decreased levels of these ions, and can raise the awareness of the patients in relation to the symptoms of these deficiencies. In this survey study, we determined that 38% of the children have anemia. The reason of the anemia might be the decrease in the level of iron due to chelation therapy, but we did not find a statistically significant correlation (p=0,9877).

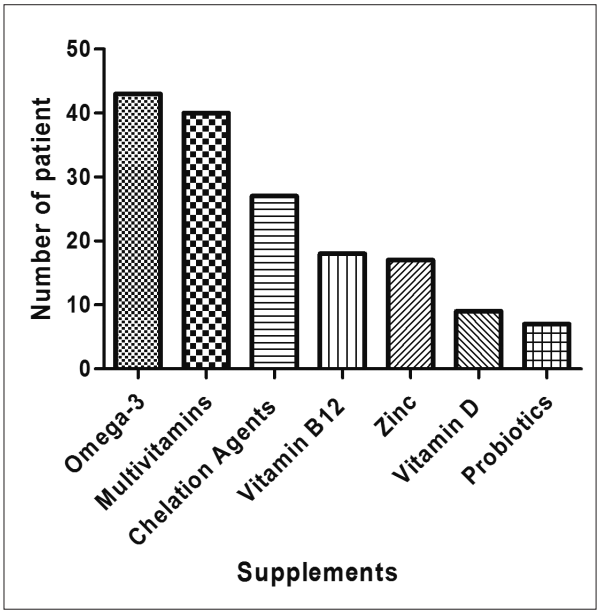

Figure 3. Rates of supplemental therapy used in this study.

Table 2. Prevalence of Comorbid Disorders of ASD

	N	Percentage (%)
• Bowel disorders		
Yes	26	52
No	24	48
• Fungal infections		
Yes	23	46
No	27	54
• Sleep disorders		
Yes	38	76
No	12	24
• Comorbid psychiatric or neurological disorder		
Yes	17	34
No	33	66
• Intellectual disability		
Yes	16	32
No	34	68
• Anemia		
Yes	19	38
No	31	62
• Heavy metal assessment (mercury, lead)		
Done (High)	33	89
Done (Normal)	4	11

Another issue that is discussed regarding the children with autism is the elimination diets. Although this is not a proven case, most families apply an elimination diet for their children. We determined that many of the ASD parents that participated in our survey study, applied gluten and casein-free diet (n=23) or the sugar diet (n=15), especially in children with autism who also have fungal infections. Dietary approaches do not treat the symptoms of ASD but they can benefit by normalizing symptoms related to gastrointestinal dysfunction and therefore, improve overall well-being in ASD patients (Kawicka and Regulska-Ilow, 2013). It should be noted that ASD children may have malnutrition risks due to low energy intake, gastrointestinal problems and malabsorption of the nutrients. Thus, ASD children are required to be followed-up for special nutrition by dieticians or doctors in order to evaluate the nutrient status, especially in terms of vitamin D, calcium, potassium, iron and fiber intake (Kawicka and Regulska-Ilow 2013; DeFilippis 2019; Sharma et al. 2018).

In this survey study, we noticed that, besides a pharmacological treatment, 54% of the parents applied psycotherapy to their children. In addition, hyperbaric oxygen therapy is another additional treatment preferred in some of the ASD children in this study. Although 64% (n=32) of the children with autism have pharmacological therapy, 90% (n=45) of the children were preferably given food supplementation such as omega-3 (n=43), multivitamins (n=40) and heavy metal chelation agents (n=27). In addition, vitamin B12 (n=18), zinc (n=17) vitamin D (n=9), and probiotics (n=7) were also used as well (Figure 3). Höfer at al. (2019) recently showed that almost half of the parents of the children with ASD in Germany reported that they use or have used complementary and alternative medicines for their children. It seems that this is a global problem, and pharmacists should be aware of the significant prevalence of the use of these supplementary medicines. In this context,

pharmacists can play a crucial role in informing the caregivers of children and adolescents with ASD about the effectiveness and potential side effects of these supplementary products.

Almost all of the parents in this survey study stated that they received the first information about the use of drugs in ASD from their doctors. Taking into account the long-term use of medicines in children with ASD and the possibility of polypharmacy, the interest of pharmacists in monitoring the patient is very important to ensure maximum benefit from the pharmacological treatment (Wongpakaran et al. 2017, Sharma et al. 2018). It is the responsibility of the pharmacist to inform the family about ASD, to explain the use of drugs in the correct manner, to warn about important side effects of drugs, to emphasize the important points in their nutrition and to inform the parents about the dietary supplements. Pharmacists' responsibility is, in fact, the part of pharmaceutical care provided to the patients and their relatives.

Concerning the families of autistic children, they need to be consulted on the process of this disease, the problems that can accompany the disease, the approach and follow-up to the child, the aim and effectiveness of the drugs and alternative therapies, the possible side effects of these therapies and the precautions to be taken, the route and duration of use of the drugs and alternative therapies (Wongpakaran et al. 2017).

Concerning the role of pharmacists, as a health professionals, they should be informed about the general characteristics and current treatments of ASD, in order to provide early awareness to the families of children who may be autistic, and to direct them to the physician in the proper time. They should contribute to the positive outcomes of the treatment by ensuring the correct use of the drugs prescribed to the children and adolescents diagnosed with autism (Wongpakaran et al. 2017; Luleci et al. 2016).

CONCLUSION

In this study, it was revealed that pharmacists should be able to inform the parents of children and adolescents diagnosed with autism about the general characteristics of ASD, provide an early awareness to their families about the disease, and direct them to the physician in the appropriate time, to inform the parents about new treatment approaches and ensure the rational use of prescribed drugs in children and adolescents with autism.

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Original Article

Anatomical features of *Salvia potentillifolia* Boiss. & Heldr. ex Benth. and *Salvia nydeggeri* Hub.-Mor. (Lamiaceae)

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ABSTRACT

The genus *Salvia* is represented by 107 taxa in Turkey and 54 of them are endemic. The genus has been used medically since ancient times and it is known as "adaçayı", "şalba", "dağ çayı", "çalba" in Turkish. Traditional uses of *Salvia potentillifolia* are reported for cold and flu in Turkey. In this study leaf, petiole and stem anatomy of two endemic species, *Salvia potentillifolia* and *S. nydeggeri*, are compared. The study found that the mesophyll type is bifacial and the leaf is of amphistomatic type in both species. In the study we observe morphologically different types of glandular hairs. There are also dense eglandular hairs. The study also observes starch grains in petioles. The vascular bundles are of the collateral type. Both species have similarities in leaf and stem anatomy. The anatomical differences and similarities of these two morphologically similar species are given in detail.

Keywords: *Salvia potentillifolia, Salvia nydeggeri*, anatomy, Turkey

INTRODUCTION

Lamiaceae is the third largest family of flowering plants in Turkey. The genus *Salvia* L. is represented by 107 taxa in Turkey, 54% of which are endemic (Celep and Dirmenci 2017). A project entitled "Taxonomic revision of the genus *Salvia* L. in Turkey" was carried out in 2008 by Doğan et al. (2008). As a part of this project the anatomy of some *Salvia* species was studied and the importance of anatomical studies was emphasized for the taxonomy of the genus *Salvia*. The authors of the above-mentioned study indicated that anatomical studies is a good tool for grouping the species. Recently several anatomical studies on *Salvia* species have been done (Özdemir and Şenel 1999, Kandemir 2003, Kaya et al. 2007, Özkan and Soy 2007, Baran et al. 2008, Özkan et al. 2008, Aktas et al. 2009, Özdemir et al. 2009, Kahraman and Doğan 2010, Kahraman et al. 2009a, 2009b, Koyuncu et al. 2009, Kahraman et al. 2010a, 2010b, 2010c, Bagherpour et al. 2010, Polat et al. 2010, Büyükkartal et al. 2011, Celep et al. 2014, Polat et al. 2017).

Salvia species has been used medically since ancient times and has different traditional uses such as antipyretic, antiseptic, antimicrobial, diuretic, stimulant, laxative and for abdominal pain, common cold, flatulence, rheumatism, tonsillitis, warts, stomachache, wounds (Yeşilada et al. 1993, Fujita et al. 1995, Ulubelen et al. 1997, Sezik et al. 1997, 2001, Baytop 1999, Topçu and Gören 2007, Gürdal and Kültür 2013, Tan et al. 2016 and 2017, Yeşil and İnal 2019). Traditional uses of *S. potentillifolia* Boiss. & Heldr. ex Benth are reported for cold and flu (Baser et al. 2006). In Turkey *Salvia* species is known as "adaçayı", "şalba", "dağ çayı", "çalba" (Baytop 1994, Tuzlacı 2006).

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Salvia potentillifolia and *S. nydeggeri* Hub.-Mor. grow in southwest Anatolia and have similar morphological characteristics. They differ from each other in terms of the size of terminal foliol and stems (erect or procumbent). In this paper the anatomical characteristics of endemic *Salvia potentillifolia* and *S. nydeggeri* have been given for the first time.

MATERIALS AND METHODS

Plant specimens were collected from Antalya during a field trip of the Tübitak Project (Project No. 114S734). Specimens were collected from *S. nydeggeri* - Antalya, Elmalı, Elmalı Mountain, Kışlaköy village, 29.06.2015, Sat-175, ISTE 107562; *S. potentilifolia* - Antalya, Cedar Research Forest, 28.06.2015, Sat-166, ISTE 107560. Voucher samples were kept in ISTE (Herbarium of Faculty of Pharmacy, Istanbul University).

Plants materials were fixed in 70% alcohol. Cross and surface sections of the plant parts (leaves, petiole, and stem) were taken by free-hand and stained with Sartur solution. Anatomical features of plants were studied by Olympus BX-53 light microscope. Photos were taken with Olympus E330 digital camera.

RESULTS AND DISCUSSION

Stem anatomy

Salvia potentillifolia: The stem is more or less angled-circular in transverse section. The epidermis is covered by relatively thick cuticle with eglandular and glandular trichomes. The epidermis is composed of a single layer of oval-rectangular cells. The collenchyma tissue is located under the epidermis which is 4-7 layered. The cortex tissue is paranchymatous and 3-5 layered. Sclerenchyma groups are present above the phloem. Cambium is not distinguishable. The xylem part is larger than the phloem part. The pith consists of paranchymatous cells which are large and polygonal or orbicular in shape (Figure 1a).

Salvia nydeggeri: In the transverse section of the stem, the epidermis consists of oblong- rectangular cells and is covered by cuticle with eglandular and glandular trichomes. The collenchyma tissue which is 3-5 layered is located under the epidermis. The cortex tissue is paranchymatous and 2-5 layered. Sclerenchyma are present above the phloem. Cambium is not distinguishable. The xylem part is larger than the phloem part. The pith comprises polygonal parenchyma cells (Figure 1b).

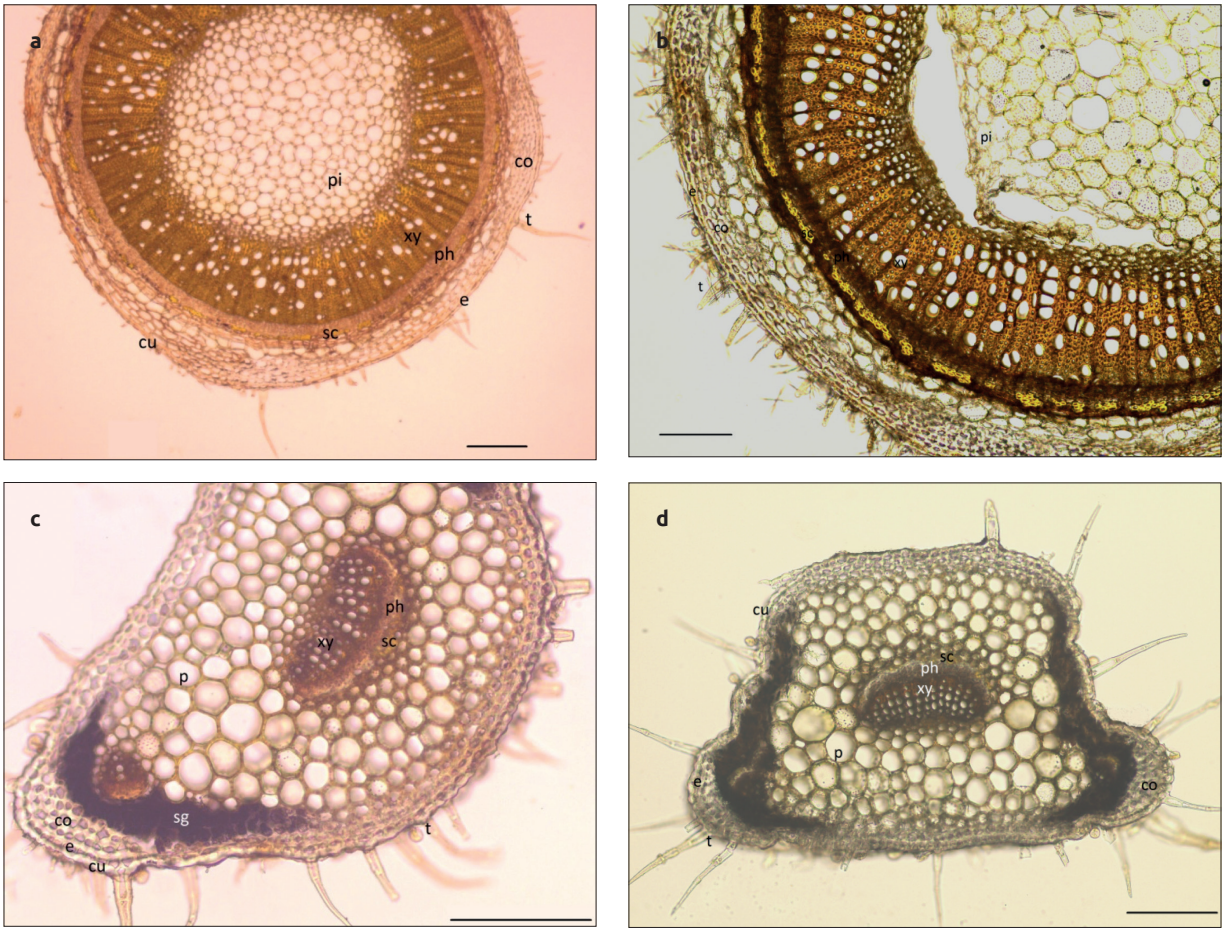

Figure 1. a-d. *Salvia potentillifolia* stem, b) *S. nydeggeri* stem, c) *S. potentillifolia* petiole, d) *S. nydeggeri* petiole.

e: epidermis, cu: cuticle, t: trichome, co: collenchyma, sc: sclerenchyma, xy: xylem, ph: phloem, pi: pith, p: parenchymatous cells, sg: starch grains. Scale: 0.2 µm

Petiole anatomy

Salvia potentillifolia: In the transverse section of the petiole, the abaxial surface is flat and the adaxial surface is convex. The epidermis is composed of uniseriate, rectangular or oval cells. There are eglandular and glandular trichomes on the epidermis. The collenchyma cells are 3-4 layered on the corner of the petiole and 1-2 layered under the epidermis. There is a large vascular bundle in the center with small bundles on the corners. The vascular bundle is of collateral type and surrounded by 1 layered sclerenchyma tissue. Several parenchymatous cells contain starch grains in the corners (Figure 1c).

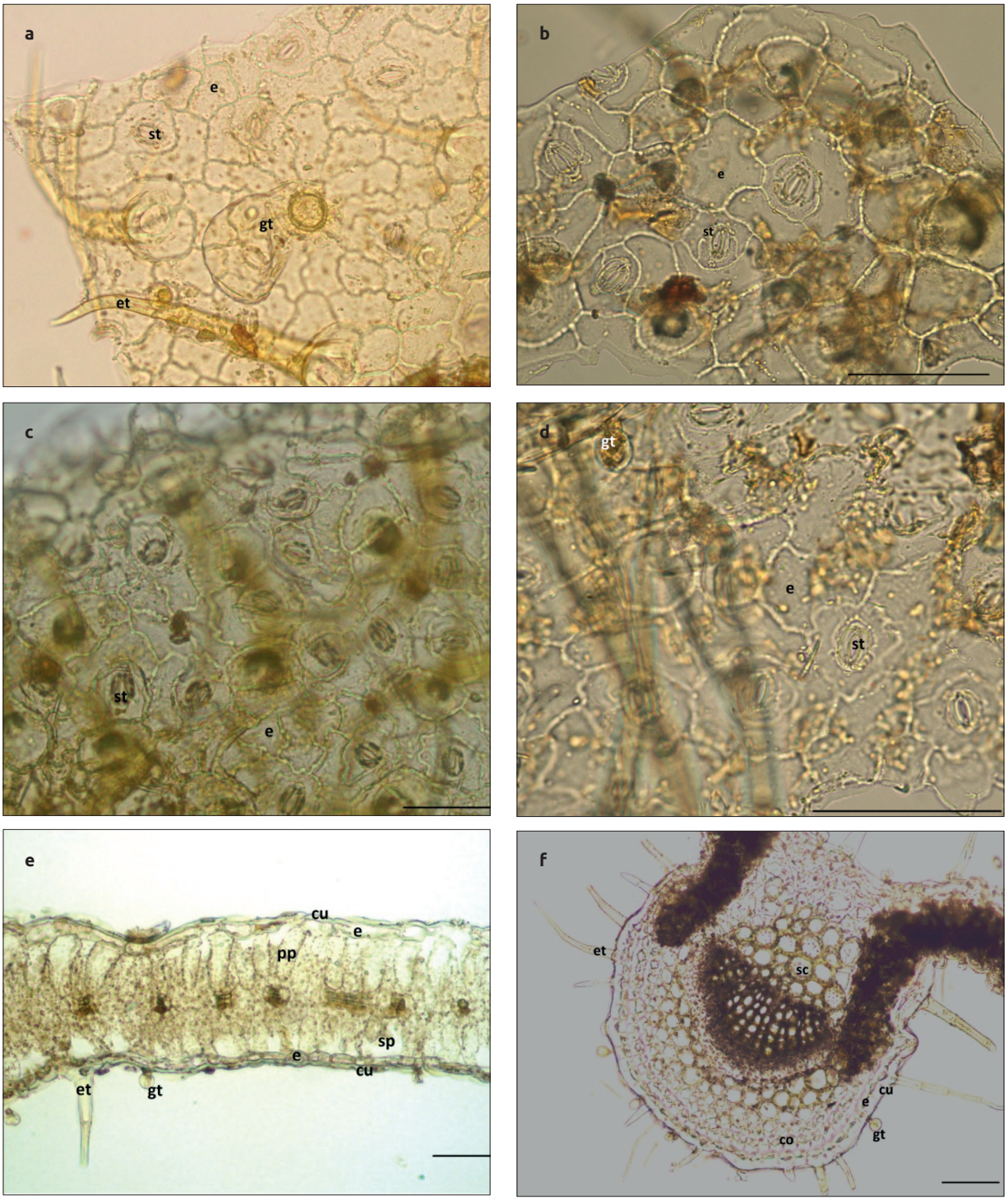

Figure 2. a-f. Leaf sections a) *Salvia potentillifolia* upper epidermis, b) *S. nydeggeri* upper epidermis, c) *S. potentillifolia* lower epidermis, d) *S. nydeggeri* lower epidermis, e) *S. potentillifolia* mesophyll tissue in leaf cross section, f) *S. nydeggeri* midrib area in leaf cross section.

e: epidermis, cu: cuticle, et: eglandular trichome, gt: glandular trichome, co: collenchyma, sc: sclerenchyma, pp: palisade parenchyma, sp: spongy parenchyma Scale: 0.1 mm

Salvia nydeggeri: In the transverse section of the petiole, the abaxial surface is almost flat and the adaxial surface is convex. The epidermis is composed of uniseriate, rectangular or oval cells with thick cuticle layer. There are eglandular and glandular trichomes on the epidermis. The collenchyma cells are 3-4 layered on corner of the petiole and 2-3 layered under the epidermis. The vascular bundles are of collateral type. There is a large vascular bundle in the center with small bundles on the corners (Figure 1d).

Leaf anatomy

Salvia potentillifolia: In the transverse section of the lamina, the upper and the lower epidermis are covered with a thick cuticle layer. There are eglandular and glandular trichomes on both sides. Both epidermis consist of a single layer, rectangular or squarish cells. The stomata cells are present on both the upper and lower epidermis. The stoma type is diacytic. The leaf is of the bifacial. The palisade cells are elongated rectangularly, 1-2 layered and the spongy parenchyma cells are 2-3 layered with large intercellular spaces. The vascular bundle is the collateral type (Figure 2).

Salvia nydeggeri: In the transverse section of the lamina, the upper and the lower epidermis are covered with a cuticle layer. There are eglandular and glandular trichomes on both sides. Both epidermis cells are of a single layer, rectangular or squarish. The stomata cells are present on the upper and lower epidermis. The stoma type is diacytic. The leaf is of the bifacial. The palisade cells are elongated rectangularly, 1-2 layered and the spongy parenchyma cells are 3-4 layered. The vascular bundle is collateral type (Figure 2).

Discussion and Conclusion

According to Metchalf and Chalk (1950), the arrangement of the collenchyma in the stem is of diagnostic value and also the vascular structure of the petiole is of taxonomic interest for the family Lamiaceae. Recently several anatomical studies have been conducted about some species of the genus *Salvia* in Turkey. Some anatomical properties show differences between the species.

Salvia potentillifolia and *S. nydeggeri*, which are our studied specimens, belong to Sect. *Salvia* Hedge. In the literature some species in the same section are studied. The leaf of *S. macrochlamys* Boiss. & Kotschy is of equifacial type and amphistomatic. The petiole of *S. macrochlamys* has one large vascular bundle in the middle and also two small vascular bundles in the petiolar wings (Kahraman et al. 2010). Petiole of *S. ballsiana* (Rech.f.) Hedge has one central vascular bundle and 4-6 small bundles in the wings (Kahraman et al. 2010c). In the petiole of *S. quezelii* Hedge & Afzal-Rafii there is a central vascular bundle and 4 small bundles in the petiolar wings (Celep et al. 2014). *S. tchihatcheffii* (Fisch. & C.A.Mey.) Boiss. Has one large vascular bundle on the median region and small vascular bundles on the end part of the petiole (Aktas et al. 2009). In our study, *S. potentillifolia* and *S. nydeggeri* also have one large vascular bundle in the center and small bundles on the corners of the petiole. When comparing the members of sect. *Salvia*, they have one central vascular bundle and in addition the number of vascular bundles in the petiolar wings varies.

Özkan and Soy (2007) mentioned that the stoma type of *Salvia blepharochlaena* Hedge & Hub.-Mor. leaf is diacytic and in petiole one vascular bundle is on the median part and a small bundle is near these bundles. In another study conducted by Özkan et al. (2008), the petiole of *S. cadmica* Boiss. has one large vascular bundle in the center and two small bundles at both ends. *S. cadmica* and *S. euphratica* Montbret & Aucher ex Benth.are members of Sect. *Hymenosphace*.

Polat et al. (2017) compared the anatomical features of three endemic *Salvia* species (*S. euphratica*, *S. divaricata* Montbret & Aucher ex Benth. and *S. hypargeia* Fisch. & C.A.Mey.) in different sections. All three species have bifacial mesophylls. Vascular bundles in the petiole show differences between these three species. *S. divaricata* and has one median vascular bundle and a single bundle at the wings of the petiole. *S. euphratica* has 4 median bundles and one small vascular bundle in each of the petiolar wings. *S. hypargeia* has 4-5 median bundles and one small bundle in the petiolar wings. *Salvia euphratica* is in Sect. *Hymenosphace*, *S. divaricata* in sect. *Salvia*, *S. hypargeia* in Sect. *Aethiopis*.

In the literature, the anatomical characteristics of some species in Sect. *Aethiopis* are studied. Kandemir (2003) showed that petiole of *S. hypargeia* has two large vascular bundles on the median part and a small one is present near these. In the leaf anatomy of *S. hypargeia* stoma type is diacytic and the leaf is bifacial. There are 5 or 7 large vascular bundles of *S. argentea* L. in the center and 3-5 small ones in the ends of petiole (Baran et al. 2008). In petiole of *Salvia vermifolia* Hedge & Hub.-Mor. there are three large vascular bundles in the middle and three more small ones located in each wing (Bagherpour et al. 2010). As regards the leaf anatomy of *Salvia indica* L., stomata are diacytic and the leaf is bifacial (Kahraman et al. 2009b). For *Salvia chrysophylla* Stapf, 2-3 large vascular bundles are in the center and 2-4 small subsidiary bundles are in the petiolar wings (Kahraman et al. 2010a). Polat et al. (2010) compared *S. aethiopis* L. and *S. argentea* in Sect. Aethiopis with *S. viridis* L. in Sect. *Horminum*. They showed that the leaf anatomy of them is similar to each other.

Kaya et al. (2007) studied the anatomy of *Salvia halophila* Hedge. Their results show that the stomata are diacytic and the epidermis is amphistomatic. The leaf is of the monofacial centric type. Six vascular bundles are present in the center of the petiole and three small vascular bundles are present in each of the petiolar wings. Leaf of *Salvia verticillata* L. subsp. *verticillata* (in Sect. *Hemisphace*) is bifacial and has anisocytic type of stomata (Koyuncu et al. 2009).

According to the studies in the literature, the difference between the species is shown particularly in the size and in the number of the vascular bundles in the petiole. In many studies, cambium is indistinguishable in the stem anatomy of *Salvia* species (Bagherpour et al. 2010, Kahraman et al. 2010, Polat et al. 2010, Koyuncu et al. 2009, Aktas et el. 2009). It is also the same in our study. Büyükkartal et al. (2011) studied the mericarp anatomy of *Salvia hedgeana* Dönmez, *S. huberi* Hedge and *S. rosifolia* Sm. and showed differences in the exocarp, mesocarp, sclerenchyma region and endocarp.

In conclusion, the stem, petiole and leaf anatomy of *Salvia nydeggeri* and *S. potentillifolia* are studied in this study for the first time. These two species are closely related to each other. Both of them

have pinnate leaves and yellow flowers. *S. nydeggeri* differs from *S. potentillifolia* by its caespitose habit, size of terminal foliol and procumbent stem. According to our data and the literature, anatomy studies can be a useful tool in the taxonomy of the genus *Salvia*.

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Review

Plants used in traditional treatment of prostate diseases in Turkey

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ABSTRACT

Turkey has a very rich flora and also many medicinal plants, due to its geography. The aim of this study was to provide information about the plants used for prostate diseases in Turkey. Ethnobotanical studies made in various parts of the Turkey have been researched and 107 taxa used in the treatment of prostate diseases have been recorded. These plants belong predominantly to the Rosaceae (15 taxa), Asteraceae (13 taxa), Lamiaceae (10 taxa), Poaceae (4 taxa), Anacardiaceae (3 taxa), Apiaceae (3 taxa), Brassicaceae (3 taxa) families. The plant parts used for treatment are leaves, aerial parts, flowers, roots and seeds. Flavonoids, essential oils, tannins, saponins, alkaloids, and steroids are the most common chemical compounds in these plants. Activity studies have been found which support the use of 17 taxa in prostate diseases. Similar activity studies can be carried out for other plants and so new drugs can be developed for the treatment of prostate diseases.

Keywords: Prostate diseases, medicinal plants, traditional treatment, Turkey

INTRODUCTION

The prostate gland is mainly an organ of the male reproductive system. It is located just below the bladder and surrounds the urethra. It secretes a special secretion that helps sperm transport and fertilization (Guyton 2007).

Prostate diseases can be listed under three main headings. These are prostate inflammation, benign prostatic hypertrophy and prostate cancer.

Prostate inflammation (prostatitis) is usually a disease caused by bacteria and reduces the quality of life due to symptoms. Clinical trials show that 50% of men have prostatitis at least once in their lifetime (Internet source 1).

Benign prostatic hyperplasia (BPH), known as benign prostate enlargement, can be described as compression of the urinary tract caused by the prostate gland is enlarged. Prostate growth is a common, non life threatening disease that reduces the quality of life of the patient (Internet source 2).

Prostate cancer is one of the most common types of cancer among men. It can be described as an uncontrolled proliferation of the prostate gland cells. Prostate cancer symptoms may not be felt for a long time or symptoms may be confused with other diseases. Prostate cancer may spread to other organs at later stages and treatment may become difficult. For this reason, men over the age of 40 must have prostate examinations at certain intervals (Internet source 3).

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Turkey has a very rich flora due to its geography. Approximately eleven thousand plant species are growing in our country, and three thousand of them are endemic (Güner et al. 2000; Özhatay et al. 2013, 2015, 2017). Traditional treatments using these plants in each region of our country can treat many diseases and protect against diseases. In the research carried out, many herbal remedies used against prostate diseases in Turkey were encountered.

MATERIALS AND METHODS

This study was prepared screening the National Thesis Center of the Council of Higher Education and electronic sources (ScienceDirect, PubMed, Google Scholar). The plants used for treating prostate diseases were searched in the ethnobotanical articles, MSc and PhD theses. Then, for each of these plants, activity studies to support the use of prostate diseases were investigated.

RESULTS

In the Table 1, information concerning the botanical and local names, used parts, usage, and chemical compositions of plants used for prostate diseases in traditional treatment in Turkey are given.

Table 1. The list of plants used in traditional treatment for prostate diseases in Turkey

Botanical name	Family	Local name	Chemical composition	Plant part used	Usage	References
Achillea biebersteinii Afan.	Asteraceae	Sarı civanperçemi	Essential oil (Mirahmadi et al. 2017), germacranolid derrivs, coumarin, monoterpene and flavonoid (Mohamed et al.2015)	Whole plant	Dec. Int.	Karakurt 2014
A. millefolium L.	Asteraceae	Sarı civanperçemi	Terpenoid, flavonoid (Shari Moghadem et al. 2017)	Whole plant	Dec. Int.	Karakurt 2014
A. wilhelmsii C. Koch	Asteraceae	Sarı civanperçemi	Essential oil (Baytop 1984, Güner et al. 2000), alkaloid, flavonoid, sesquiterpene (Güner et al. 2000)	Whole plant	Dec. Int.	Karakurt 2014
Acorus calamus L.	Acoraceae	Eğir kökü, Hazanbel	Carbohydrates, calcium and potassium salt (Baytop 1984)	Root	Inf. Int.	Akan and Bakır Sade 2015; Korkmaz and Karakurt 2014; Fakir et al. 2009
Agrimonia eupatoria L.	Rosaceae	Tırtıklı aslan pençesi	Catechical tannins (PDR 2007)	Whole plant	Inf. Int.	Tetik 2011
Agropyron repens L.	Poaceae	Ayrık otu	Mucilage, carbohydrates, calcium and potassium salt (Baytop 1984)	Whole plant	Inf. Int.	Karakurt 2014, Akan et al. 2015
Allium cepa L.	Liliaceae	Soğan	Alliin, essential oil, peptide, flavonoids (Çubukçu et al. 2002)	Leaf	Dec. Int.	Sağıroğlu et al. 2013
Alnus glutinosa (L.) *Gaertner subsp. glutinosa*	Betulaceae	Kızılağaç	Shikimic acid, epigallocatechin gallate (Altınyay et al. 2016)	Male flower	Dec. Int.	Ecevit Genç and Özhatay 2006
Alyssum sibiricum Willd.	Brassicaceae	Prostat çiçeği	-	Aerial part	Inf. Int.	Özdemir and Alpınar 2015
Anchusa undulata L.	Boraginaceae	Sığır dili, Ballıbaba	Triterpene glycosides, flavone glycosides (Koz et al. 2009)	Root	Dec./ Inf. Int.	Oral 2007
Anthemis cotula L.	Asteraceae	Beyaz papatya, Papatya	Essential oil, organic acids, glycosides and alkaloid (Baytop 1999)	Capitulum	Inf. Int.	Tuzlacı 2006
A. kotschyana Boiss. var. *kotschyana*	Asteraceae	Papatya	-	Aerial part	Inf. Int.	Özdemir and Alpınar 2015
Apium graveolens L.	Apiaceae	Kereviz	Essential oil, coumarins, flavonoids (Çubukçu et al. 2002)	Seed Whole plant	Dec. Int. +Honey, Int. Dec. Int.	Polat 2010 Akan and Bakır Sade 2015 Polat and Satıl 2012
Arbutus unedo L.	Ericaceae	Andrana, Kocayemiş, Dağyemişi	Sugars, tannins, vitamin (Baytop 1963; 1999), phenolic acids, aucubin glycosides, diterpenoid, triterpenoid (Baytop 1999, Evans 2002)	Fruit	Eaten	Kızılarslan and Özhatay 2012

Table 1. The list of plants used in traditional treatment for prostate diseases in Turkey (continued)

Botanical name	Family	Local name	Chemical composition	Plant part used	Usage	References
A. andrachne L.	Ericaceae	Dağ çileği, Sandal ağacı	Organic acids, sugars, phenolic components (Serçe et al. 2010)	Fruit	Eaten, Inf.	Polat et al. 2015, Güzel et al. 2015
Arum sp.	Araceae	Yılan burçağı	Alkaloids, essential oil (Azab 2017)	Fruit	Eaten	Şenkardeş and Tuzlacı 2014
Asparagus acutifolius L.	Liliaceae	Kediyen, Tilki kuyruğu	Steroid saponins (Gürdal 2010)	Whole plant Aerial part Flowering branches, leaf	Inf. Int. Dec. Int. Oinment, Inf.	Sargın et al. 2013 Güzel et al. 2015 Sargın et al. 2015
Brassica oleracea L. var *italica*	Brassicaceae	Brokoli	Sulforaphane, phenolic compounds, carotenoids, vitamin C and K (RX Media Pharma 2017)	Fruit	Raw, Eaten Dec. Int.	Eşen 2008 Metin 2009
Cannabis sativa L.	Cannabinaceae	Kenevir otu, Aptal otu	Resin, essential oil, oil (2008a)	Seed	Cooked, Int.	Birinci 2008
Carduus nutans L.	Asteraceae	Eşek çalısı, Eşek dikeni	Sterols, triterpenes, flavonoids (Abdallah et al. 1989)	Flowering branch	Dec. Int.	Tuzlacı et al. 2010
C. pycnocephalus L. subsp. *albidus* (Bieb.) Kazmi	Asteraceae	Eşek dikeni, Kangal	-	Flowering branch	Dec. Int.	Doğan 2014
Carthamus tinctorius L.	Asteraceae	Aspir	Pigments (Baytop 1984)	Flower	Not specified	Akan and Bakır Sade 2015
Cedrus libani A.Rich.	Pinaceae	Katran	Terpenic acids (Avcıbaşı et al. 1988)	Flower, Laef, Sprout	Not specified	Özçelik and Balabanlı 2005
Centaurea glastifolia L.	Asteraceae	Tahlişk	Guaianolides (Oksuz and Tupcu 1994)	Aerial part	Dried, Dec. Int.	Kaval et al. 2014
Cerasus mahalep (L.) Miller var. *mahalep*	Rosaceae	Mahlep	Oil, coumarin (Tetik 2011)	Seed	Inf. Int. Powder	Altundağ and Öztürk 2011 Akbulut and Bayramoğlu 2013
C. microcarpa (C. A. Meyer) Boiss. subsp. *tortuosa*	Rosaceae	Zerdali	-	Fruit	Eaten	Doğan 2014, Kaval et al. 2014
Ceratonia siliqua L.	Fabaceae	Harnup, Keçiboynuzu	Sugars, oil, pectin, tannin, vitamin, mineral, protein (Çubukçu et al. 2002, Baytop 1999)	Fruit	Molasses, Eaten	Gürdal and Kültür 2013
Ceterach officinarum DC.	Aspleniaceae	Altınotu	Essential oil, tannin, mucilage (Baytop 1999)	Aerial part	Inf./ Dec. Int.	Oral 2007
Cichorium intybus L.	Asteraceae	Kaniş	Coumarin glicoside, sesquiterpenes, caffeic acid, polysaccharides, tannin (PDR 2007, Çubukçu et al. 2002, Baytop 1999, Tyler et al. 1988, Tanker and Tanker 2003, Trease and Evans 2009)	Aerial part	Dec. Int.	Kaval et al. 2014
Cistus laurifolius L.	Cistaceae	-	Diterpene, glucosides (Sadhu et al. 2006, Joaquin et al. 1986), inositol (Joaquin et al. 1986)	Leafy branch	Dec. Int.	Tuzlacı 2006
Convolvulus arvensis L.	Convolvulaceae	Tarla sarmaşığı	Alkaloids, phenolic compounds, sugars, mucilage, sterols, resin, tannins, unsaturated sterols/ triterpenes, lactones, proteins (Al-Snafi 2016)	Leaf and flower	Inf. Int.	Fakir et al. 2009
Corylus avellana L.	Corylaceae	Fındık, Yabani fındık	Tannin, resin, protein, starch, oil, salt, vitamin, flavonoid (2008a)	Pericarp	Dec. Int.	Kültür 2007
C. maxima Miller	Corylaceae	Fındık	Oil, protein, sugars, phosphor, calcium (Baytop 1999)	Sprout	Dec. Int.	Tuzlacı 2006

Table 1. The list of plants used in traditional treatment for prostate diseases in Turkey (continued)

Botanical name	Family	Local name	Chemical composition	Plant part used	Usage	References
Cotinus coggyria Scop.	Anacardiaceae	Tetre, Tetere	Tannin, flavonoid (Baytop 1999)	Leaf	Inf. Int.	Tuzlacı and Alparslan 2007
Crataegus monogyna Jacq. subsp. *monogyna*	Rosaceae	Kırmızı alıç	Sugars, organic acids, phenolic compounds (Edwards et al. 2012)	Leaf anf flower	Inf. Int.	Fakir et al. 2009
Crataegus orientalis Pallas ex Bieb. var. *orientalis*	Rosaceae	Sarı alıç	Phenolic compounds Çalışkan 2015; Melikoğlu (et al. 1999)	Leaf anf flower	Inf. Int.	Fakir et al. 2009
Cucurbita maxima Duchesne	Cucurbitaceae	Kabak	Proteins, minerals, sugars, tocopherols, β-sitosterol, phenolic acids (Rezig et al. 2012)	Seed	Eaten	Güzel et al. 2015
C. pepo L.	Cucurbitaceae	Kabak	Steroids, Δ-7 stigmasterol, tocopherol, oil (Çubukçu et al. 2002)	Seed	Dec. Int.	Kahraman and Tatlı 2004
Cupressus sempervirens L.	Cupressaceae	Andız	Tannin, essential oil (Baytop 1999)	Leaf	Int.	Gürdal and Kültür 2013
Cynara scolymus L.	Asteraceae	Enginar	Caffeic acid derivatives, sesquiterpene lactone, flavonoid (Çubukçu et al. 2002)	Leaf	Dried, Dec. Int.	Sargın et al. 2013
Cynodon dactylon (L.) Pers.	Poaceae	Ayrık otu	Calcium and potassium salts, polysaccharides, saponin, essential oil (Baytop aerial 1963; 1999)	Root Root and part	Dec./ Inf. Int. Dec. Int.	Oral 2007, Bulut et al. 2017, Kartal and Güneş 2017 Güneş 2017
C. dactylon (L.) Pers. var. *villosus* Regel	Poaceae	Ayrıkotu	-	Rhizome	+Fruits of *Hordeum vulgare* and root of *Eryngium campestre* var. *virens* Dec., Int.	Şenkardeş 2014
Dracunculus vulgaris Schott	Araceae	Yılanbıçagı, Yılanburçagı	Fatty acids (Saglik et al. 2002)	Fruit	Eaten	Bulut et al. 2017
Elaeagnus angustifolia L.	Elaeagnaceae	İğde	Flavonoids, terpenoid compounds, saponins, sugars, phenolic acid, tannins, amino acids, carotenoids, vitamins (Hassanzadeh and Hassanpour 2018)	Fruit	Eaten	Güzel et al. 2015
Epilobium angustifolium L.	Onagraceae	Yakı otu, Çayır gülü	Provitamin A, karotenoid, steroids, barbituric acid derivatives, flavonoids (Sayık 2007)	Flower, leaf	+Water Int.	Akan and Bakır Sade 2015
Equisetum arvense L.	Equisetaceae	Ekli ot	Minerals, flavonoids, dicarboxylic acid (Çubukçu et al. 2002)	Whole plant	Dried, Dec. Int.	Kayabaşı 2011
E. giganteum L.	Equisetaceae	Kırkkilit otu, kavakotu	Phenolic compounds (Francescato et al. 2013)	Aerial part	Dec. Int.	Güneş 2017
E. telmateia Ehrh.	Equisetaceae	Çam otu, Kırkboğum	Alkaloids, silicic acid, saponin, tannin (Baytop 1999), flavone, sugars, mineral (Baytop and Gürkan 1972)	Aerial part	Dec. Int.	Ezer and Arısan 2006
Eryngium campestre var. *virens* (Link) Weins	Apiaceae	Boğa dikeni, Eşek dikeni, Kenger, Kuşkonmaz	Triterpene saponins, coumarins, monoterpene glycosides, caffeic acid esters, oligosaccharide, tannin (PDR 2007, Baytop 1999)	Root	Dec. Int. +Fruits of *Hordeum vulgare* and rhizome of *Cynodon dactylon* var. *villosus* Dec., Int.	Saglik et al. 2002

Table 1. The list of plants used in traditional treatment for prostate diseases in Turkey (continued)

Botanical name	Family	Local name	Chemical composition	Plant part used	Usage	References
Fraxinus angustifolia Vahl. subsp. *oxycarpa*	Oleaceae	Dışbudak	Secoiridoids (Çalış et al. 1996)	Flowering branch	Inf. Int.	Bulut 2008
Galium verum L.	Rubiaceae	Yoğurt otu	Hydroxycinnamic acid derivatives and glycosides, flavonoid, phenolic acid and glycosides (Jaiswal et al. 2014)	Leaf	Dec./ Inf. Int.	Oral 2007
Helichrysum armenium DC.	Asteraceae	Yayla çiçeği, Altınotu, Ölmez çiçek	Flavonoids (Çubukçu and Yüksel 1982)	Whole plant	Inf. Int.	Korkmaz and Karakurt 2014
Helichrysum plicatum subsp. *plicatum* DC.	Asteraceae	Yayla çiçeği, Altınotu, Ölmez çiçek	Flavonoids, saponin, catechical tannin (Keklik 1990), essential oil (Sezik and Aslan 1997)	Flower	Inf. Int.	Karakurt 2014
Hypericum perforatum L.	Hypericaceae	Kantaron, Seker otu	Flavonoids, phloroglucinol, catechic tannin, naftodiantron deriv (hypericin) (Bisset 1994), essential oil (Erken et al. 2001)	Aerial part Flowering branch	Dec. Int. Oleat, . Int	Tuzlacı and Alparslan 2007 Bulut et al. 2017
Juniperus oxycedrus L. subsp. *oxycedrus*	Cupressaceae	Ardıç	Essential oil, resin, bicyclic sesquiterpene, phenol deriv. (Baytop 1999, Tyler et al. 1988, Tanker and Tanker 2003, Trease and Evans 2009)	Cone	Inf. Int.	Tuzlacı and Alparslan 2007
Lallemantia iberica (M.Bieb.) Fisch et C.A.Mey.	Lamiaceae	-	Carbonhydrates, essential oil (Badawy et al. 2013), phenoilic glycoside (Dehaghi et al. 2012)	Whole plant	Inf. Int.	Doğan 2014
Lamium purpureum L.	Lamiaceae	Ballıbaba	Iridoid glycosides, essential oil (Flamini et al. 2005)	Aerial part	Inf. Int.	Uzun et al. 2004
Laurus nobilis L.	Lauraceae	Defne, Lüks	Starch, oil, tannin, essential oil (Baytop 1999), alkaloid, flavonoid (Topaloğlu 1987), sesquiterpenes (Glasby 1991)	Seed	Crushed, Inf. Int.	Sarıkan 2007
Lavandula stoechas L. subsp. *stoechas*	Lamiaceae	Karabaş otu, Karahan	Glycoside, saponin, essential oil (Baytop 1999)	Aerial part	Inf. Int.	Gürdal and Kültür 2013
Linum mucronatum Bertol. subsp. *mucronatum*	Linaceae	Sarı çiçek	Aryl-tetralin lignans (Koulman et al. 2005)	Aerial part	Inf. Int.	Doğan 2014
Malva neglecta Wallr.	Malvaceae	Ebegümeci	Mucilage, tannin, flavonoid (Bisset 1994)	Leafy branch	Dec. Int.	Koyuncu 2005
M. slyvestris L.	Malvaceae	Ebegümeci, Devetabanı, Katırtırnağı	Alkaloid (Topaloğlu 1987), anthocyanidins, flavonoids, tannin (Çubukçu et al. 2002), sugars, polysaccharides (Baytop 1999)	Leafy branch	Dec. Int.	Koyuncu 2005
Marrubium vulgare L.	Lamiaceae	Köpeksiyan otu	Diterpenes, essential oil, tannin (Çubukçu et al. 2002)	Aerial part	Dec. Int.	Tuzlacı 2006
Mentha spicata L. subsp. *spicata*	Lamiaceae	Yarpuz, Nane	Essential oil (Baytop 1999), flavonoids, caffeic acid (Brendler et al. 2003)	Leaf	Cooked or raw, Eaten	Koçak 1999
Micromeria cristata subsp. *orientalis* P.H. Davis	Lamiaceae	Kekik	-	Leaf, Flower	Inf. Int.	Karakurt 2014
Myrtus communis L.	Myrtaceae	Murt, mersin, yaban mersini	Essential oil, phenolic compounds, fatty acids (Mahboubi 2017)	Leaf, Fruit	Eaten, Fruit juice, Dec. Int.	Sargın 2015; Bulut and Tuzlaci 2013

Table 1. The list of plants used in traditional treatment for prostate diseases in Turkey (continued)

Botanical name	Family	Local name	Chemical composition	Plant part used	Usage	References
Nasturtium officinale R. Br.	Brassicaceae	Sukumbi	Sulfuric glycosides, vit A, C, D, essential oil (Baytop 1999)	Aerial part	Not specified	Saraç et al. 2013
Ononis spinosa subsp. *leiosperma* (Boiss.) Sirj.	Fabaceae	Sabankıran	Sugars, tannin, essential oil, flavonoids, minerals, organic acids, saponin (PDR 2007, Dermirezer 2011, Gürkan 2007)	Root	Dec. Int.	Doğan 2014
Ornithogalum umbellatum L.	Hyacinthaceae	Sakarca	Rhodexin A, rhodexoside (Smith and Paterson 1967), cardenolide glycosides, steroids (Tang et al. 2013, Şabudak and Oyman 2002), glcosides, saponin (Baytop 1999)	Whole plant	Inf.	Polat et al. 2015
Paliurus spina-christi Mill.	Rhamnaceae	Çaltı, muska otu	Flavonoids, tannins, amino acids, alkaloids, sterols (Brantner et al. 1996, Baytop 1999)	Seed	Dec. Frankincense	Sargın 2015
Peganum harmala L.	Zygophyllaceae	Üzerlik	Harmala alkaloids (Küçükbezirci 1967), musilage (Akalın and Alpınar 1994)	Sprout	Dec. Int. Not specified	Özgökçe and Özçelik 2004 Öztürk et al. 2013
Petroselinum crispum L.	Apiaceae	Maydanoz	Essential oil, flavonoid heteroside, furanocoumarins, phtalide, steroids, vit C, oil (Çubukçu et al. 2002, Baytop 1999, Tyler et al. 1988, Tanker and Tanker 2003)	Whole plant	Dec. Int., Eaten	Karakurt 2014
Phlomis russeliana Poiret	Lamiaceae	Laden	Essential oil, sesquiterpene (Demirci et al. 2008)	Leaf	Inf. Int.	Demirci and Özhatay 2012
Pinus nigra J.F.Arnold	Pinaceae	Selvi, Çam kozalağı	Essential oil (Sezik et al. 2010)	Cone	Dec. Int.	Akan and Bakır Sade 2015
Pistacia atlantica Desf.	Anacardiaceae	Çitlenbik ağacı, Menengeç	Essential oil (Trabelsi et al. 2012)	Leafy sprout	Inf. Int.	Tuzlacı 2006
P. terebinthus subsp. *paleaestina* (Boiss.) Engler	Anacardiaceae	Çitlenbik ağacı, Menengeç	Resin, tannin, essential oil, oil (Baytop 1999), flavonoids (Topaloğlu 1987)	Leafy sprout	Dec. Int.	Tuzlacı 2006
Plantago lanceolata L.	Plantaginaceae	Damar otu	Iridoid glycosides, flavonoids, coumarin, saponin, alkaloids, silicic acid, sugar, organic acids, mucilage, tannin (Çubukçu et al. 2002)	Leaf	Dec. Int.	Arslan 2005
P. *major* L. subsp. *major*	Plantaginaceae	Sinirli ot, Sinirotu, Yara otu	Polysaccharides, lipids, caffeic acid derivatives, flavonoids, iridoid glycosides, terpenoids, vitamins, organic acids (Samuelsen 2000)	Leaf	Inf. Int.	Güneş 2017
Platanus orientalis L.	Platanaceae	Çınar	Tannin, triterpenes, flavonoids (Bulut 2008)	Stem bark Leaf	Dec. Int. Inf. Int. Mash, Ext..	Koyuncu 2005 Güneş et al. 2017
Polygonum aviculare L.	Polygonaceae	Keçi memesi	Tannins, triterpenoids, anthraquinones, coumarins, phenylpropanoides, lignans, flavonoids (Granica et al. 2013)	Aerial part	Dec. Int.	Güzel et al. 2015
Polypodium vulgare L. subsp. *vulgare*	Polypodiaceae	Tatlı papra	Saponin, essential oil, tannin (Baytop 1999)	Aerial part	Inf. Int.	Kültür 2007
Prunus armeniaca L.	Rosaceae	Kayısı	Oil, amygdalin (Baytop 1999)	Leaf	Inf. Int.	Uzun et al. 2004

Table 1. The list of plants used in traditional treatment for prostate diseases in Turkey (continued)

Botanical name	Family	Local name	Chemical composition	Plant part used	Usage	References
Quercus cerris var. *austriaca* (Willd.) Loudon	Fagaceae	Palamut meşesi, meşe	Triterpenes (Sen et al. 2015)	Leaf	Dec. Int.	Kültür 2007
Rosa canina L.	Rosaceae	Köpek gülü, Kuşburnu	Phenolic compounds, minerals, vitamins, sugar, pectin, organic acid, essential oil, tannin (Demir and Özcan 2001)	Fruit	Dec., Inf. Int.	Güneş 2017
Rubus canescens DC.	Rosaceae	Böğürtlen	-	Root, Fruit Root	Dec. Int., Pickles, jam, molasses +*Urtica urens* roots + *Juglans regia* leaves	Sargın 2015 Polat and Satıl 2012
R. canescens var. *glabratus* (Godr.) Davis et Meikle	Rosaceae	Böğürtlen, Garantı	-	Root	+*Urtica* sp., leaf of *Juglans regia, Secale cereale,* Int.	Polat 2010
R. discolor Weihe and Nees	Rosaceae	Karamuk, Böğürtlen	Anthocyanins, ascorbic acid (Dujmović Purgar et al. 2012)	Root	Dec. Int.	Kültür 2007
R. hirtus Waldst. and Kit.	Rosaceae	Böğürtlen	Flavonoids, triterpenes, gallic tannin (Mercan 2006)	Root	Dec. Int.	Tuzlacı 2006, Kültür 2007
R. sanctus Schreber	Rosaceae	Böğürtlen, Garantı	Tannin, organic acid, sugar (Baytop 1999)	Root Leaf and Flower Root, Fruit	Inf. Int. +*Urtica* sp., *Juglans regia*, *Secale cereale*, Int. +*Urtica urens* roots+ *Juglans regia* leaves Dec. Int. Dec. Int., Pickles, jam, molasses	Tetik 2011, Tetik et al. 2013 Polat 2010 Polat and Satıl 2012 Güzel et al. 2015 Sargın 2015
R. saxatilis L.	Rosaceae	Böğürtlen	Sugars, pectins, anthocyanins, catechins, vitamin C, phenolic acids, flavonoids, tannins, fatty acids (Tomczyk and Gudej 2005)	Root, Fruit	Dec. Int., Pickles, jam, molasses	Sargın 2015
Sambucus nigra L.	Caprifoilaceae	Mürver, Milver, Patlanguç, Özübüyük	Caffeic acid, mucilage, potassium salts, glycosides, steroids, tannin, triterpenes, essential oil (Çubukçu et al. 2002)	Fruit	Eaten Dec. Int. Mac. Int.	Kültür 2007 Fujita et al. 1995 Sargın et al. 2013
Satureja cuneifolia Ten.	Lamiaceae	Kekik, Keklik otu, Dağ kekiği	Essential oil, terpenes (Oke et al. 2009)	Aerial part	Dec. Int.	Kartal and Güneş 2017; Güneş 2017
Sorbus aucuparia L.	Rosaceae	Üvez	Anthocyanins (Isakina et al. 2015)	Leaf	Dec. Int.	Kültür 2007

Table 1. The list of plants used in traditional treatment for prostate diseases in Turkey (continued)

Botanical name	Family	Local name	Chemical composition	Plant part used	Usage	References
S. domestica L.	Rosaceae	Üvez, Börtlücan	Tannin, essential oil, sorbitol, organic acids, pigments (Baytop 1999)	Leaf	Dec. Int.	Kültür 2007
Telephium imperati subsp. *lorientale* (Boiss.) Nyman	Caryophyllaceae	Sidik zoru otu, Siğil otu	-	Aerial part	Inf. Int.	Tuzlacı and Erol 1999
Thymus sibthorpii Bentham	Lamiaceae	Kekik	Phenolic acids, flavone glycosides (Raudone et al. 2017)	Aerial part	Inf. Int.	Ecevit Genç and Özhatay 2006
T. sipyleus subsp. *sipyleus* Boiss.	Lamiaceae	Kekik	Essential oil, sesquiterpenes, monoterpenes (Tanker and Tanker 2003, Tepe et al. 2005)	Leaf and flower	Inf. Int.	Karakurt 2014
Tribulus terrestris L.	Zygophyllaceae	Demir dikeni	Saponin, amid, alkaloid (Koçyiğit 2005)	Aerial part	Dec. Int.	Doğan 2014; Güzel et al. 2015
Urtica dioica L.	Urticaceae	Isırgan	Ca, K, silicic acid salts, organic acids, biogen amids, sitosterols, lectin, terpenes, triterpenes, flavonoids, lignans, essential oil, coumarins, Vit C (PDR 2007, Çubukçu et al. 2002, Baytop 1999, Trease and Evans 2009, Demirezer 2011, Gürkan 2007)	Aerial part Leaf	Inf. Int. +*Petroselinum crispum* Dec. Int. +Olive oil, impasted, eaten Dec. Int.	Bulut et al. 2017 Karakurt 2014 Yeşilyurt et al. 2017; Korkmaz and Alpaslan 2014
U. urens L.	Urticaceae	Dırık, Isırgan ve Pırıke	Phenolic compounds (Carvalho et al. 2017), vitamins, essential oil (Mzid et al. 2018)	Leaf	Dec. Int.	Korkmaz and Alpaslan 2014
Verbascum cheiranthifolium var. *cheiranthifolium* Boiss.	Scrophulariaceae	Sığır kuyruğu	Saponin, mucilage, resin, bitter compound (Baytop 1999, Keskin 2011)	Leaf	Inf. Int.	Keskin 2011
V. pycnostachyum Boiss. and Heldr	Scrophulariaceae	Sığır kuyruğu	Iridoid glycosides, phenylethanoid glycosides (Tatlı et al. 2007)	Leaf and flower	Dec. Int.	Oral 2007
Viburnum lantana L.	Adoxaceae	Girebolu	Glycosides (Calis et al. 1995)	Fruit	Juice, Int.	Karakurt 2014
V. opulus L.	Adoxaceae	Gilaburu, Girebolu	Organic acids, phenolic compounds, vitamins, terpenoids (Sarıözkan et al. 2017)	Meyve	Juice, Int.	Korkmaz and Karakurt 2014
Viola odorata L.	Violaceae	Tırtılsız aslan pençesi, Kokulu menekşe	Alkaloid, glycoside, saponin, methyl silicate, gum (Demiray 2013), β-ionone (Ansari and Emami 2016)	Flower and leaf	Inf. Int.	Tetik 2011
Viscum album L subsp. *austriacum* (Wiesb.) Vollman	Loranthaceae	Burç, Ahlat purçu, Çam burcu, Ökse otu	Alkaloids, glycosides, lectines, polypeptides, triterpenes, phenolic acids, organic acids, biogen amin, mucilage, lignans, resin, tannin (PDR 2007, Çubukçu et al. 2002, Baytop 1999, Tyler et al. 1988, Tanker andTanker 2003, Demirezer 2011, Gürkan 2007, Ergün and Deliorman 1995)	Leaf Aerial part, branches, leaf	Dec. Int. Dec., Mac., Int.	Sargın et al. 2013 Sargın et al. 2015
Zea mays L.	Poaceae	Mısır	Bitter compounds, amins, phytosterol, starch, pigments, resin, saponin, vitamin C,K (Barnes et al. 2002), potassium salt, oil, flavonoids, mucilage, sugar, tannin, essential oil (Çubukçu et al. 2002), maysin (Lee et al. 2014)	Corncob	Dried, Dec. Int. Dec., Inf. Int.	Tuzlacı 2006; Güneş and Özhatay 2011 Tetik 2011

Dec: Decoction; Inf: Infusion; Mac: Maceration; Int: Internal

CONCLUSION

In this study, 107 taxa which have traditionally been used in the treatment of prostate diseases in Turkey have been recorded. These plants are predominantly from the Rosaceae (15 taxa), Asteraceae (13 taxa), Lamiaceae (10 taxa), Poaceae (4 taxa), Anacardiaceae (3 taxa), Apiaceae (3 taxa), Brassicaceae (3 taxa) families.

Commonly used taxa in different regions of Turkey are *Apium graveolens, Asparagus acutifolius, Cynodon dactylon*, *Rubus sanctus*, *Sambucus nigra* and *Urtica dioica*. The parts of the plants that are used for treatment are the leaves, aerial parts, flowers, roots and seeds. Flavonoids, essential oils, tannins, saponins, alkaloids, steroids, sugars, and minerals are the most common chemical compounds in plants.

Activity studies have been found which support the use of 17 taxa in prostate diseases. These plants are; *Achillea wilhelmsii, Acorus calamus*, *Allium cepa.*, *Brassica oleraceae* var. *italica*, *Cannabis sativa*, *Carthamus tinctorius*, *Cucurbita pepo*, *Epilobium angustifolium*, *Hypericum perforatum*, *Mentha spicata* subsp. *spicata, Nasturtium officinale*, *Prunus armeniaca*, *Tribulus terrestris*, *Urtica dioica, Viburnum opulus*, *Viola odorata* and *Zea mays*. The scientific studies on these plants are as follows:

Volatile oil of *Achillea wilhelmsii* has a cytotoxic effect against prostate cancer cells. No cytotoxic effect for normal cells (Guatam et al. 2014).

The ethanolic extract of the *Acorus calamus* root was found to suppress cell proliferation and angiogenesis and stimules early apoptosis in LNCaP (prostate left supraclavicular lymph node carcinoma) prostate cancer cell lines, depending on dose and time (Koca et al. 2018).

Consumption of onion (*Allium cepa*) and garlic slows down the formation of BPH (Galeone et al. 2007).

It has been stated that Sulforaphane (one compound of *Brassica oleracea* L. var *italica*) reduces prostate cancer formation by induces Phase 2 Enzymes in human prostate cells (Brooks et al. 2001).

It has been identified that cannabinoid (one compound of *Cannabis sativa*) receptors are more abundant in cells with prostate cancer than normal prostate cells, especially in men with bone metastatic prostate cancer, it is thought that cannabinoids are effective in improving analgesia of bone pain and improving the quality of life of the patient (Ramos and Bianco 2012).

It has been determined that the ethanolic extract of *Carthamus tinctorius* is an effective 5α-reductase inhibitor and hair growth promoter (Kumar et al. 2012). 5α-reductase is an important enzyme in the conversion of testosterone (T) to the more potent androgen dihydrotestosterone (DHT) in androgens metabolism. Dihydrotestosterone is effective in the growth of the prostate gland. It is thought that *Carthamus tinctorius* may be used as a 5α-reductase inhibitor to stop the growth of the prostate gland.

β-Sitosterol in *Cucurbita pepo* oil has been shown to be a potent inhibitor of prostaglandin biosynthesis in prostate tissue of patients with BPH (Nakic et al. 2006). It is thought that *Cucurbita pepo* may be used in the treatment of inflammation and complaints depending on prostaglandin as a consequence of inhibites prostaglandin biosynthesis. A multicenter clinical trial was conducted with 2245 BPH patients. Urinary symptoms were recorded using the International Prostate Symptom Score (I-PSS) according to the American Urological Association and the effect on quality of life was also recorded using the Life Quality questionnaire (LQ Index). The patient was treated with capsules containing 500 mg pumpkin seed extract. I-PSS decreased by 41.4% during treatment and the quality of life score increased by 46.1% at the end of treatment. More than 96% of the patients reported no side effects in this treatment (Younis et al. 2000).

Oenothein A and oenothein B, which are macrocyclic elagitannins of *Epilobium angustifolium*, inhibit the activity of 5-alpha-reductase and aromatase enzymes, which play a key role in the formation of benign prostate growth (BPH), and so prevent benign prostate growth (Şığva 2012).

In another study, it was reported that photodynamic therapy with hypericin (one compound of *Hypericum perforatum*) is an alternative approach to the treatment of prostate tumors and may be useful in tumors that do not respond to androgen therapy (Colasanti et al. 2000).

In the experiment on male rats, the anti-androgenic effect of *Mentha spicata* on testis was determined (Kumar et al. 2008). It is thought that *Mentha spicata* subsp. *spicata* can be used in the treatment of prostate diseases due to its anti-androgen effect.

Nasturtium officinale contains significant amounts of β-Phenethyl isothiocyanate (PEITC). It has been shown that at the progression phase of carcinogenesis in cell lines of prostate, leukemia, colon, lung and liver cancer, PEITC can inhibit proliferation and induces apoptosis (Pappa et al. 2006).

It was observed that the *Prunus* shell in different species was significantly inhibitory for testosterone-induced BPH. The most effective species were identified as *P. domestica*, *P. persica*, *P. amygdalus*, P. *cerasoides* and *P. armeniaca* (Jena et al. 2016).

The in vivo study of *Tribulus terrestris* extract and *Cornus officinalis* extract showed a marked enhancement of ICP and cAMP. Accordingly, it has been determined that a mixture of *T. terrestris* extract and *C. officinalis* extract may enhance erectile function (Kam et al. 2012). Reduction of erectile dysfunction, one of the symptoms of BHP in the treatment of prostate diseases, is thought to improve patient quality of life.

Urtica dioica extract (UR102) inhibits 5 α-reductase enzyme activity depending on concentration. UR102 can affect enzyme activity only at high concentrations (≥ 12 mg/mL) and it was calculated to ED50 of 14.7 mg/mL (Hartmann et al. 1996). *Urtica dioica* is thought to be potentially useful in the treatment of prostate diseases by inhibiting 5-α-reductase enzyme.

Gilaburu (*Viburnum opulus*, Glb) fruit extract is effective on testis and sperm damage induced by docetaxel (DTX) and paclitaxel (PTX) chemotherapeutics in rats. According to the study, DTX and PTX caused significant decreases in absolute and relative weights of all reproductive organs, testosterone level, sperm motility, concentration, Bcl-2 anti-apoptotic immunopositive cell scores of testes and spermatozoa as well as catalase activity in epididymal tissue, superoxide dismutase and glutathione peroxidase activities of testicular and epididymal tissues when compared with the control group. However, Glb consumption mitigated the PTX-induced decreases in the absolute weights of the epididimis, seminal vesicles, ventral prostate and both taxanes-induced disturbances in sperm characteristics, imbalances in oxidant/antioxidant system, increments in germ cell apoptosis and testicular histo-and cyto-pathological damage. It was concluded that long-term Glb consumption alleviates the taxanes induced damage in the reproductive system of male rats (Sarıözkan et al. 2017).

Viola odorata is known to contain β-ionone (Ansari and Emami 2016). In studies on the cytotoxic activity of β-ionone, it has been shown to inhibit the proliferation of cancer cells in a concentration-dependent manner on β-ionone on DU145, LNCaP (human prostate carcinoma cells), and PC-3 (prostate adenocarcinoma cells) (Jones et al. 2013).

It was identified that maysin, an important flavonoid in the maize tassel (*Zea mays*), inhibits the growth of PC-3 (androgen-independent human prostate cancer cell) by induction of mitochondrial-dependent apoptotic cell death and it has a strong therapeutic potential in the treatment of chemotherapy-resistant or androgen-independent human prostate cancer (Lee et al. 2014). Purple corn is a pigmented variety of *Zea mays* grown for hundreds of years in the Andean region of South America (Paucar-Menacho et al. 2017). It has been determined that purple corn is effective in the inhibition of prostate carcinogenesis in animal studies. Three main anthocyanins (cyanidin-3-glucoside, pelargonidin-3-glucoside and peonidin-3-glucoside) of purple corn, have been tested with LNCaP (Prostate left supraclavicular lymph node carcinoma) cells. The results showed that cyanidin-3-glucoside and pelargonidin-3-glucoside were the active compounds (Long et al. 2013).

Similar activity studies can be conducted for other plants and so new drugs can be developed for the treatment of prostate diseases.

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