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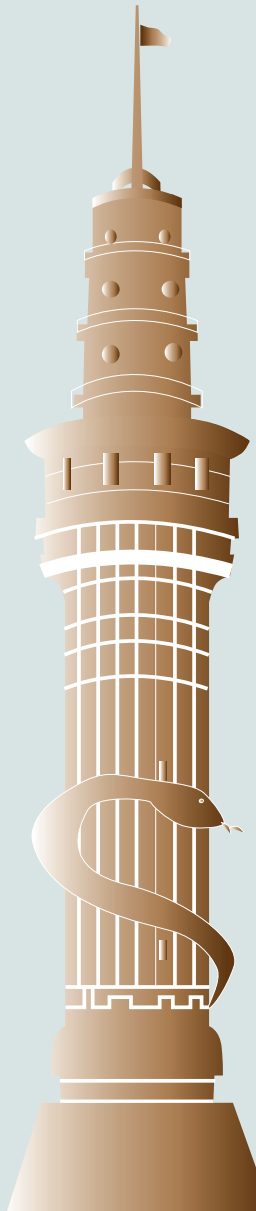

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Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations 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.

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

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

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

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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.

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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.

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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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CONTENTS

ORIGINAL ARTICLES

REVIEWS

Istanbul J Pharm 49 (2): 45-52
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Original Article

Evaluation of biological activity of *Diplotaenia cachrydifolia* Boiss. that medicinal plant

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ABSTRACT

The aim of this study is to evaluate the antimicrobial, antioxidant, and antigenotoxic activity of extracts obtained from *Diplotaenia cachrydifolia* Boiss, which is traditionally used for food and medical treatment by the local people of Hakkâri Province (Turkey). As a result of this study, the extracts of *D. cachrydifolia* exhibited a broad antimicrobial spectrum and high antimicrobial activity. It was also observed that the extracts had a high antioxidant activity. The DPPH IC_{50} values of the extracts were 2.5234 µg/mL, 6.0075 µg/mL, and 29.1974 µg/mL for ethanol, acetone, and hexane, respectively. The highest phenol content was found to be 55.36±0.035 µg/mL for ethanol. Furthermore, the ethanol extract was found to have a protective effect against mitomycin C-induced genotoxicity. *D. cachrydifolia* has a high biological activity; therefore, it is anticipated that future pharmacological studies will shed more light on this plant.

Keywords: Biological activity, genotoxicity, *Diplotaenia cachrydifolia*, DPPH, phenolic component

INTRODUCTION

In a modern context, at least 25% of the active ingredients of pharmacologically produced medicines are derived from plants. In addition, the active ingredients of many synthetically produced medicines are similar to chemicals that were first isolated from medicinal plants (Berber et al. 2013). The active ingredients in medicinal plants have been shown to exhibit high biological capability (Bhouri et al. 2010; Kilani-Jaziri et al. 2011) and these plants seem to be the best resources for the discovery of novel bioactive chemicals in the design of new drugs. Recently, there has been considerable interest in the antimicrobial (Rates 2001), antioxidative (Kilani-Jaziri et al. 2011) and antigenotoxic activities (Amkiss et al. 2013) of medicinal plants.

At present, alternative antimicrobial medicines are needed, in particular, for the treatment of infectious diseases. Mounting resistance of microorganisms to many antibiotics has led to increasing difficulties in the treatment of infectious diseases. In this context, local medicinal plants are known to be rich in antimicrobial properties and thus, a source of novel antimicrobials (Rates 2001; Kilani-Jaziri et al. 2011). Furthermore, some medicinal plants are reported to exhibit high antioxidant activity (Kilani-Jaziri et al. 2011), and plant phenolics are generally the source of these natural antioxidant properties (Atoui et al. 2005; Cavlak and Yagmur 2016). Antioxidants prevent the formation of free radicals produced routinely in all cells as part of their normal function; however, free radical accumulation in cells may play a role in many diseases (Young and Woodside 2001). Recently, there has been a growing interest in natural antioxidants of plant origin. Natural antioxidants obtained from medicinal plants can prevent the formation of free radicals, thus indicating their potential as novel therapeutic agents (Nogochi and Nikki 2000; Chaabane et al. 2012). Of late, considerable research has been focused on the antigenotoxic activity of medicinal

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plants (Amkiss et al. 2013). With the biosphere progressively accumulating chemical compounds that possess mutagenic properties (Karamova et al. 2011) and the increase in health problems associated with human exposure to environmental mutagens, the importance of antigenotoxicity has been highlighted (Demir et al. 2013). Moreover, recent studies seem to indicate that medicinal plants hold promise for the development of antigenotoxic agents.

Medicinal plants seem to be the best resources for the discovery of new drugs and so novel medicinal plants are needed for the control of different diseases. In this context, the main aim of this work was to reveal the biological importance of *D. cachrydifolia* Boiss. which is known as a medicinal plant. This plant, known locally as "siyabu", flourishes at high altitudes and grows wild in Hakkâri Province (Turkey). *D. cachrydifolia* has been used by the local inhabitants for medicine as treating diabetes, rheumatism and scorpion stings and food for many years and is considered to be one of the most important plant species (Kaval et al. 2014; Uce and Tuncturk 2014). Kaval et al. (2014) were the first to report the medicinal uses of *D. cachrydifolia* Boiss. The root and aerial parts of the plant are used for medical applications among local people (Agaoglu et al. 2005; Uce and Tuncturk 2014) and according to them, the plant has properties as an anti-snake venom properties. The parts growing above the ground are used for food, while the roots are used for medicine. Locals have reported that, in addition to snakebites, *D. cachrydifolia* is used traditionally for treating diabetes, rheumatism and scorpion stings (Agaoglu et al. 2005; Mükemre et al. 2015). Consequently, in this study, the use of this plant was evaluated from different pharmacological perspectives. There is also the possibility that this plant will gain value for use in different pharmacological areas.

MATERIALS AND METHODS

Plants Used in the Study

In previous studies, the potential medical properties of *Diplotaenia cachrydifolia* Boiss were identified through its use among the population (Kaval et al. 2014; Uce and Tuncturk 2014). In April-May 2016, it was purchased from the local public markets in Yüksekova, Hakkâri. The plant materials were identified by S. Semra Candar Erol, in the Traditional and Complementary Medicine Research Center, Düzce University. The freshly obtained *D. cachrydifolia* samples were minced and dried in a drying oven with the temperature kept under 40°C and then pulverized in a grinder. The powdered plants were then mixed at a 1:10 ratio with hexane, ethanol and acetone solvents, consecutively and extruded at 150-170 rpm for 24 h at room temperature. After each extraction, filtration was carried out and the remaining plant residue was dried for use in the next solvent. The obtained extracts were evaporated under vacuum at 80-150 rpm at about 30°C. The quantities of the extracts obtained after evaporation were determined and dissolved in dimethyl sulfoxide (DMSO, Merck). DMSO is commonly used to prepare stock solutions of samples in a solvent in which is highly soluble because it is a polar and aprotic solvent. These samples were stored at +4°C for further use in subsequent stages of this research (Baravalia et al. 2009).

Percentage of extract yields

The yield of the extracts was calculated using the following % yield formula:

$$\% \, Yield = [(w_2 - w_1) / w_0] \times 100$$

w_0 = the initial weight of the dried plant sample, w_1 = the weight of the cabinet and w_2 = the weight of the remaining extract after evaporation (Anokwuru et al. 2011).

Microorganisms used in the study

The microorganisms used in the antimicrobial activity test studies were provided by Düzce University Faculty of Agriculture and Natural Sciences, Field Crops Department Microbial Biotechnology Laboratory and the Faculty of Medicine Microbiology Laboratory, and included: *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Yersinia pseudotuberculosis* ATCC 911, *Proteus vulgaris* ATCC 13315, *Salmonella typhimurium* ATCC 14028, *Enterococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 35218. *Listeria monocytogenes* ATCC 7644 and *Candida albicans* ATCC 90028. The microorganisms used in the study were grown at 37°C for 16-18 h using Mueller Hinton broth (MHB) or Mueller Hinton agar (MHA) media as described for use in antimicrobial activity tests (Sen and Batra 2012).

Antimicrobial activity test

The antimicrobial activity of the plant extracts was determined using the agar well diffusion method (Chung et al. 1990). Initially, test microorganisms were inoculated into the MHB medium and incubated for 16-18 h at 37°C in a shaking water bath. At the end of the incubation period, the absorbance of the microorganism cultures was measured in a spectrophotometer (Mapada, UV3100PC) at a wavelength of 600 nm. The cultures were then diluted with sterile dH_2O to about 1×10^7-1×10^8 CFU/mL. The 100 µL diluted microorganism samples were spread onto plate containing the MHA growth medium and then wells were opened on these plates. And finally, 100 mL of the hexane, ethanol and acetone extracts were added to each well. An antibiotic (Streptomycin, 10 µg/disc) was used as a positive control and dimethyl sulfoxide (DMSO) was used as a negative control (Arullappan et al. 2009). DMSO, which is not toxic for microorganisms, was used for preparation of test samples (Sokmen et al. 2013).

DPPH radical scavenging activity

The radical scavenging activity assay was performed using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical (Brand et al. 1995; Ertürk et al. 2014). For the study, the methanol solution of the DPPH radical was freshly prepared, and 1500 µL of the DPPH solution was added to 750 µL of plant extracts prepared at concentrations of 20-100 µg/mL. Absorbance values were measured after 30 minutes at 517 nm in the spectrophotometer. Butylated hydroxyanisole (BHA, Aldrich) was used as the positive control (standard). As the negative control, a mixture of 1500 µL of DPPH and 750 µL of methanol was used, while methanol was used as the blank. The process was repeated in duplicate. The DPPH radical scavenging activity was calculated using the following formula:

DPPH Radical Scavenging Activity (%) = $[(A_0-A_1) / A_0] \times 100$

A_0 = the negative control absorbance value and A_1 = the standard and sample absorbance value.

Total phenolic component analysis

The Folin-Ciocalteu reagent was used to determine the total phenolic component content of the extracts. The amounts of phenolic compounds corresponding to the gallic acid standard of the extracts were determined in the study (Döger 2010). The work was performed in triplicate at two different times.

Allium cepa assay and treatments

Healthy *A. cepa L.* (2 n=16), approximately equal in size, were used as the test material in the study. The outer dry skin and the old roots were carefully removed from the onions, then washed, dried and kept refrigerated at 4°C until the study began. Five onions were used for each concentration, including a negative and positive concentration. Three types of treatment groups were created: pre-, post- and simultaneous. In the pre-treatment group, the roots were first treated with *D. cachrydifolia* ethanol extracts at different concentrations (10, 100, 1000 µg/mL) and after 24 h were treated with Mitomycin C (MMC) at 5×10^{-5} M concentration. In the post-treatment group, the roots were first treated with MMC at a concentration of 5×10^{-5} M for 24 h, and then with the plant extracts at different concentrations (10, 100, 1000 µg/mL) for 24 h. In the simultaneous treatment group, MMC at a concentration of 5×10^{-5} M was added to each concentration of the extract. In this study, MMC at a concentration of 5×10^{-5} M was used as the positive control and distilled water as the negative control.

Allium cepa chromosomal aberration assay

With all treatments, root tips were fixed in Carnoy's solution (3 acetic acid:1 methanol) for 24 h, placed into 70% alcohol and stored at 4°C. To prepare the formulations, the root tips were passed through distilled water, and then hydrolyzed with 1 N HCl for 15 min at 60°C. The root tips were stained with aceto-orcein for 20 min and crushed samples were prepared (Darlington and La Cour 1979). At least 1000 cells per well were examined for each concentration and for the controls. To determine the mitotic index, 3000 cells were counted, and the ratio of mitotic cells per 100 was calculated.

The percent of inhibition was calculated according to the formula:

PI = a-b / a-c * 100

where a is the number of aberrant cells caused by the positive control, b is the number of aberrant cells caused by the ethanol extract of *D. cachrydifolia*, and **c** is the number of aberrant cells caused by the negative control (Kumari et al. 2013). The linear relationship between the effects and the doses of the *D. cachrydifolia* via Pearson regression and correlation analyses is shown in Figure 1.

Statistical Analysis

The data obtained as a result of genotoxicity and cytotoxicity studies were analyzed by using The Statistical Package for the Social Sciences (SPSS) 20 for Windows (IBM Corp.; Armonk, NY, USA) and results obtained were expressed as mean±standard error (SE) The Kruskal-Wallis test was carried out followed by the Mann-Whitney U test to compare the statistical significance of the differences between the treated and control groups. The dose response relationship was determined using Pearson correlation analysis. A value of $p<0.05$ was considered significant. The value for all statistics was $p<0.05$ and for Mann Whitney U test, Bonferroni correction was selected as alpha/number of comparisons = 0.05/10 = 0.005. The dose response relationship was determined using Pearson correlation analysis.

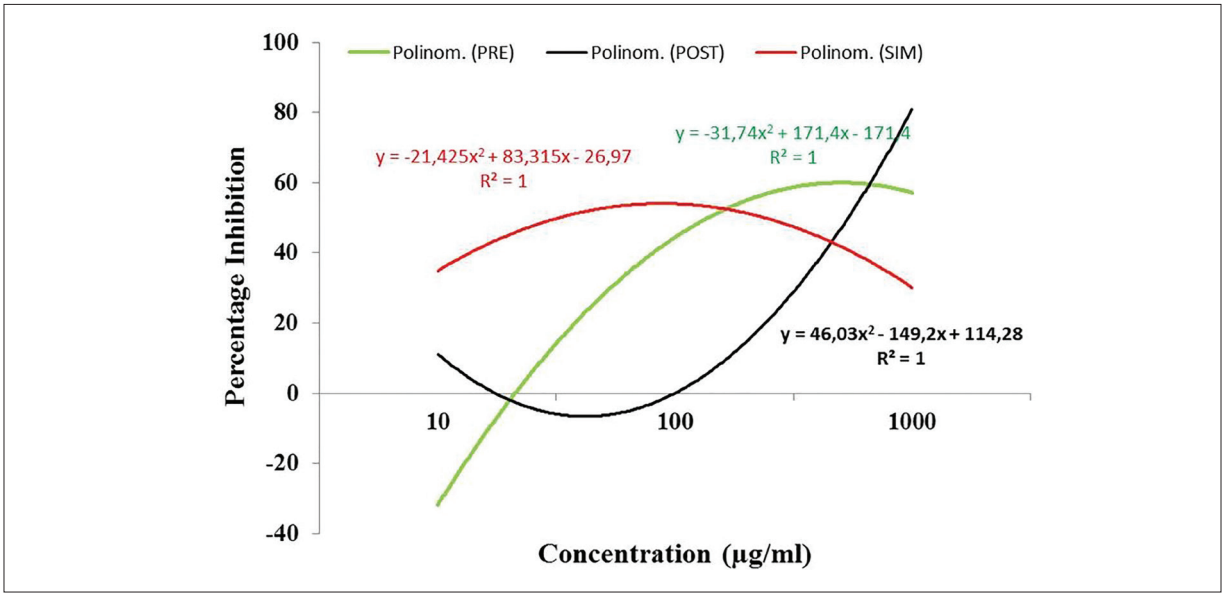

Figure 1. Percentage of inhibition of the genotoxic effect induced by MMC in the A. cepa chromosomal aberration method and the relationship between different concentrations of *D. cachrydifolia* ethanol extracts.

RESULTS

The percentage yields of the extracts obtained as a result of the study, which are given in Table 1, show the highest yield was achieved with the ethanol extract. Total phenolic component contents of the plant extracts are shown in Table 1 as the gallic acid equivalent (GAE, µg/mL). The total phenolic component content was found to be highest in the ethanol extract of the plant.

The zones of antimicrobial activity of the extracts against the test microorganisms are shown in Table 2 and Figure 2. The plant ethanol extract showed high antimicrobial activity against all test microorganisms used in the study. The acetone extract showed a high activity against all microorganisms except for *P. vulgaris*. The antimicrobial activity against *P. vulgaris* was relatively low. When the plant extracts were evaluated, high antimicrobial activity was observed against *K. pneumoniae, L. monocytogenes, S. typhimurium, Y. pseudotuberculosis, P. aeruginosa, E. cloacae, S. epidermidis* and *C. albicans*.

The DPPH radical scavenging activity of the extracts is shown as inhibition % in Figure 3. The sequence of the control and

Table 1. Percentage yield and total phenolics content of extracts

Extracts	Yield (%)	Total Phenolic Content *
Hexane	2.1205	43.59±0.657
Ethanol	3.5972	55.36±0.035
Acetone	0.3253	16.29±0.006

* µg gallic acid equivalent

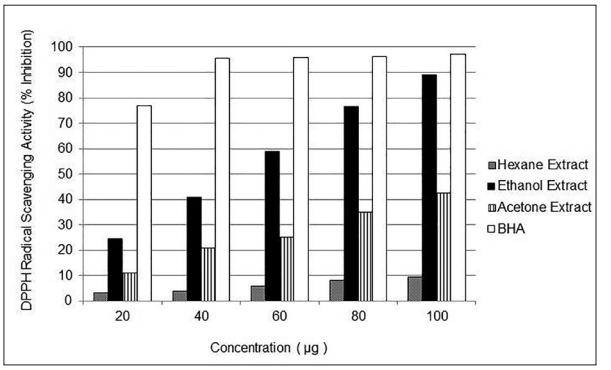

Figure 3. DPPH radical scavenging activity.

Table 2. Antimicrobial activity test

	Extracts / Inhibition zones*				
Microorganisms	Hexane	Ethanol	Acetone	S	DMSO
S. typhimurium	21.0±2.83	22.5±3.54	24.5±2.12	22.0±2.83	-
K. pneumoniae	22.0±2.83	32.0±2.83	27.0±7.07	22.5±6.36	-
E. coli	05.0±7.07	22.5±4.95	17.0±8.49	14.0±0.41	-
P. vulgaris	15.5±0.71	19.5±0.71	15.5±2.12	-	-
L. monocytogenes	19.0±4.24	22.0±2.83	20.0±4.24	-	-
E. faecalis	09.5±13.4	23.5±0.71	20.0±2.83	-	-
Y. pseudotuberculosis	17.5±7.78	24.0±5.66	18.5±6.36	21.5±7.78	-
P. aeruginosa	17.0±7.07	22.5±2.12	17.5±7.78	16.0±5.66	-
S. epidermidis	19.0±5.66	20.5±6.36	21.5±6.36	06.0±1.41	-
S. aureus	16.0±1.41	23.5±4.95	19.0±2.83	12.5±2.12	-
E. cloaceae	18.5±7.78	23.5±3.54	23.5±3.54	-	-
B. subtilis	16.0±2.83	18.5±0.71	18.5±3.54	19.0±0.00	-
C. albicans	26.5±4.95	23.0±1.41	23.5±0.71	-	-

*: millimeter values; S: Streptomycin (positive control); DMSO: Dimethyl sulfoxide (negative control)

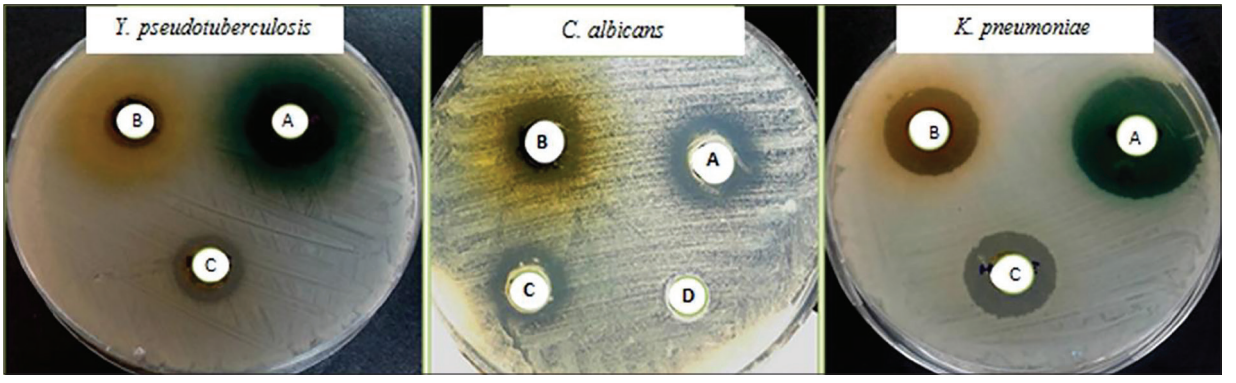

Figure 2. a-d. The inhibition activity of extracts from *D. cachrydifolia* against the microorganisms (a), and different samples (b, c and d).

Table 3. Cytological effects of *D. cachrydifolia* ethanol extract on *A. cepa* root cells

Groups	Types of abnormalities							MI (%)
	Irregular Metaphase	Stickiness	C-Mitosis	Polar deviations	Micronucleus	Lagging chromosomes	Bridges	Total aberrant cells	
Negative control	1.00±0.00	1.00±0.00**	0.50±0.50	0.00±0.00	0.00±0.00**	0.00±0.00	0.00±0.00***	2.50±0.50***	2.93**
Positive control	8.66±0.66	9.00±2.00	1.00±0.00	1.00±0.57	2.33±0.33	1.67±0.33	1.66±0.33	25.33±1.45	1.08
PRE10	12.50±1.32	7.75±0.75	1.50±0.28	0.25±0.25	0.00±0.00**	0.00±0.00	0.00±0.00***	22.00±1.91	1.11
PRE100	3.80±1.62	2.40±1.02**	0.80±0.48	0.20±0.20	0.60±0.24	0.20±0.20	0.00±0.00***	8.00±2.07***	2.27**
PRE1000	5.66±0.88	2.66±0.33	0.00±0.00	0.33±0.33	1.00±1.00	0.00±0.00	1.50±0.50	10.66±0.33	2.09
POST10	12.00±3.46	5.66±0.33	0.66±0.33	0.00±0.00	1.00±0.57	1.00±0.57	0.00±0.00***	20.33±3.75	1.54
POST100	5.25±2.86	4.25±1.84	6.00±2.67	0.00±0.00	0.50±0.50	1.00±0.70	0.00±0.00***	17.00±4.20	1.77
POST1000	2.66±0.66	1.33±0.66**	1.00±0.57	0.00±0.00	0.33±0.33	0.33±0.33	0.00±0.00***	5.66±0.88***	2.79**
SIM10	4.00±0.94	4.60±1.20	0.60±0.24	0.00±0.00	0.00±0.00**	0.00±0.00	0.00±0.00***	9.20±1.93***	2.06*
SIM100	4.33±0.88	4.66±1.45	0.33±0.33	0.00±0.00	0.33±0.33	1.33±0.88	0.33±0.33***	11.33±2.60	1.85
SIM1000	5.60±1.63	3.80±0.96	0.20±0.20	0.00±0.00	0.00±0.00**	0.00±0.00	0.20±0.20***	9.80±2.59***	2.31*

*p≤0.05; **p≤0.01; ***p≤0.001 when compared to positive control

the extract activity was found as: BHA > ethanol extract > acetone extract > hexane extract. According to the inhibition % data, the DPPH radical scavenging activity closest to the standard BHA was observed in the ethanol extract of the plant. The IC_{50} values were calculated with linear regression analysis as 29.1974 µg/mL for hexane extraction, 2.5234 µg/mL for ethanol extraction and 6.0075 µg/mL for acetone extraction. When the results of the antioxidant activity analysis were evaluated, the radical scavenging activities of the ethanol and acetone plant extracts were high. Specifically, when the results of DPPH analyses were evaluated, it was seen that the second place in the order, in which ethanol extract had the highest antioxidant activity, was taken by the acetone extract.

The data obtained as a result of genotoxicity and cytotoxicity studies are shown in Table 3. Using the *A. cepa* chromosomal aberration method, the antigenotoxic effect of different concentrations of *D. cachrydifolia* against genotoxicity induced by MMC was evaluated. The MMC caused a variety of chromosome aberrations such as irregular metaphase, stickiness, c-mitosis, pole deviation, micronucleus, lagging chromosome and bridge (Figure 4). When all treatment models of *D. cachrydifolia* extracts were compared with the positive control group, there were reductions at the chromosomal aberration frequency. According to statistical analyses, statistical differences in the percentage of the mitotic index were found in the 100 µg/mL and 1000 µg/mL pre-treatment, and in the post-treatment highest concentration of 1000 µg/mL, while in the simultaneous treatment, there were a number of aberrations at all concentrations. Among all treatment models, it was determined that the maximum reduction in chromosomal aberration frequency occurred with the highest concentration (1000 µg/mL) of the post-treatment. Pearson correlation analysis showed that the percentage of inhibition of chromosomal aberration was linked to the concentration (Figure 3).

DISCUSSION

The availability of more than half a million identified plants in the world is promising because the medicinal activities of most plants have not yet been studied. Treatments stemming from current and future research will be determined by the properties of these medicinal plants (Rasool Hassan 2012).

As a result of this study, *D. cachrydifolia* was seen as a good antimicrobial source. Furthermore, the radical scavenging activities of the ethanol extracts were high. The Folin– Ciocalteu assay, based on the chemical structure of the phenolics (Atoui et al. 2005), found the total phenolic component content to be highest in the ethanol extract of the plant. Thus, it can be said that the radical scavenging activity of these plant extracts can indicate their total phenolic content. Furthermore, this is consistent with the results of the antioxidant activity and antimicrobial activity of the plant extracts.

Results showed the ethanol extract of *D. cachrydifolia* to have the highest biological activity. There are very few studies in the literature on the chemical characteristics of *D. cachrydifolia*, and no studies on its biological activity or antimicrobial and antioxidant properties. A previous study investigated the essential oil yield

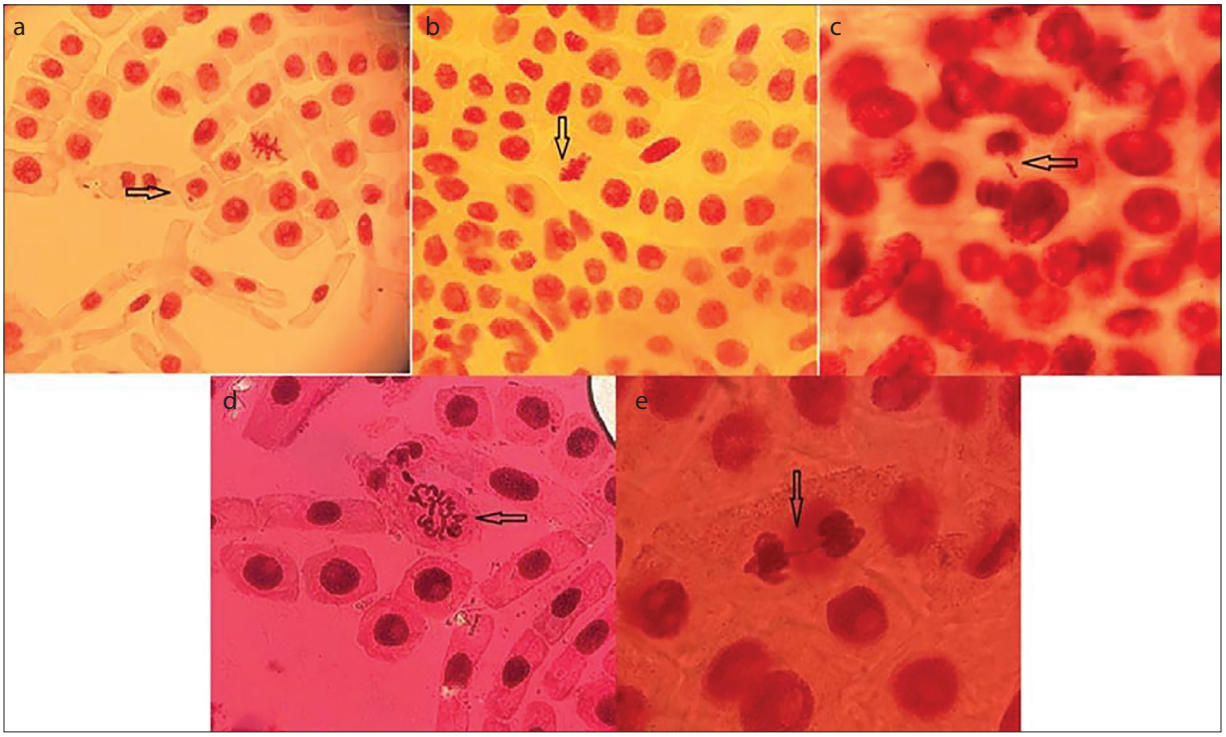

Figure 4. a-e. Types of abnormalities: a) micronucleus, b) stickiness, c) lagging chromosome, d) c-mitosis, e) bridge.

and composition of the aerial parts of *D. cachrydifolia* and reported that the high amount of the oil in its green state is a factor for decreasing plant palatability (Azarnivand et al. 2009). Harkiss and Salehy Surmaghy determined the chemical composition of *D. cachrydifolia* (Harkiss and Salehy Surmaghy 1988), while the antimicrobial activity of the essential oils obtained from the leaves, root and the seeds of *D. damavandica* was determined in another study. That study reported that the essential oil from the leaves displayed the highest antimicrobial activity against all test microorganisms including *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae* (Eftekhar et al. 2005). This study also reported that *D. cachrydifolia* exhibited high antimicrobial activity against all test microorganisms including *K. pneumoniae, L. monocytogenes, S. typhimurium, Y. pseudotuberculosis, P. aeruginosa, E. cloacae, S. epidermidis* and *C. albicans*. These findings raise the possibility that members of this genus may be good sources of antimicrobial agents.

In yet another previous study, the DPPH radical scavenging activity of some species of the Apiaceae family was tested (Coruh et al. 2007). Since *D. cachrydifolia* is a member of the same family (Khajeh et al. 2012), the results are important for the present study. It was reported in another earlier work that the antioxidant capacities of *P. ferulacea*, *H. persicum* and *C. macropodum* had been determined for DPPH radical scavenging as IC_{50} values of 0.242 mg/ml, 0.438 mg/ml and 0.623 mg/ml, respectively (Coruh et al. 2007). In the present study, the antioxidant capacities of *D. cachrydifolia* were calculated as IC_{50} values of 29.1974 µg/mL, 2.5234 µg/mL and 6.0075 µg/mL for the hexane, ethanol and acetone extractions, respectively. The antioxidant activity of *D. cachrydifolia* was much higher compared with the results of the previous study. Furthermore, in the previous study, the total phenolic content of *P. ferulacea*, *C. macropodum*, and *H. persicum* was reported as 65.1±6.4, 34.0±7.0 and 59.6±2.8 µg/mg, respectively (Coruh et al. 2007). In the present study, the total phenolic content of *D. cachrydifolia* was calculated as 43.59±0.657, 55.36±0.035 and 16.29±0.006 µg/mg for the hexane, ethanol and acetone extractions, respectively, and it was observed that these results were very close to each other. It can be said that the antioxidant activity of *D. cachrydifolia* was higher because its active molecules were different from those of the phenolic substances.

The antigenotoxic activity of *D. cachrydifolia* was determined with the *A. cepa* chromosomal aberration assay according to Lima et al. (2017), compounds that prevent mutagenic or genotoxic events from affecting DNA can act by various mechanisms which are usually detected in pre-treatment or co-treatment. Other substances are effective by promoting DNA repair after damage has occurred and can be detected in post-treatment. No study is found in the literature that deals with the antigenotoxic potential of *D. cachrydifolia*. To detect the activity mechanisms of *D. cachrydifolia*, three different treatment groups were used in the present study. A protective effect was observed at these concentrations. The establishment of a protective effect indicated the presence of antimutagenic components in the *D. cachrydifolia* ethanol extract that acted as desmutagens. In other words, antimutagenic substances acted directly on the compounds that cause DNA mutations. This can also be accomplished by chemical or enzymatic means by inhibiting the metabolic activation of the pro-mutagenic or reactive molecules (Kada et al. 1978). Many antimutagenic components found in foods have antioxidant properties and work with pre- or simultaneous treatment to differentiate the free oxygen radicals that cause DNA mutations (Silva et al. 2013).

It should be mentioned in particular that plants from the family Apiaceae are commonly used as food and for flavoring as well as for medical purposes (Christova-Bagdassarian et al. 2013; Christova-Bagdassarian et al. 2014) and that scientific studies have supported the medical use of *D. cachrydifolia*.

CONCLUSION

This study showed that the extracts of *D. cachrydifolia,* specifically an ethanol extract, have a high antimicrobial and antioxidant activity. Therefore, it can be used as a natural source in different areas of the medicine and food industries. Furthermore, this work showed that the ethanol extract of *D. cachrydifolia* may act as desmutagens due to antimutagenic components. The results of this study have revealed the potential of the scientifically proven biological properties of *D. cachrydifolia,* which is routinely used for food and medical treatment by the local population of Turkey. In this respect, it can light the way for future pharmacological research on this plant.

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Original Article

Effects of common variations of NOS3 and CAV1 genes on hypercholesterolemic profile in coronary heart disease

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ABSTRACT

Caveolin-1 (CAV-1) plays a crucial role in endothelial-nitric oxide synthase (eNOS) enzymatic activity. Therefore, CAV-1 and eNOS interactions have a significant impact on endothelial dysfunction, cholesterol levels, and atherosclerosis. We investigated the critical variations in *NOS3* and *CAV1* genes in this case–control study to determine the relations between the coronary heart disease (CHD) risk factors. The NOS3-rs1799983, CAV-1 rs3840634, and rs3807990 variations were analyzed in 76 CHD patients and 91 controls using the polymerase chain reaction. Mean serum Total-cholesterol levels were significantly higher in CHD patients with the CAV-1 rs3807990-T allele than in patients with CC genotype (p=0.017). There was a statistically significant correlation between the rs3807990-T allele and hypercholesterolemia in the CHD group (p=0.008). The multivariate analysis confirmed that the CAV-1 rs3807990-T allele (p=0.011) is a risk factor for hypercholesterolemia. Moreover, the serum HDL-Cholesterol level was detected to be higher in patients carrying both CAV1-rs3807990-T and NOS3-rs1799983-T alleles than those with the CAV-1 rs3807990-CC/NOS3-rs1799983-GG genotype subgroup (p=0.013). These results suggested that the genetic variations of CAV-1 rs3807990 and NOS3-rs1799983 may contribute to the increased hypercholesterolemia risk and thus on the development of CHD.

Keywords: Cav1, NOS3, gene, hypercholesterolemia, lipid, CHD

INTRODUCTION

Coronary heart disease (CHD) was among the leading causes of mortality in worldwide. Endothelial dysfunction plays a central role in atherosclerosis pathogenesis that leads to CHD (Foy and Grant 1997; Hadi and Suwaidi 2007). Main causes of endothelial dysfunction are impaired anticoagulant and antiplatelet mechanisms, increased production of cellular adhesion molecules and increased vascular tone due to reduced bioavailability of endothelial-derived vasodilatory nitric oxide (NO) (Lahera et al. 2007). In the mammalian blood vessels, most of the NO production is mediated by calcium-calmodulin controlled endothelial nitric oxide synthase isoenzyme (eNOS). Caveolin-1 (Cav-1) encoded by the Cav-1 gene (Grilo et al. 2006), is a permanent regulator of the eNOS enzyme activity (Ju et al. 1997; Blair et al. 1999). Cav-1 converts the inactivated form of eNOS by binding directly to the oxygenase domain and Ca^{+2}/CaM activate the eNOS in the endothelial cells (Ju et al. 1997).

Cav-1 is found in macrophages, and endothelial and vascular smooth muscle cells (VSMC) take part in the atherosclerosis process (Couet et al. 1997). A remarkable decrease was observed in the extent of atherosclerotic lesions in a study on $ApoE^{-/-}$ $Cav\text{-}1^{-/-}$ double knockout mice (Puglielli et al. 1995). In contrast, it was reported that the reduced Cav-1 expression in VSMCs of atheroma,

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which suggested that it might have an anti-atherogenic effect in VSMCs (Williams and Lisanti 2004). Cav-1 functions as a scaffold protein for the organization and activation of proteins such as Src-like kinase, G proteins and eNOS in caveolae membrane (Marsden et al. 1993; Chen et al. 1996). It was shown that Cav-1 may cause atherosclerosis by inactivating eNOS in endothelial cells in several experiments on mice. Endothelial nitric oxide synthase enzyme encoded by the *NOS3* gene has "N-terminal oxygenase" and "C-terminal reductase" domains and the Ca^{+2}/ calmodulin (CaM) binding region is located between these two domains (Venema et al. 1996; Yoshimura et al. 1998).

Cav-1 and caveolae also have roles in maintaining cholesterol homeostasis. While Cav-1 interacts with the sterol carrier protein-2 (SCP-2) involved in the delivery of newly produced cholesterol from the smooth endoplasmic reticulum to the cell membrane (Chen et al. 1996; Lusis 2000), caveolae are effective in taking the released free cholesterol to plasma by the HDL and cholesterol esters from plasma (Frank and Lisanti 2004). Both the amino-terminal and carboxy-terminal cytoplasmic regions of Cav-1 and the oxygenase domain of eNOS are involved in this interaction. Recent studies have reported that the deficiency of Cav-1 in endothelial cells (ECs) impairs LDL transendothelial migration. Therefore, the pro-atherogenic effects of Cav-1 in endothelial cells are mainly attributed to its effects on LDL transcytosis. Unlike ECs, Cav-1 deficiency in macrophages causes the foam cells formation by stimulating the accumulation of cholesterol esters. This observation suggests that it has an antiatherogenic effect (Hassan et al. 2004). Therefore, it was suggested that the Cav-1 had differently influenced the development of atherosclerotic vascular disease depending on cell type and metabolic pathway.

Studies regarding the association between the Cav-1 gene variants with cardiovascular diseases and lipid metabolism are scarce (Chen et al. 2005; Schwencke et al. 2005; Carey et al. 2012). Chen et al. 2005, reported that rs3807989 at the Cav-1/Cav-2 locus was associated with significant risk of coronary artery disease (CAD) and myocardial infarction (MI) by increasing Cav-1 expression. They also suggested that "A" allele of the *CAV1* rs3807989 gene is correlated with a decreased LDL cholesterol level.

There is transversion mutation (G>T) at nucleotide 1917 in exon 7 of the *NOS3* gene, causing the glutamic acid to aspartic acid substitution at codon 298 (Glu298Asp, rs1799983) (Huang et al. 1995; Conde et al. 2006). The Glu298Asp mutation has been shown to be related to atherosclerotic coronary events (Huang et al. 1996; Freedman et al. 1999; Lefer et al. 1999; Huang 2000). Although the interactions of Cav-1 and eNOS activity have been shown in previous studies (Chen et al. 1996; Venema et al. 1996), there are limited studies concerning the association of the genetic alterations in these genes. Joshi et al. have shown that the Cav-1 - NOS3 complex is dissociated to notably much more in the Glu/Glu wild type ECs than in the Asp variants. They suggested that NOS3 Glu298Asp variation changed caveolar localization and damaged endothelial response to shear in human endothelium (Joshi et al. 2007).

In a recent study, the rs3840634 (2 bp deletion) and rs3807990 C>T variants of Cav-1 together with *NOS3* rs1799983 were shown to be related to colorectal cancer susceptibility (Conde et al. 2006). Shyu et al. (2017) demonstrated the minor alleles of genotypic polymorphisms of the *NOS3* rs1799983 G>T (Glu-298Asp), Cav-1 rs3807987 G>A and rs7804372 T>A are related to the increased stroke risk of large artery among Han Chinese. Due to the relationship between Cav-1 and eNOS, we suggest that functional genetic variations of these proteins could be an important risk factor for cardiovascular events. Thus, the aim of this study was to examine the association between the *NOS3*-rs1799983, Cav-1 rs3840634, and rs3807990 gene variations and the risk of CHD with respect to both of individual and combined effects of lipid profiles and the other atherosclerotic risk factors in the Turkish population. Regarding Cav-1, this is the first study investigating the effects of the Cav-1 rs3807990 SNP on risk parameters in CHD patients.

MATERIALS AND METHODS

Subjects

This study was a case-control investigation. Control group consisted of 91 healthy individuals with no family history and any signs of diabetes mellitus, renal failure, hypertension, or dyslipidemia. The patient group consisted of 76 patients diagnosed with coronary heart disease followed by the Department of Cardiology, İstanbul University Faculty of Medicine between the period from 2013 to 2014. All the patients were receiving statin for lipid-lowering effects based on conventional therapy.

Criteria for angiography were 50% stenosis at least in one main coronary artery caused by atherosclerosis, and a vascular case, described as myocardial infarction, percutaneous transluminal coronary angioplasty, or coronary artery bypass grafting. All subjects gave the answers for the full histories with special emphasis on coronary risk factors like family history, diabetes mellitus, hyperlipidemia, hypertension, and smoking. Percentages of patients with hypertension, type 2 diabetes and left ventricular hypertrophy were 43.4%, 43.4%, and 39.6%, respectively.

This study was arranged in accordance with the "World Medical Association Declaration of Helsinki" and written consents were obtained from all participants. This study was authorized by the Ethics Committees of the Faculty of Medicine, İstanbul University.

Genotyping

Genomic DNA was isolated from the peripheral blood samples collected in EDTA tubes by using a DNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). *CAV1* rs3840634 (AC deletion) genotyping was carried out by LightCycler real-time PCR (Roche, Germany) via commercial LightSNiP assays from TIB-MolBiol (Germany), in accordance with the company's procedures. Melting curve analysis of the PCR products qualified the genotypes of the rs3840634 as homozygote major allele (TT), heterozygote (CT) and homozygote minor allele (CC). Tm values were 56.98 and 63.77 for the T and C alleles, respectively.

Cav-1 rs3807990 (C>T) and *NOS3* rs1799983 (G>T) polymorphisms were determined by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method using the MspI and BanII restriction enzymes, respectively. MspI

Table 1. Characteristics of the study groups

	Control n=91	CHD n=76	p
Age (year)	59.10±9.85	59.49±12.38	0.822
Sex (Women/Men) (n)	38/53	24/52	0.175
SBP (mmHg)	123.99±12.27	135.95±32.38	0.003
DBP (mmHg)	73.91±8.54	83.99±17.18	0.0041
TC (mmol/L)	4.72± 0.96	5.28± 1.40	0.003
TG (mmol/L)	1.45± 0.66	1.77± 1.80	0.123
HDL-C(mmol/L)	1.22± 0.34	1.03± 0.21	0.001
LDL-C (mmol/L)	2.86± 0.87	3.23± 0.81	0.006
VLDL-C (mmol/L)	0.71± 0.39	0.72± 0.32	0.819
BMI (kg/m2)	25.83± 2.33	25.58± 4.20	0.669
Smoking (%)	21.8%	59.5%	0.001
Alcohol consumption (%)	4.7%	16.7%	0.030
CHD Family history (%)	21.7%	33.9%	0.285

Values were derived by using independent-samples. The results are shown as mean ± SD. Bold values of p indicates statistical significance. TC, total cholesterol; TG, triglyceride; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; VLDL-C, VLDL-cholesterol; TC/HDL-C, total cholesterol/ HDL-cholesterol; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; n, number of individuals.

Table 2. Genotype distributions of the *CAV1* and *NOS3* variations in the study groups

CAV1 and *NOS3* variations		Study Groups	
CAV1 rs3807990		Controls (n=91)	CHD patients (n=73)
Genotypes	CC	49 (53.8%)	46 (63%)
	TT	8 (8.8%)	5 (6.8%)
	CT	34 (37.4%)	22 (30.1%)
	HWE	p=0.552 (p>0.05)	p=0.300 (p>0.05)
Alleles	C	132 (72.5%)	114 (78.1%)
	T	50 (27.5%)	32 (21.9%)
CAV1 rs3840634			
Genotypes	TT	91 (100%)	73 (100%)
	CC	0 (0%)	0 (0%)
	CT	0 (0%)	0 (0%)
	HWE	Uncountable	Uncountable
NOS3 rs1799983			
Genotypes	GG	44 (48.35%)	33 (44%)
	TT	7 (7,69%)	8 (10.7%)
	GT	40 (43.96%)	32 (45.3%)
	HWE	p=0.726 (p>0.05)	p=0.826 (p>0.05)
Alleles	G	122 (67.03%)	98 (67.12%)
	T	54 (29.67%)	48 (32.88%)

n: number of samples, HWE: Hardy-Weinberg Disequilibrium.

digestion resulted in fragments of 122 and 121 bp for the C allele of *CAV1* rs3807990 (C>T). PCR product of the *CAV1* rs3807990 (C>T) (243 bp) was not digested with MspI in the presence of the T allele. The *NOS3* gene rs1799983 (G>T) Glu298Asp missense mutation causes a BanII recognition site, that digests the PCR product to fragments of 198 and 122 bp in the presence of the G allele and a single fragment of 320 bp for the T allele. The digestion products were separated by 3% agarose gel electrophoresis. Homozygous normal GG genotype had two bands (198 and 122 bp) while the homozygous mutant TT genotype had a 320 bp band after visualized under UV. Heterozygous GT genotype has three bands like 320 bp, 198 bp, and 122 bp.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) software package version 20.0, (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Values of $p<0.05$ were accepted statistically significant. The odds ratio and 95% confidence interval were worked out to examine relative risks among the study groups. Comparing the quantitative data among groups were carried out by applying Student's t-Test and ANOVA test, for more than two variables. Compatibility with the Hardy-Weinberg equilibrium (HWE) of the comparisons of genotype and allele were analyzed by chi-square test. Gene counting methods were used for calculating the allele frequencies.

The Binary logistic regression analysis was used to assess the effects of the *CAV1* rs3807990 SNP on hypercholesterolemia in patients with CHD (Table 6). In the regression analysis, the *CAV1* rs3807990 SNP T allele, sex, and Type 2 Diabetes mellitus and smoking were used as independent variables. The multivariate regression model including hypercholesterolemia status as the dependent variable was used.

RESULTS

Clinical Investigation

Demographic and biochemical characteristics of the study groups are given in Table 1. As expected, the CHD group had a higher prevalence of conventional cardiovascular diseases risk factors such as systolic blood pressure (SBP) (p=0.003), diastolic blood pressure (DBP) (p<0.001), Total-cholesterol (Total-C) (p=0.003), LDL-cholesterol (LDL-C) (p=0.006), smoking (p<0.001) and alcohol consumption (p=0.03) and lower HDL-cholesterol (HDL-C) levels (p<0.001). There were no statistical differences in age, sex, body mass index (BMI), triglyceride and

VLDL-cholesterol (VLDL-C) levels and family history of CHD between the patient and control groups (p>0.05).

Distributions and Metabolic Effects *CAV1* rs3807990 and *NOS3* rs1799983 Genotypes

As shown in Table 2, the Cav-1 rs3807990 and *NOS3* rs1799983 gene variations were concordant with HWE in both groups (p>0.05). We also analyzed the Cav-1 gene rs3840634 AC deletion. However, the rare C allele of the Cav-1 rs3840634 was not found in either group and all individuals were carrying the normal TT genotype. Therefore, compliance with HWE was not evaluated for the Cav-1 rs3840634 variants.

When the effects of the Cav-1 rs3807990 genotypes on serum lipid profile, BMI, and blood pressures were investigated in study groups, BMI levels were higher in individuals with the CT genotypes than with the TT genotype in the control group (p=0.046) and DBP value was lower in Cav-1 rs3807990 normal C allele carriers than in non-carriers (TT genotype) (C allele: 73.42±8.65, TT genotype: 79.67±4.32, p=0.014) (Table 3). In the patient group, CT genotype carriers had higher serum Total-C levels than CC genotype carriers (p=0.008, one way Anova test) (Table 3). In addition, it was found that Total-C were higher in patients with the T allele than in those with the CC genotype (T allele: 5.78±1.88, CC genotype: 4.98±0.90, p=0.017, Student's t test) (Table 3). Therefore, increased levels of Total-C observed in the patients carrying the CT genotype can be attributed to the T allele.

When the effects of the *NOS3* rs1799983 genotypes on serum lipid profile, BMI, and blood pressures in the study groups were examined, BMI value was found to be higher in controls with the TT genotype as compared to the GG and GT genotype carriers (p=0.003) and G allele carriers (p=0.002). However, in the patient group, there were no statistically important effects of the *NOS3* rs1799983 genotypes on clinical or biochemical parameters (p>0.05) (Table 4).

The combined effects of Cav-1 rs3807990 and *NOS3* rs1799983 genotypes were also evaluated on serum lipids, BMI, and blood pressures which are given in Table 5. In CHD patients with the Cav-1 rs3807990 rare T allele and the *NOS3* rs1799983 rare T allele higher HDL-C levels were observed than patients with the common genotypes of Cav-1 rs3807990 (CC) / *NOS3* rs1799983 (GG) (1.12±0.12 vs. 1.01± 0.22, p=0.013). There were no significant differences found among the rare T alleles of Cav-1 rs3807990 / *NOS3* rs1799983 haplotype and the Cav-1 rs3807990 CC/*NOS3* rs1799983 GG haplotype in terms of serum lipid profile in the controls (p>0.05).

The association between the Cav-1 rs3807990 SNP and hypercholesterolemia (serum Total-C above 5.18 mmol/L) in the patients with CHD is shown in Figure 1. A significant association was observed between the rs3807990 rare T allele and hypercholesterolemia (chi-square= 6.951, p=0.008). However, we didn't observe any effect of this SNP on hypercholesterolemia in the controls (p=0.161), as it was observed in CHD patients.

Table 3. Metabolic parameters for the CAV-1 rs3807990 genotypes among study groups

Groups	rs3807990				
	CC	CT	TT	C allele (CC+CT)	T allele (TT+CT)
Control	n=49	n=34	n=8	n=83	n=42
Age	59.78±10.36	58.71±9.60	56.63±8.02	59.34±10.01	58.31±9.27
Glucose	94.64±10.10	96.94±12.19	105.00±7.55	95.48±10.85	98.04±11.87
Total-C (mmol/L)	4.66±1.06	4.84±0.88	4.59±0.61	4.74±0.99	4.80±0.84
TG (mmol/L)	1.38±0.55	1.57±0.80	1.41±0.62	1.46±0.66	1.54±0.77
HDL-C (mmol/L)	1.20±0.28	1.19±0.36	1.42±0.52	1.20±0.31	1.23±0.40
LDL -C(mmol/L)	2.79±0.91	3.03±0.84	2.47±0.56	2.89±0.88	2.93±0.82
VLDL-C(mmol/L)	0.69±0.42	0.74±0.38	0.70±0.34	0.71±0.40	0.73±0.37
BMI (kg/m2)	25.94±1.96	26.05±2.51*	24.23±3.17	25.99±2.19	25.71±2.71
SBP (mmHg)	123.29±12.84	125.27±12.11	122.67±9.93	124.09±12.50	124.83±11.67
DBP (mmHg)	73.02±7.50	74.00±10.19	79.67±4.32	73.42±8.65¥	74.97±9.65
CHD Patients	n=46	n=22	n=5	n=68	n=27
Age	61.46±10.75	58.50±14.30	54.60±15.82	60.50±11.99	57.78±14.35
Glucose	179.35±121.15	174.72±130.63	223.33±184.81	177.83±123.13	181.67±134.99
Total-C (mmol/L)	4.98±0.90	5.92±2.04&	5.13±0.63	5.29±1.43	5.78±1.89∑
TG (mmol/L)	1.55±0.67	2.23±3.13	1.89±1.10	1.77±1.9	2.16±2.85
HDL-C (mmol/L)	1.03±0.20	1.06±0.16	1.12±0.21	1.04±0.19	1.07±0.17
LDL -C(mmol/L)	3.07±0.82	3.47±0.69#	3.33±0.81	3.20±0.79	3.44±0.70 Ω
VLDL-C(mmol/L)	0.72±0.31	0.72±0.30	0.72±0.49	0.72±0.30	0.72±0.34
BMI (kg/m^2)	25.75±4.07	24.96±3.43	22.91±3.70	25.47±3.85	24.52±3.51
SBP (mmHg)	133.64±25.78	145.0±41.94	116.0±37.82	137.42±32.30	139.62±42.10
DBP (mmHg)	82.84±17.13	88.41±17.42	74.0±15.17	84.69±17.29	85.74±17.68

The results are shown as mean ± SD. *, p=0.046; ¥, p=0.014; &, p=0.008; ∑, p=0.017; #, p=0.054; Ω, p=0.053.

Table 4. Metabolic parameters for the NOS3 rs1799983 genotypes among study groups

GROUPS	rs1799983				
	GG	GT	TT	GG/GT	TT/GT
CONTROL	n=44	n=40	n=7	n=84	n=47
Age	58.49±9.96	59.66±9.66	56.67±12.48	59.04±9.76	59.18±10.02
Glucose	97.06±11.96	94.73±12.62	99.00±7.53	95.80±12.20	95.48±11.87
Total-C	4.65±1.15	4.80±0.64	4.95±1.00	4.72±0.94	4.82±0.69
TG	1.35±0.49	1.48±0.78	1.36±0.81	1.41±0.64	1.46±0.76
HDL-C	1.29±0.35	1.20±0.38	1.15±0.31	1.25±0.36	1.19±0.37
LDL -C	2.84±0.99	2.94±0.62	2.80±0.95	2.89±0.83	2.91±0.67
VLDL -C	0.62±0.25	0.74±0.42	0.85±0.82	0.67±0.34	0.76±0.49
BMI	25.44±1.99	25.35±2.70	28.59±2.19*	25.40±2.34¥	25.86±2.89
SBP	122.72±11.80	125.13±13.14	113.60±10.71	120.03±12.50	123.48±13.33
DBP	72.40±7.65	77.03±9.37	72.60±9.15	74.92±8.86	76.40±9.34
CHD PATIENTS	n=33	n=32	n=8	n=65	n=40
Age	61.09±11.84	59.24±13.63	56.38±6.93	60.15±12.72	58.69±12.61
Glucose	194.48±134.92	191.19±140.49	135.50±71.01	192.86±136.36	180.75±131.22
Total-C	5.29±1.64	5.24±1.27	5.35±1.07	5.27±1.45	5.27±1.23
TG	1.65±0.81	1.94±2.54	1.50±0.67	1.80±1.90	1.85±2.31
HDL-C	1.01±0.23	1.05±0.19	1.12±0.17	1.02±0.21	1.06±0.18
LDL -C	3.26±0.95	3.20±0.72	3.15±0.59	3.23±0.84	3.19±0.69
VLDL -C	0.74±0.36	0.68±0.27	0.75±0.34	0.72±0.32	0.70±0.28
BMI	26.16±4.48	24.98±4.00	26.46±4.03	25.53±4.23	25.32±4.00
SBP	142.34±31.90	134.09±33.66	121.25±26.42	138.15±32.82	131.59±32.49
DBP	86,72±16,29	83,18±16,85	78,13±22,35	84,92±16,55	82,20±17,86

The results are shown as mean ± SD. *, p=0,003; ¥. p=0.002.

Table 5. Combined effects of CAV-1 rs3807990 and NOS3 rs1799983 on metabolic parameters

GROUPS	CAV-1 rs3807990 and NOS3 rs1799983 Haplotypes		
	eNOS T allele/CAV-1 T allele	eNOS GG/CAV-1 CC	P value
CONTROL	n=22	n=69	
Glucose (mg/dL)	99.36±13.37	95.05±10.07	0.250
Total-C (mmol/L)	4.77±0.66	4.75±1.04	0.943
TG (mmol/L)	1.62±0.91	1.39±0.54	0.288
HDL-C (mmol/L)	1.19±0.44	1.23±0.30	0.586
LDL -C(mmol/L)	2.91±0.62	2.89±0.94	0.920
VLDL -C(mmol/L)	0.79±0.43	0.68±0.39	0.293
BMI (kg/m2)	25.27±3.14	25.87±2.01	0.432
SBP (mmHg)	126.26±14.77	123.27±11.81	0.380
DBP (mmHg)	76.53±10.94	73.65±7.29	0.206
CHD PATIENTS	n=16	n=56	
Glucose (mg/dL)	194.75±141.84	186.02±132.13	0.842
Total-C (mmol/L)	5.81±1.60	5.12±1.34	0.084
TG (mmol/L)	2.44±3.66	1.57±0.71	0.357
HDL-C (mmol/L)	1.12±0.12	1.01±0.22	0.013
LDL -C(mmol/L)	3.53±0.64	3.13±0.84	0.089
VLDL -C(mmol/L)	0.70±0.32	0.71±0.32	0.885
BMI (kg/m2)	25.25±3.62	25.75±4.43	0.693
SBP (mmHg)	129.38±39.41	139.75±30.38	0.313
DBP (mmHg)	81.56±18.05	85.27±17.01	0.451

The results are shown as mean ± SD. Bold values of p indicates statistical significance.

Table 6. Evaluation of risk factors associated with hypercholesterolemia by Binary logistic regression analysis in CHD patients

Variables	p value	B	SE	Exp (B)	OR (95% CI) for Exp (B)
Sex	0.353	0.582	0.627	1.790	0.524-6.114
Type 2 Diabetes mellitus	0.480	-0.448	0.553	0.639	0.184-2.217
Smoking	0.850	0.133	0.700	1.142	0.290-4.504
CAV1 rs3807990 T Allele	0.011	-1.412	0.726	0.244	0.082-0.721

SE: Standard Error; OR: odds ratio; CI: Confidence Interval; (level of significance: p<0.05).

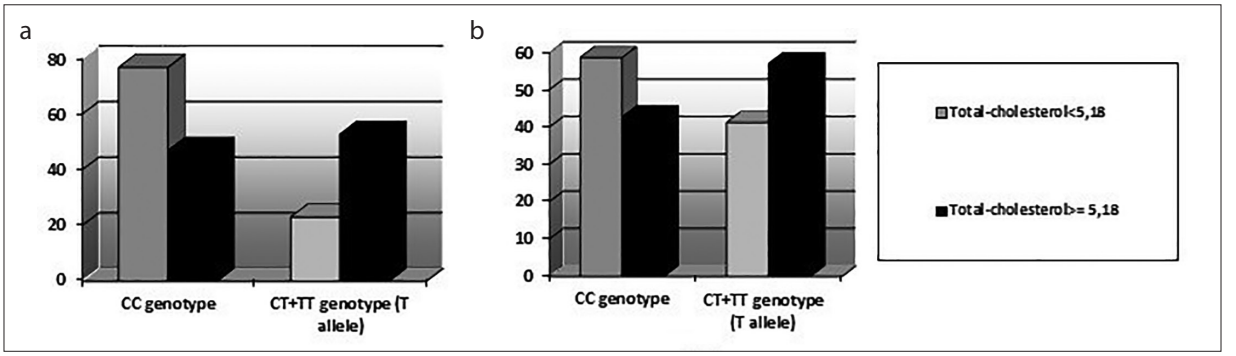

Figure 1. a, b. The association between the CAV1 rs3807990 SNP and hypercholesterolemia in (a) the patient and (b) the control group. *, chi-square= 6.951, p=0.008.

The effects of the risk parameters that we observed on the development of hypercholesterolemia in the CHD group were further evaluated by Binary logistic regression analysis (Table 6). The Cav-1 rs3807990 T allele, sex, diabetes mellitus, and smoking took part in the categorical variables in the risk analysis for hypercholesterolemia. As a result, it was confirmed that the Cav-1 rs3807990 variation was a risk of hypercholesterolemia in the patient group using logistic regression analysis (p=0.011).

DISCUSSION

Endothelial dysfunction is associated with cardiovascular risk factors like dyslipidemia, arterial hypertension, hyperglycemia, and diabetes mellitus (Foy and Grant 2007; Hadi and Suwaidi 2007; Lahera et al. 2007). Reduction of nitric oxide (NO) bioavailability underlies on the basis of the endothelial dysfunction (Drab et al. 2001). NO, synthesized from L-arginine by eNOS enzyme in the endothelial cell membrane, is an important factor blocking the atherosclerosis pathogenesis (Blair et al. 1999; Foy and Grant 2007). Several studies examined the effects of the eNOS gene variations on endothelial dysfunction and atherosclerosis pathogenesis in eNOS gene-deficient mice. Absence of the eNOS gene was shown to lead to lack of EDRF (Endothelial-derived releasing factor) activity, hypertension (Razani et al. 2001), leukocyte-endothelial adhesion (Hingorani et al. 1999), increment of platelet aggregation (Casas et al. 2004), vascular smooth muscle cell proliferation (Razani et al. 2001; Fedele et al. 2013), inclination to thrombosis, stroke (Colombo et al. 2002) and atherosclerosis (Shyu et al. 2017).

The eNOS enzyme activity is transcriptionally regulated by interaction with the presence of the substrate, calcium, calmodulin, enzyme cofactors such as FAD, FMN, NADPH, BH4 and with proteins such as Hsp90 and caveolins in various steps (Atochin et al. 2007). Direct interaction of eNOS and caveolin-1 inhibits the eNOS activity. It was shown in studies on mice lacking the Cav-1 gene that the eNOS activity was increased due to the lack of inhibition that should have been created by the interaction between Cav-1 and eNOS (Granath et al. 2001; Karvonen et al. 2002).

Much is known about the association of the *NOS3* rs1799983 (Glu298Asp) genotypes with the risk of atherosclerotic coronary events in humans, but the results are controversial. Many studies have suggested a relationship between this gene variation and cardiovascular risk (Huang et al. 1995; Huang et al. 1996; Lefer et al. 1999). In a meta-analysis including 26 studies and a total of 23028 samples, Casas et al. (2004) suggested that the risk of ischemic heart disease might be high in individuals with *NOS3* Asp298 and intron-4a homozygous genotypes. However, some studies yielded conflicting results (Huang 2000). Also, in our study, the *NOS3* genotype distribution was similar between the study groups (p>0.05). These different findings might be due to ethnic differences between populations and interactions between the G894T polymorphism and other polymorphisms. Additionally, these findings caused by other factors determining the activity of the eNOS enzyme may show a difference in various populations. When we examined the effects of *NOS3* rs1799983 variant on biochemical parameters such as serum lipid profile and blood pressure, we also found no significance between individuals with different genotypes in both groups. In the control group, BMI was observed higher in TT genotype carriers than in GT and GG genotype carriers. As a result of this study, we determined that the *NOS3* rs1799983 polymorphism individually could not lead to CHD or its risk factors.

There are only a few studies in the literature that have examined the *NOS3* and Cav-1 gene variations in cardiovascular diseases (Grilo et al. 2006; Joshi et al. 2007; Shyu et al. 2017). It was reported that the Cav-1/NOS3 complex is dissociated to a notably greater extent in the common Glu/Glu ECs than in the Asp variants (Joshi et al. 2007). They suggested that the *NOS3* Glu298Asp variation altered caveolar localization and impaired response to shear in human endothelium. Shyu et al. suggested that the minor alleles of the *NOS3* rs1799983 G>T, *CAV1* rs3807987 G>A and rs7804372 T>A are associated with the increased risk of large artery atherosclerotic stroke among Han Chinese (Shyu et al. 2017). Moreover, Grilo et al. (2006) showed an association between the Cav-1 rs3807990 mutant T allele and high systolic blood pressure, while they did not find any association with serum lipids and lipoprotein levels in their study. They also suggested that there was no gene-gene interaction between the *NOS3* and Cav-1 genes with regard to metabolic syndrome in hypertensive patients. However, Razani et al. (2001) have determined that Cav-1 - / - mice had higher plasma triglyceride levels compared to mice with normal genotype. Frank et al. (2006) investigated the relationship between Cav-1 and cellular cholesterol homeostasis and found that the Cav-1 molecule had a minimal effect on HDL and apolipoprotein A-mediated cholesterol efflux. They also found that the Cav-1 molecule had a crucial effect on the cellular cholesterol homeostasis regulation (Hassan et al. 2004). Smart et al. (1996) similarly suggested that the Cav-1 molecule may be an intracellular cholesterol raft. Formation of Caves and the expression of the Cav-1 protein depend on cholesterol. Caveolin proteins also take a hand in the intracellular cholesterol balance (Chen et al. 1996; Lusis 2000). Cav-1 protein has been shown to interact with SCP-2, which is responsible for the newly synthesized cholesterol delivery from the endoplasmic reticulum to the cell membrane (Chen et al. 1996). Also, Caves function in the process of taking up excess free cholesterol released to plasma into HDLs, the reverse transport of cholesterol, and also the removal of cholesterol esters from plasma (Frank et al. 2004). Our findings confirm that caveolin-1 may have an effect on serum lipid/lipoprotein levels. In our study, BMI was higher in controls with the Cav-1 rs3807990 CT genotype than the TT genotype (p= 0.046) and the normal C allele was observed to be correlated with low diastolic blood pressure (p=0.014).

Serum total-C levels have been shown to be higher in patients with both the CT genotype (p=0.008) and the T allele (TT genotype + CT genotype) (p=0.017) compared to the CC genotype. Therefore, our results indicate that the Cav-1 rs3807990 rare T allele may contribute to the high total–C (p=0.017) levels in patients with CHD.

We also determined that the *CAV1* rs3807990 rare T allele is associated with hypercholesterolemia (serum total-C>5.18 mmol/L) (p=0.008). In addition, logistic regression analysis confirmed the Cav-1 rs3807990 T allele was a significant risk factor in the development of hypercholesterolemia in CHD subjects. In this context, our study findings also support the association between serum cholesterol levels and the Cav-1 gene variations. Moreover, we investigated the combined effects of the NOS3 and Cav-1 gene polymorphisms in the present study. In CHD patients who had both the Cav-1 rs3807990 rare T allele and the *NOS3* rs1799983 rare T allele, the serum HDL-C level was found to be higher in the than the Cav-1 rs3807990 common CC/ *NOS3* rs1799983 common GG genotype carriers (1.12±0.12 vs. 1.01± 0.22, p=0.013). However, this association needs to be verified in larger study groups. We believe that our findings will be more meaningful and more concrete in a study group on a wider scale, and will guide the future work in this context.

The limitation of our study was the relatively small study groups (n=177). Secondly, some effects of Cav-1 polymorphisms on serum lipid levels may be masked because the individuals in the patient group received statin therapy in this study, Therefore, we suggest that the effects of Cav-1 gene variations will be more definitive in future studies in which we will investigate the effect of Cav-1 gene polymorphisms on serum lipid levels in patients with CHD receiving with and without statin therapy.

As a conclusion, a better understanding of endothelial dysfunction in the pathogenesis of atherosclerosis is essential for the development of new treatment modalities. Therefore, we investigated the critical variations in *NOS3* and Cav-1 genes in this study in order to determine the relations between the CHD risk factors and NO production in the endothelial function process. Our study indicated that the Cav-1 rs3807990 T allele may be one of the risk factors for the development of hypercholesterolemia and coronary heart disease.

Ethics Committee Approval: This study protocol was approved by the Ethics Committees of the Istanbul Faculty of Medicine, Istanbul University (approval number:2011/1276-643, date: 27th July, 2013).

Informed Consent: All participants received medical approval from their personal physicians and gave written, informed consent prior to giving their blood sample. This study protocol was arranged according to the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects".

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – H.Y.A., S.İ.; Design - H.Y.A., Z.B.; Supervision - H.Y.A.,O.Ö., Z.B.; Resource - Z.B.; Materials - Z.B., S.I., Ö.K.G.; Data Collection and/or Processing - E.C., S.I., A.P.E.; Analysis and/or Interpretation - H.Y.A., O.Ö., E.C.; Literature Search - S.I., A.P.E., Ö.K.G.; Writing - H.Y.A., S.I., A.P.E., Ö.K.G; Critical Reviews - O.Ö., Z.B.

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Original Article

A new approach for the synthesis of *N,N'*-bis(phenoxyacetyl)hydrazines

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ABSTRACT

This report is about an unexpected formation of *N,N'*-bis(substituted phenoxyacetyl) hydrazine derivatives. The reaction between *N'*-(4-ethylcyclohexylidene)phenoxyacetohydrazide with sulfanyl acids to obtain 4-thiazolidinones s, gave an unexpected product, *N,N'*-bis(phenoxyacetyl)hydrazine, with high purity instead of the expected cyclocondensation product. When repeating the reaction with p-toluenesulfonic acid under the same conditions, the reaction resulted in the same compound. This indicates that two molecules of *N'*-(4-ethylcyclohexylidene)phenoxyacetohydrazide were involved in the reaction. The reaction can be applied as a different and new procedure for the synthesis of several *N,N'*-bis(substituted phenoxyacetyl) hydrazine derivatives.

Keywords: Diacylhydrazines, sulfanyl acid, reaction mechanism

INTRODUCTION

Diacylhydrazines have attracted considerable attention recently due to their simple structure, low toxicity, and high insecticidal selectivity. Their synthesis can be achieved by the reaction of hydrazine with acyl chlorides or anhydrides of carboxylic acids (Patai 1970; Wang et al. 2011; Sun and Zhou 2015). When the operation is applied by the reaction of esters of carboxylic acids, monoacyl hydrazine derivatives are generally obtained and subsequent insertion of a second acyl moiety is very difficult.

During our studies on the synthesis of 4-thiazolidinone derivatives, we obtained an incidental product, *N,N'*-bis(phenoxyacetyl) hydrazine with high purity and yield. The reaction of phenoxyacetohydrazide hydrazones with sulfanyl acids resulted in the same product.

In this paper will examine the interconversion of phenoxyacetohydrazide hydrazones, mainly *N'*-(4-ethylcyclohexylidene)phenoxyacetohydrazide, into *N,N'*-bis(phenoxyacetyl)hydrazine, through a new synthetic route for *N,N'*-bis(phenoxyacety)hydrazine derivatives.

MATERIALS AND METHODS

For our study the chemicals were obtained from Merck and Aldrich. Melting points (mp) were determined on a Buchi 530 melting point apparatus in open capillaries and are uncorrected. Microanalysis and mass spectrum of *N,N*-bis(phenoxyacetyl)hydrazine was performed on a Thermo Finnigan Flash EA 1112 elemental analyzer and Agilent 6460 TripQuad LC-MS/MS mass spectrometer. IR spectra were recorded in KBr discs (ν_{max} in cm^{-1}) on a Shimadzu IR Affinity-1 FTIR spectrophotometer. ^{1}H-NMR (DMSO-d_6) spectra were obtained on a Varian Mercury-400 MHz and PULSAR NMR Spectrometer-60 MHz instruments. *(E,Z)-N'*-(4-ethylcyclohexylidene)

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phenoxyacetohydrazide was recorded in SciFinder Scholar database but no analytic or spectral data have been given.

Synthesis of *(E,Z)-N'*-(4-ethylcyclohexylidene)phenoxyacetohydrazide

A solution of 2-phenoxyacetohydrazide and 4-ethylcyclohexanone (0.01 mol) in 20 mL of ethanol (96%) was refluxed for 1 hour and then the solution was allowed to cool. The solid thus formed was filtered off, dried and directly used in the next step.

White powder (Yield 72%); mp: 113-115°C; IR (KBr): υ max 3327, 3180 (N-H), 3072 (Ar-H), 1683 (C=O). ^{1}H-NMR (DMSO-d_6/60 MHz): 0.81-3.07 (m, cyclohexane $C_{2,6}$-H, $C_{3,5}$-H, C_2H_5 and DMSO), 4.66, 5.00 (2H, 2s, OCH_2), 6.65-7.80 (5H, m, phenyl C_{2-6}-H), 10.38, 10.51 (1H, 2 broad s, NH).

***N,N'*-bis(phenoxyacetyl)hydrazine as a by-product during the synthesis of 4-thiazolidinones**

0.005 mol (1.37 g) *N'*-(4-ethylcyclohexylidene) substitueted phenoxyacetohydrazide and (0.005 mol) sulfanyl acids (sulfanylacetic acid or 2-sulfanylpropanoic acid) or p-toluenesulfonic acid was refluxed in 30 mL of dried toluene for 4-6 hours using a Dean-Stark water separator. Excess toluene was evaporated *in vacuo*. The resulting mixture was treated with saturated sodium bicarbonate solution to remove the excess acid. The solid thus obtained was filtered, washed with water, dried and recrystallized from ethanol.

White powder (Yield 56%); mp: 168-170°C; IR (KBr): υ max 3450 (O-H), 3228 (N-H), 1703, 1660 (C=O). ^{1}H-NMR (DMSO-d_6/400 MHz): 4.60 (4H, s, OCH_2), 6.95-7.00 (6H, m, phenyl $C_{2,4,6}$-H), 7.28-7.32 (4H, m, phenyl $C_{3,5}$-H), 10.18 (2H, s, NH). MS (ESI-) m/z (%): 299 $[M-H]^-$ (100). Anal. calcd. for $C_{16}H_{16}N_2O_4.H_2O$: C: 60.37, H: 5.70, N: 8.80. Found: C: 59.82, H: 5.72, N: 8.80 (mp 164°C; Baltazzi and Delavigne 1955).

RESULTS AND DISCUSSION

There are several methods for the synthesis of 4-thiazolidinone derivatives and their mechanism of cyclization have been reported previously (Singh et al. 1981, Hamama et al. 2008; Tripathi et al. 2014, Kocabalkanlı et al. 2017). One of the most common methods for obtaining 4-thiazolidinone ring is refluxing equimolecular amounts of aldimines or ketimines with alkanoic acids in dried aprotic solvents. In the course of our studies in the synthesis of spiro derivatives of 4-thiazolidinone, the reaction of *N'*-(4-ethylcyclohexylidene)phenoxyacetohydrazide with sulfanylacetic acid or 2-sulfanylpropanoic acid did not give the desired spirothiazolidinones, *N*-(8-ethyl-3-oxo-1-thia-4-azaspiro[4.5]dec-4-yl) or *N*-(8-ethyl-2-methyl-3-oxo-1-thia-4-azaspiro[4.5]dec-4-yl)-2-phenoxyacetamides. Surprisingly, an unexpected product was formed instead. The product was confirmed as *N,N'*-bis(phenoxyacetyl) hydrazine by IR, ^{1}H-NMR, mass spectrum and microanalysis. When the same study was repeated with *N'*-(4-propylcyclohexylidene) phenoxyacetohydrazide the main product was again *N,N'*-bis(phenoxyacetyl)hydrazine. In the cases of 4-methylcyclohexylidene, 4-phenylcyclohexylidene or 4-*tert*-butylcyclohexylidene derivatives of phenoxyacetohydrazide, *N,N'*-bis(phenoxyacetyl) hydrazine was isolated as by-products with different yields together with the targeted products, spirothiazolidinones.

N,N'-bis(phenoxyacetyl)hydrazine was firstly reported in 1955 (Baltazzi and Delavigne 1955). According to this study, the condensation of phenoxyacetohydrazide with some aldehydes or cyclic ketones such as cyclohexanone, cyclopentanone in aqueous acetic acid, the corresponding hydrazone derivatives were formed. However, when acetone or methyl propyl ketone was used, *N,N'*-bis(phenoxyacetyl)hydrazine was isolated. The proposed mechanism (A) is shown in Figure 1.

In a study performed with a series of aryloxyacetic acids, it was reported that monocyclic hydrazides were obtained by heating methyl or ethyl esters of the acids with hydrazine in ethanol, whereas conducting the reaction without ethanol resulted in a mixture of monocyclic and *N,N'*-bis(phenoxyacetyl) hydrazines (Mirek 1958).

It was also reported that the reaction of ethyl acetoacetate with hydrazides of phenoxyacetic or 4-nitrophenoxyacetic acids did

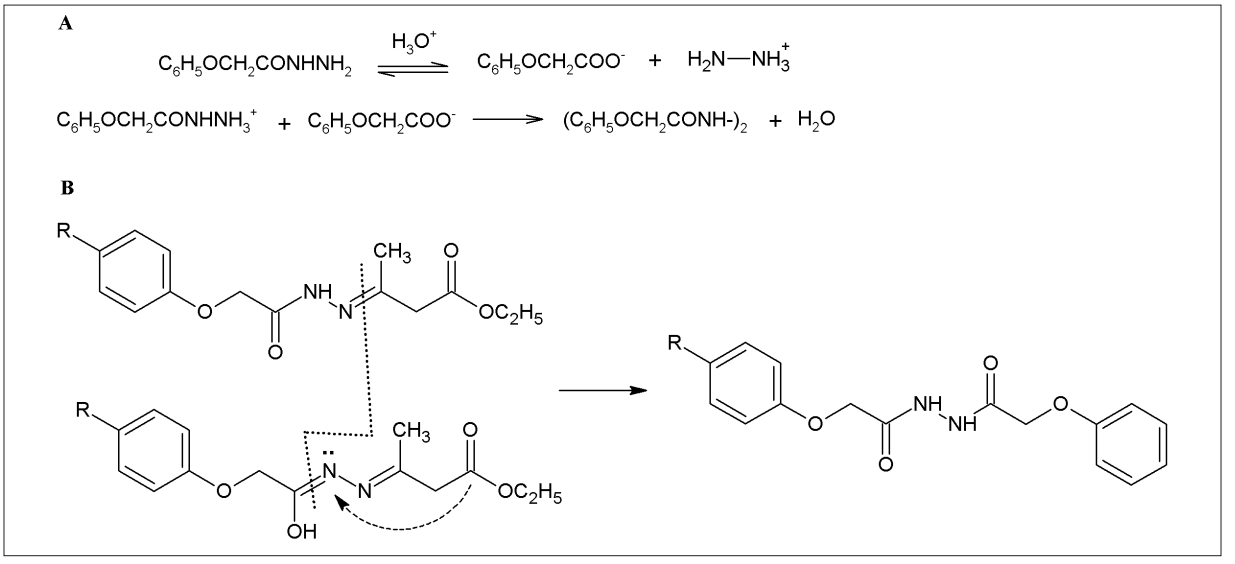

Figure 1. Suggested mechanisms for the unexpected synthesis of *N,N'*-bis(diacyl)hydrazines.

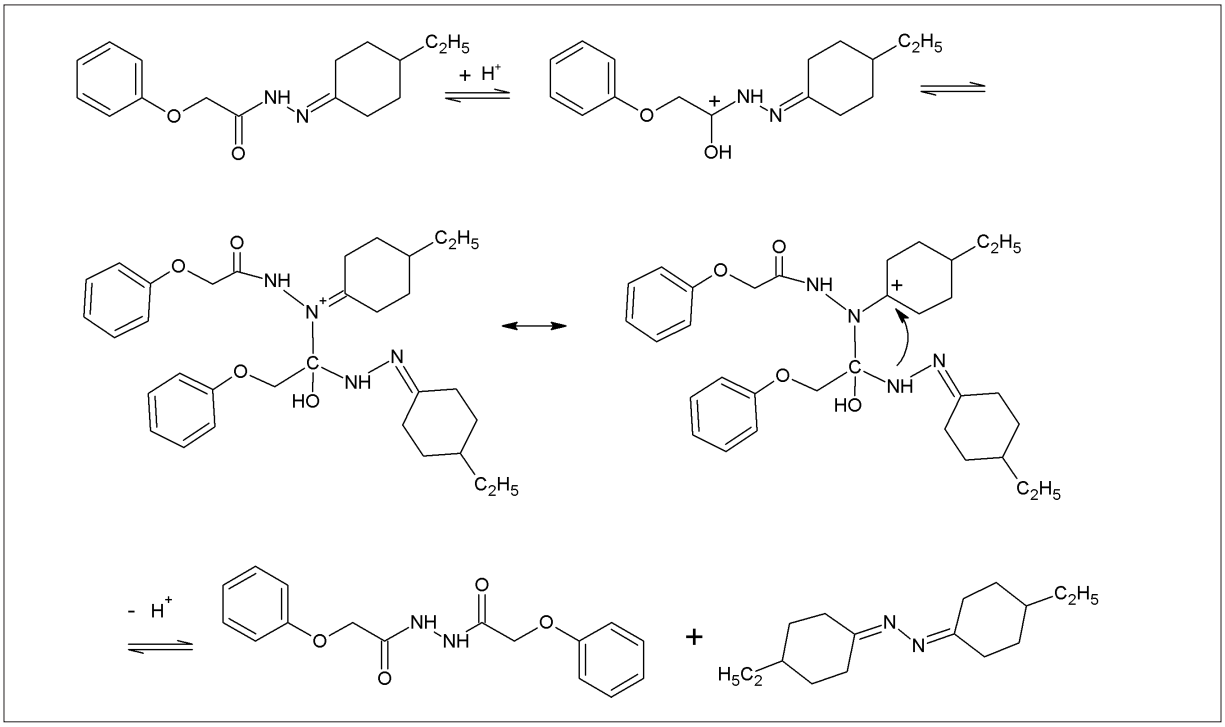

Figure 2. Proposed mechanism for the reaction of *N,N'*-bis(diacyl)hydrazine.

not result in the targeted N^1-acylpyrazolones. Other products, mainly *N,N*-bis(diacyl)hydrazines were formed (Zawadowsca 1961). The suggested mechanism for the unexpected synthesis of *N,N'*-bis(diacyl)hydrazines (B) is presented in Figure 1.

In general reaction conditions the acidic properties of the alkanoic acids we used did not promote the cycloaddition reaction in anhydrous toluene. *N,N*-bis(phenoxyacetyl)hydrazine occured directly from hydrazide-hydrazone derivatives. Repeating the reaction with p-toluenesulfonic acid again yielded the same derivative, which indicates that two molecules of phenoxyacetohydrazide hydrazone were involved in the reaction. The possible mechanism of the reaction can be explained by the action of the oxygen atom of the phenoxy group. The electrophilic nature of the carbonyl carbon atom of hydrazide-hydrazone is being increased by the oxygen atom in the phenoxy residue inductively. Then the hydrazone group of the second molecule attacks the imine nitrogen. This directly results in a balance towards *N,N'*-bis(diacyl)hydrazine direction. And finally, *N,N*-bis(phenoxyacetyl) hydrazine occurs through a transition intermediate and electron shifts. Because the reaction proceeds under anhydrous conditions, an acid catalyzed hydrolysis possibility is excluded. Secondary product, probably azine, was not isolated. The proposed mechanism for the reaction is illustrated in Figure 2.

The reaction can be applied as a different and new procedure for the synthesis of several *N,N'*-bis(substituted phenoxyacety) hydrazine derivatives.

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Original Article

Evaluation of the association of SNP in carboxylesterase enzyme (CES1) with pharmacokinetic and adverse effects of capecitabine in breast and colorectal cancer patients

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ABSTRACT

Capecitabine is an oral prodrug and converted to 5-fluorouracil using three-step enzymatic pathways which include carboxylesterase (CES). Interindividual differences in the activities of drug-metabolizing enzymes may affect efficacy and toxicity. The aim of this study is to evaluate the association of Single nucleotide polymorphisms (SNP) in CES1 with the pharmacokinetic and adverse effects of capecitabine. Plasma samples were obtained from 7 breast and colorectal cancer patients who were treated with capecitabine-based chemotherapy (1000-1250 mg/m^2) at 0.5, 1, 2, 3 and 4 hours following drug administration on their first day of the first cycle. The plasma concentrations of the capecitabine were determined by using a high-pressure liquid chromatography-UV detector. SNP (rs8192950) was genotyped using the reverse transcription-polymerase chain reaction. Patients were found to have heterozygote (57%), wild (29%), and mutant (14%) distributions of genotypes (p=0.909). The mean plasma area under the curve (AUC_{0-4h}) was 4.60±2.25 µg.h/mL, and maximum plasma concentration (C_{max}) was 3.19±2.5 µg/mL. There were no statistically significant differences between genotypes and AUC values (p=0.2236) and the most frequently observed side effects were diarrhea (p=0.1028), asthenia (p=0.6456), anemia (p=0.6456), emesis (p=0.3499). This is the first study evaluating an association of genetic variation in CES1 (rs8192950) with pharmacokinetic and adverse effects of capecitabine. Therefore, additional study in larger groups of patients is required to support our study.

Keywords: Capecitabine, carboxylesterase, pharmacogenetics, breast cancer, colorectal cancer

INTRODUCTION

Cancer is a major worldwide public health concern and is the second leading cause of death in the world with colorectal and breast cancer being among the most common cancers in the world (Siegel et al. 2019). Breast cancer is the most common type of cancer and the leading cause of cancer related deaths in women while colorectal cancer is the third most common cancer among women and men in Turkey (TC Ministry of Health Turkey Cancer Statistics 2018).

Capecitabine is used orally in the treatment of breast, colorectal and gastric cancer. Capecitabine is a precursor and converted to 5-fluorouracil (5-FU) (which is a cytotoxic agent) in the tumor. 5-FU is one of the oldest and the most widely used antimetabolite and cytotoxic agents (Reigner et al. 2001).

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Capecitabine is extensively metabolized to 5-FU using three-step enzymatic pathways. After oral administration, it is converted into 5'-deoxy-5-fluorocytidine (5'-DFCR) in the liver by carboxylesterase (CES). The 5'-DFCR is then converted to 5'-deoxy-5-fluorouridine (5'-DFUR) by cytidine deaminase (CDA) which is found in the liver and tumor tissues. 5-FU formation occurs on the tumor area with a basis of thymidine phosphorylase (TYMP) and it minimizes the systemic exposure of 5-FU to healthy body tissue. Capecitabine becomes cytotoxic after conversion to 5-FU and its metabolites. 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPYD) and transforms to the inactive metabolites dihydro-5-fluorouracil (FUH_2), 5-fluoro-ureido propionic acid (FUPA) and α-fluoro-β-alanine (FBAL) (Reigner et al. 2001; Thorn et al. 2011; Daniele et al. 2013). Dose limiting toxicities of capecitabine include diarrhoea, abdominal pain, nausea, stomatitis and hand-foot syndrome (hand-foot skin reaction, palmar-plantar erythrodysesthesia) (Xeloda® Roche 500 mg Summary of Product Characteristics). Pharmacokinetics, efficacy and adverse effects incidence from using oral capecitabine also are vary in individuals in the treatment of breast and colorectal cancer when administered as a monotherapy or combination therapy. Expression differences of enzymes involved in the metabolism of capecitabine may be the reason in the interindividuals' variation (Reigner et al. 2001; Thorn et al. 2011).

Mammalian carboxylesterases (CEs) are key enzymes from the serine hydrolase superfamily. Two carboxylesterases (CES1 and CES2) have been identified in the human body and extensively studied over the past decade. CES1 are expressed mainly in the liver while CES2 mostly in the intestine. The key roles of CES are xenobiotic and drug metabolism. To date, a number of functional genetic variants of CES1 and CES2 have been reported, which may be associated with substantial individual variations in the responses to pharmacologic therapies (Wang D et al. 2018). It has been found that is an association between efficacy of CES enzymes substrates such as clopidogrel (Lewis et al. 2013; Zhao et al. 2016; Xiao et al. 2017), dabigatran (Pare et al. 2013), enalapril (Tarkiainen et al. 2015), imidapril (Geshi et al. 2005), oseltamivir (Tarkiainen et al. 2012) and CES polymorphisms. It has been shown that polymorphisms in the enzyme CES 2 (Ribelles et al. 2008; Martin et al. 2015) and CES 1 (Hamzic et al. 2017) altered the efficacy and toxicity of capecitabine but no pharmacokinetic evaluation has been made. In this study, the association of SNP (rs8192950) in CES1 (which metabolizes capecitabine to 5'-DFCR), with the pharmacokinetic and adverse effects of capecitabine were analyzed.

MATERIALS AND METHODS

Study Protocol

A routine standard chemotherapy regimen (which uses capecitabine) was administered to the patients who signed the voluntary informed consent form in Acibadem Maslak Hospital Oncology Clinic, Istanbul, Turkey. Patients meeting the inclusion criteria and enrolling in the study received capecitabine at 1000-1250 mg/m^2, perorally twice daily for 14 days followed by a 7-day rest period, over a total of 6 months. Blood samples (5 mL) were collected for genotype analysis in EDTA coated tubes on day 1 of the first cycle before capecitabine application. During the same day, genomic DNA was isolated from whole blood for further analysis of the genetic variants and stored at -80°C. Blood samples (3 mL) were taken for pharmacokinetic analysis at 0.5, 1, 2, 3 and 4 hours after capecitabine application on day 1 of the first cycle. The blood samples in the tetrahydrouridine tube were centrifuged immediately (10 minutes, 3000 rpm) and the plasma was stored at -80°C for later analysis.

All patients provided informed consent, and the study was conducted after ethical approval (Approval No: ATADEK 2015/9) which was provided by the Ethical Committee of Acibadem University Hospitals (ATADEK), Istanbul, Turkey).

Inclusion criteria

- Histopathologic diagnosis of breast or colon adenocarcinoma,
- Patients who had received neoadjuvant and/or adjuvant treatment for more than 6 months before,
- Adults > 18 years of age,
- Appropriate ECOG performance status (0-2),
- Patients without a prior history of severe cardiac problems (coronary artery disease, congestive heart failure, arrhythmia, etc). Normal kidney, liver and bone marrow functions.
- Ability to understand and sign a written informed consent form, which must be obtained prior to the initiation of the study procedure.

Exclusion criteria

- Pregnant or breastfeeding women,
- Patients who refuse to use an acceptable contraception method at reproductive age,
- Diagnosis of active gastrointestinal malabsorption and/or patients with enteral nutrition (jejunostomy),
- Experience of toxicity above the second degree in previous adjuvant or neoadjuvant therapy according to the CTCAE Version 4.0 (National Cancer Institute, USA, 2010),
- Abnormal liver or kidney functions,
- Patients who have a known DPD deficiency,
- Patients who have a known or symptomatic brain metastasis,
- Diagnosis of a systemic disease (uncontrolled diabetes, congestive heart failure, etc.),
- Concomitant administration of drugs which are metabolized by the CYP2C9 enzyme system (warfarin, phenytoin, etc.),
- Patients who had undergone major surgical procedures within the last 4 weeks,
- Patients who had received radiotherapy within the last 4 weeks,
- Patients who had a known allergic reaction to capecitabine and 5-FU.

Evaluation of Side Effects

The evaluation of side effects and the collection of the demographic and clinical data were managed by an oncologist. The adverse effects recorded included anemia, abdominal pain, nausea, diarrhea, stomatitis and hand-foot syndrome. Toxicity was graded according to the CTCAE version 4.0 of the National Cancer Institute and toxicity due to capecitabine administration was managed by symptomatic treatment and/or modification of the dose (treatment interruption or dose reduction).

Pharmacokinetic Analysis

Analytical Method

Quantification of capecitabine in plasma was performed using a validated, sensitive and selective high-pressure liquid chromatography (HPLC) method which was used by Farkouh et al. (Farkouh et al. 2010). The capecitabine was provided by BioChemPartner (CAS No. 154361-50-9). Methanol Merck, Germany) and water (Milli Q-Millipore, USA) were HPLC grade. The analyses were performed on an HPLC system consisting of a Waters 2695 pump, autosampler, column heater, and Waters 2487 ultraviolet/visible (UV/VIS) detector (Waters, USA). The chromatographic conditions for the quantification of capecitabine in plasma are shown in Table 1.

Extraction

The matrix components were removed from the plasma samples by solid-phase extraction (Oasis® 1 cc/30 mg; Waters, USA). After washing the cartridges for preconditioning with methanol (1 mL) and water (1 mL) the plasma sample (1 mL) was applied to the cartridge. After washing-out the matrix components with 5% methanol (1 mL), the capecitabine was eluted from the cartridge with methanol (1 mL).

Calculation of Pharmacokinetic Parameters

In order to determine the concentrations of capecitabine in the plasma samples, the peak areas in the chromatogram were used and the calibration curve was obtained using the following equation: $y = ax + b$. The calibration curve was linear within the range of 10-0.306 µg /mL. The capecitabine plasma concentration time curve between 0 and 4 hours (t=0 and t=4h) was obtained using the GraphPad Prism 6. The area under the plasma concentration-time curve from 0 to 4 hours (AUC_{0-4h}) was calculated using the trapezoidal method. The C_{max} and t_{max} values were calculated using the plasma concentration time curve. The elimination rate constant (k_{el}) was calculated from the terminal points of the cepecitabine plasma concentration-time plot and the slope of this line was equal to k_{el}. The terminal elimination half-life ($t_{1/2}$) was calculated using log-linear approximation of the terminal points of the data. The terminal elimination half-life and elimination rate constant (k_{el}) were interconverted with the following formula: $t_{1/2} = \ln 2 / k_{el}$

Table 1. The chromatographic conditions for quantification of capecitabine in plasma

Column	Phenomenex® 250 mm x 4 mm (5 µm) C18 column
Guard column	Phenomenex® 2.1 x 3.9 mm C18 column
Mobile phase	Methanol : water = 50:50 (v:v)
Flow rate	0.6 mL /min
Column temperature	36 ºC
Injection volume	30 µL
Detection	2487 dual wavelength UV/VIS detector
Wavelength	305 nm
Retention time	20 min
Extraction	SPE Cartridges (Oasis® 1 cc/30 mg; Waters, USA)

Genotyping

Genomic DNA was extracted from whole blood using the standard phenol chloroform extraction protocol and further purification was done using High Pure PCR Product Purification Kit (Roche 11796828001; Roche, Mannheim, Germany). SNP analysis was performed using a LightCycler FastStart DNA Master HybProbe (Roche, Mannheim, Germany) and custom-designed LightSNiP assay probes (Lot 41341701, Roche, Mannheim, Germany) according to the manufacturer's instructions. In a final volume of 20 mL reaction mix per sample, the following mixtures was added: 1X FastStart DNA Master Mix, 2 mM MgCl2, 0.2 mM LightSNP HybProbe (Roche 03003248001) appropriate amount of PCR grade water and 500 ng DNA sample. The plates were sealed and centrifuged at 3000 rpm for 1 minute. CES1 SNP rs8192950 was genotyped using a Roche Light Cycler 480 (Roche, Mannheim, Germany) real-time PCR platform and melting curve analyses were performed by the Carousel-Based System PCR program.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS) version 20 (IBM Corp.; Armonk, NY, USA) was used for statistical analysis. Hardy-Weinberg equilibrium (HWE) analysis was performed using the Chi-square (χ^2) test for the analysis of genotype frequencies. According to the results of the statistical evaluation, a value of $p<0.05$ was considered statistically significant. The un-paired t test method was used to compare pharmacokinetic parameters with alleles. The relationship between side effects and genotypes was performed using the χ^2 test.

RESULTS

Clinical Study Results

Seven patients who received a routine standard chemotherapy regimen including capesitabine in the Acibadem Maslak Hospital Oncology Clinic were enrolled in this study. 71% of the patients were female and 29% of them were male. 43% of the patients were treated for breast cancer and 57% of them were treated for colon cancer. The mean age of the patients was 55±16 years. Each patient enrolled in the study received capecitabine at 1000-1250 mg/m^2, perorally twice daily for 14 days. Patients were routinely examined at the end of the first cycle of treatment by a clinician. While diarrhea and nausea - are dose-limiting side effects - were

Table 2. Pharmacokinetic parameters (mean) of capecitabine

	C_{max} (µg/ mL)	k_{el} (h^{-1})	$t_{1/2}$ (h)	AUC_{0-4h} (µg.h/ mL)	$AUC_{4h-\infty}$ (µg.h/ mL)	AUC_{total} (µg.h/ mL)
x̄	3.19	0.46	2.98	4.60	3.18	7.77
SD	2.53	0.27	3.84	2.25	3.86	4.42

x̄:mean; SD: standard deviation

observed during the first cycle, abdominal pain, stomatitis and hand-foot syndrome were not reported.

The plasma concentration time curve of the capecitabine which was obtained at 0.5, 1, 2, 3 and 4 hours after capecitabine administration are shown in Figure 1.

The C_{max}, k_{el}, $t_{1/2}$ and AUC which were calculated using plasma concentration time curve are shown in Table 2.

A comparison of AUC_{total} values of the capecitabine plasma concentrations are shown in Figure 2.

Pharmacogenetic and Pharmacokinetic Study Results

Statistical evaluation of genotyping analysis for CES1 SNP (rs8192950) was performed as indicated in the relevant section. The expected and observed values of SNP were determined using Hardy-Weinberg Equilibrium. The chi-square (χ^2, Chi-square) test was performed to check if genotype frequencies were be in Hardy-Weinberg equilibrium and the statistical test indicated that all variants within the population were in Hardy-Weinberg equilibrium (Table 3).

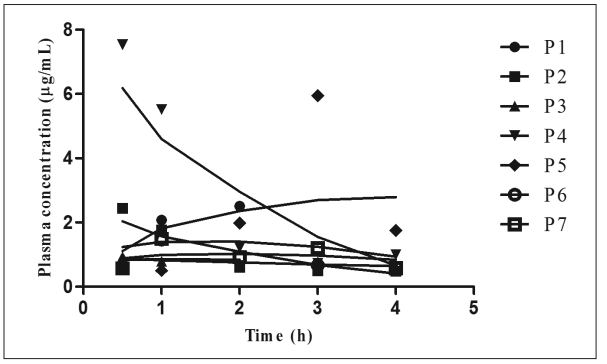

Figure 1. Capecitabine plasma concentration time curve.

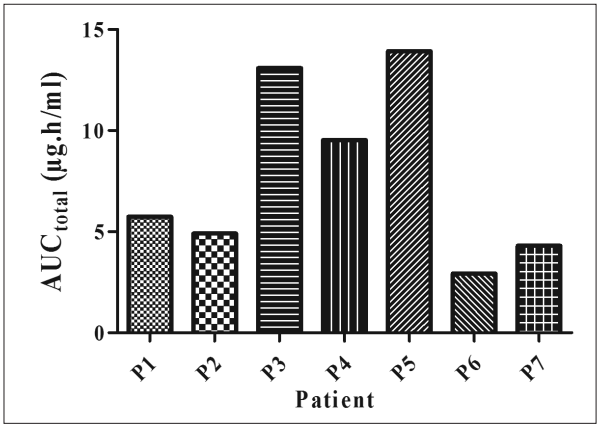

Figure 2. Comparison of AUC_{total} patient values each subject individually in the study group.
P: Patient

Heterozygotes (A/C) were observed in 4 of the 7 patients (57%) and wild type (C/C) were observed in 2 of the 7 patients (29%). CES1 gene mutation (A/A) was only detected in one patient (14%). The allele and allele frequencies that cause a change in enzyme activity in the CES1 gene are shown in Figure 3.

The mean $AUC_{0\text{-}4h}$ of the five heterozygote and mutant rs8192950 allele carriers was 5.11±1.11 µg.h/ mL, the mean $AUC_{0\text{-}4h}$ of the two non-carriers was 3.29±0.6 µg.h/ mL (Table 4). The mean AUC values of the mutant and heterozygote patients were 1.5 times more than the AUC values of the wild type. However, no statistically significant differences between CES1 gene mutation (rs8192950) and AUC values (p=0.2236) were observed (Figure 4).

The relationship between side effects and CES1 gene mutation (rs8192950) was performed using chi-square (χ^2, Chi-square) test. No statistically significant differences between genotypes and the most frequently observed side effects of diarrhea (p=0.1028), asthenia (p=0.6456), anemia (p=0.6456), emesis (p=0.3499) were observed.

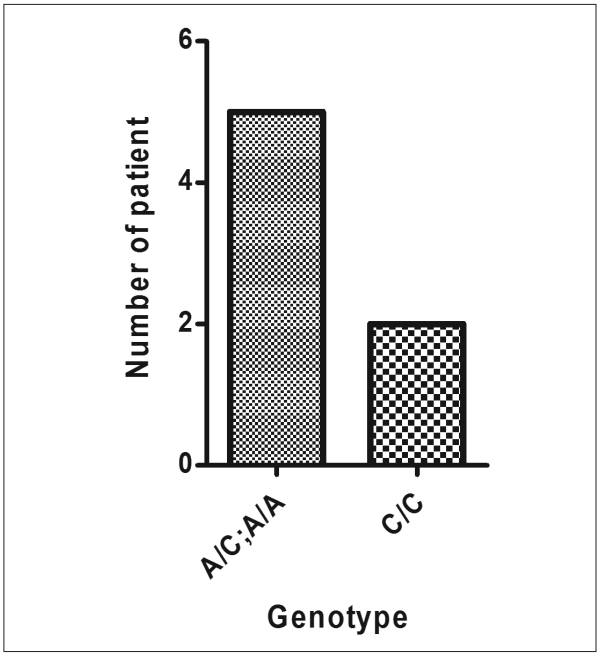

Figure 3. Alleles and allele frequencies of CES1 gene in the study population given as numbers.

Table 3. Statistical evaluation of the selected SNP in CES1

SNP	Allele	Observed (n)	Expected	X^2	p
CES1	C/C	2	2.282	0.193	0.909
	A/A	1	1.288		
	A/C	4	3.429		

Table 4. Statistical analysis for the relationship between CES1 (rs8192950) genotypes and pharmacokinetics of genotypes

CES1 (rs8192950)	N	$AUC_{0\text{-}4h}$ (µg.h/ mL)	SE	p
A/C;A/A	5	5.11	1.11	0.2236
C/C	2	3.29	0.61	

AUC0-4h as a mean; SE: standard error.

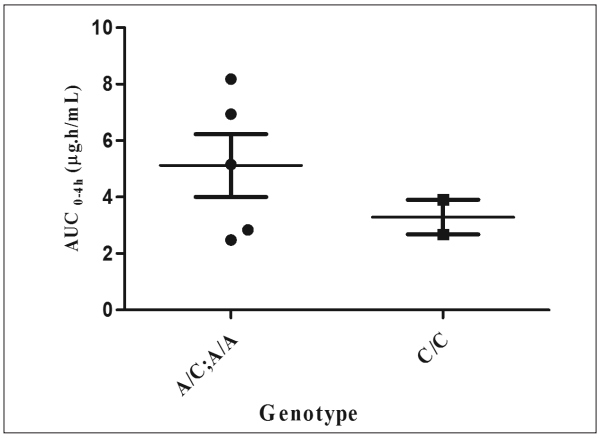

Figure 4. Boxplot showing AUC_{0-4h} values for each variants of CES1 (rs8192950) (•) indicate AUC0-4h of each subject individually in the subgroup.

DISCUSSION

Capecitabine is a tumor-selective fluoropyrimidine carbamate, an oral prodrug of 5-FU. Capecitabine is used orally in the treatment of breast, colorectal and gastric cancer. 5-FU is one of the oldest and the most widely used antimetabolite and cytotoxic agents. 5-FU, which is responsible for the anticancer effect of capecitabine, and capecitabine have narrow therapeutic indices, such as many other antineoplastic agents and high interpatient variability is observed in the pharmacokinetics parameters of capecitabine and its metabolites. Therefore, differences in efficacy and toxicity profile are indicated among individuals (Ribelles et al. 2008). We eveluated the association of SNP in CES1, involved in the metabolism of capecitabine, with pharmacokinetic and adverse effects of capecitabine in breast and colorectal cancer patients and demonstrated the effect of interindividual differences.

A routine standard chemotherapy regimen of 1000-1250 mg/ m^2, perorally twice daily for 14 days followed by a 7-day rest period, was applied to the patients. Large interpatient variability in the pharmacokinetics of capecitabine was observed despite body surface area based dosing (Rudek et al. 2013). The mean AUC_{0-4h} of the capecitabine for the seven patients was 4.60±2.25 µg.h/ mL, which was similar to previous findings. After oral administration, capecitabine was rapidly absorbed from the gastrointestinal tract and reached peak plasma concentrations at approximately 0.3-3 hours. While the C_{max} of capecitabine is 4.47 mg/ mL (Xeloda Roche 500 mg Summary of Product Characteristics; Rudek et al. 2013), in our study, the C_{max} of capecitabine was 3.19±2.5 µg/ mL.

The observed AUC_{total} value of capecitabine in our study was highly variable between patients, but it was similar to previous findings (27-89%). The reason for differences in the pharmacokinetics of the capecitabine among individuals was different expression of enzymes involved in the metabolism (Lam et al. 2016). Capecitabine is metabolized to 5-FU by three-step enzymatic pathways. It is converted into 5'-DFCR in the liver by CES. The 5'-DFCR is then converted to 5'-DFUR by CDA which is found in the liver and tumor tissues. 5-FU formation occurs on the tumor area with the basis of TYMP (Thorn et al. 2011; Daniele et al. 2013).

Genetic variation in CES enzymes may lead to changes in the inactivation of drugs and activation of prodrugs and contribute to adverse drug reaction and/or increased sensitivity/resistance to drug treatment. The majority of variations at the DNA level (over 90%) are in the form of single-nucleotide polymorphisms (Langmann et al. 1997; Kim et al. 2003; Marsha et al. 2004). The SNP rs8192950 in CES1, which has a mutation frequency of approximately 64% in the European population, was selected in this study. The mutation of rs8192950 in CES1 is considered an association with a decreased CES1 enzyme activity. It has been asserted that the mutation of CES1 rs8192950 decreases CES1 enzyme activity, resulting in more clopidogrel to be converted into active metabolites and was associated with the decreased recurrence of ischemic events (Zhao et al. 2016).

The mean AUC_{0-4h} of the four heterozygote (A/C) and mutant (A/A) rs8192950 allele carriers is 5.11±1.1 µg.h/ mL, the mean AUC_{0-4h} of the two non-carriers (C/C) is 3.29±0.6 µg.h/ mL. The mean AUC_{0-4h} values of the mutant and heterozygote patients were 1.5 times higher than the AUC_{0-4h} values of the wild type. However, no statistically significant differences between the CES1 gene mutation (rs8192950) and the AUC values were observed (p=0.2236). This is the first study in which the relationship between CES1 rs8192950 mutation and pharmacokinetics of capecitabine has been indicated.

Dose limiting toxicities of capecitabine include diarrhoea, abdominal pain, nausea, stomatitis and hand-foot syndrome (Reigner et al. 2001). At the end of the first cycle, the most frequently observed side effects were diarrhea, asthenia, anemia, emesis; however hand-foot syndrome, stomatitis were not reported in our study. No statistically significant differences between genotypes and the most frequently observed side effects were observed. The low number of patients in our study is the most important reason for the lack of statistical significance between genotypes and observed side effects.

Some previous studies have evaluated the association of SNPs in CES, involved in the metabolism of capecitabine, with efficacy and toxicity. Hamzic et al. (2017) evaluated the association of genetic variability in CES1 and CDA in 144 patients treated with capecitabine. CES1 c.690+129delC (rs3217164) and c.1165-41C>T (rs2244614) was revealed as significantly associated with overall capecitabine toxicity. This is the first study identifying an association of genetic variation in CES1 with capecitabine related toxicity (Hamzic et al. 2017). In contrast, Pellicer et al. (2017) conducted a study in which 23 selected SNPs in 8 enzymes (CDA, DPD, ENOSF1, CES1, TYMS, SLC22A7, TYMP, UMPS) were analyzed in 301 colorectal cancer patients treated with capecitabine-based chemotherapy. No association was reported between CES1 SNPs and the risk of capecitabine related toxicity (Pellicer et al. 2017). Therefore, additional studies are required to support the association of genetic variation in CES1 with the efficacy and toxicity of capecitabine, and the results will need to be confirmed by larger studies.

CONCLUSION

Based on the results of our study, no statistically significant differences between CES1 gene mutation (rs8192950) and

AUC_{total} values and the most frequently observed side effects were observed. As mentioned above, the low number of enrolling patients in our study may be the cause of the lack of statistically significant differences. This correlation could not be completely revealed in most of the published research regarding the association between efficacy and toxicity of capecitabine and other substrate drugs with SNPs in CES1 enzyme. Therefore, additional study in larger groups of patients is required to support our study. In conclusion, this is the first study evaluating an association of genetic variation in CES1 (rs8192950) with pharmacokinetic and adverse effects of capecitabine. Our study will offer an insight into the pharmacogenetic researches to personalized chemotherapy and will provide basic knowledge to related studies.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Acibadem University Hospitals (ATADEK), Istanbul, Turkey (No: ATADEK 2015/9).

Informed Consent: Written informed consent was obtained from all patients in this study.

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Original Article

Ethics in pharmacy education: a research on pharmacy students' ethical awareness and views

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ABSTRACT

This study was conducted to determine the ethical awareness of pharmacy students. Additionally, it intends to find the similarities and differences between ethical orientations of the pharmacy students having done the course "Pharmacy Deontology" or not. Moreover, the views and evaluations of the students who have completed the course are determined. Two hundred pharmacy students from Istanbul University Faculty of Pharmacy participated in the research. The research data was collected through questionnaires prepared by the researchers based on Deontology Charter by the Turkish Pharmacists' Association and the Pharmacies and Pharmacists Law no. 6197 and analyzed via t-test. The general results reflect a great deal of ethical awareness except the issues such as, providing prescription medicine and non-medical health products, promotional activities and use of advertisements. Moreover, there is a statistical significant difference between the two groups' of ethical orientations (participants having completed the course or not). As an overall evaluation, the pharmacy deontology course seems to add value for pharmacicst candidates to make ethical decisions.

Keywords: Ethics, pharmacy, pharmacy education, pharmacy ethics education, pharmacy deontology

INTRODUCTION

Pharmacy, as a profession, is one of the most important professions in the health care area. Patients can easily gain access to the pharmacists. Therefore, pharmacists as health professionals consulting directly with patients, who explain the use and storage of medicine, should know how to deal with the patients in a proper way. Today not only communication between patients and pharmacists is important, but also patients' rights. One of the most important topics is "ethics" in developing new programmes. Due to these developments, it is crucial for pharmacy students to get the necessary information regarding pharmaceutical ethics and it is a requirement for pharmacy students to have strong skills in replying to and dealing with the ethical dilemmas they may face during their careers. So the objective of this research is to determine the ethical awareness of the students and compare ethical awareness of the students having had the pharmacy deontology course or not. Moreover, the research intends to determine the course's effectiveness based on the course participants' views.

LITERATURE REVIEW

Basic Terms: Ethics, Moral and Deontology

The term "ethics" derives from the word "ethicos" in Greek. "Ethos" means character and tradition. Ethics, as a definition, is a philosophical system trying to explain morality and determine what is good and bad, right and wrong, virtuous and just (Rose 2008). In other words, ethics is a philosophy of moral.

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Morality involves all the restrictive rules of the society those are derived from, the difference between right and wrong, good and bad. Therefore, morality can be expressed as the required beliefs for a good life. The term morality is unique for human beings both consciously and emotionally. It distinguishes human beings from the other living things. The three basic approaches for morality involves the following (Öztürk 2006):

- Morality has a religious basis and derives from the nature of human beings.
- Morality has a philosophical basis and is based on senses.
- Morality has a society basis. That means people have to obey several rules in order to be legitimate from the side of the society.

In the literature, the terms "ethics" and "morality" are usually used interchangeably although they have differences. Firstly, ethics is the philosopy of moral whereas morality is the research subject of ethics. Secondly, morality deals with more tangible concepts. On the other hand, ethics has both tangible and intangible concepts. Finally, morality may change from person to person whereas ethics for the term "desired good" is universal.

The term "deontology" derives from the words "deontos" and "logos" in Greek. Deontos means duty, the things that ought to be done and logos means science. So deontology can be defined as the science of duty or responsibility.

Considering the rapid change in many working environments, including the health industry, there are plenty of unanswered ethical dilemmas professionals face. From the side of the pharmacy field, the pharmacists have to obey the rules in the Deontology Charter by Turkish Pharmacists' Association (date: 27.07.1968). Deontology is the information of liabilities which have several sanctions as a result of the current situation. On the other hand, ethics does not only look for the answers for the current situation, it also looks for the answers for the potential situations. Therefore, ethics and deontology complete each other. The relationship between ethics, morals and deontology is presented in Figure 1.

Ethical Theories

The history of ethics goes back to 2500 years ago and reaches back to Greek philosopher Aristoteles time. Ethical theories can be investigated in two categories namely "teleological" and "deontological" approaches.

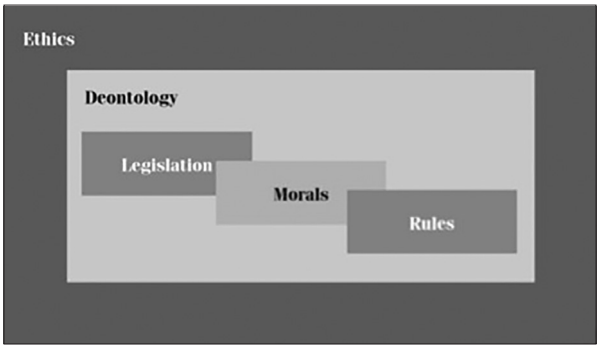

Figure 1. Relation between ethics, morals and deontology (Arda and Şahinoğlu 1995).

Teleological approach: The teleological approach primarily concerns itself with outcomes or ends since "telos" means the final purpose, issue or goal in Greek (Winstanley and Woodall 2000). According to the teleological approach, behaviors can not solely be classified as right or wrong. In order to evaluate behaviors, their results have to be evaluated. If the results are good, then the behavior is good.

A famous theory of teleological approach is utilitarianism. According to the utilitarian theory, one has a duty to calculate how much happiness various courses of action will generate, and to choose the course of action generating the most happiness (Buckley et al. 2001). In other words, utilitarianism offers the criterion of producing the greatest good for the greatest number of people (Greenwood 2002).

Deontological approach: The deontological approach involves several theories such as duty ethics, rights and justice theories. According to Kant's duty ethics, the majority of societies good can not be taken as an indicator to define behaviors as good or not (Bezci 2005). According to Kant, one must "act in such a way that you always treat humanity, whether in your own person or in the person of another, never simply as a means, but always at the same time." (Greenwood 2002)

On the other hand, rights theory involves protection of human rights on the basis of individuals or groups. Everybody has human rights and they must be treated in a respectful way (Aydın 2017).

All ethical theories intend to determine which behaviors, decisions or practices are ethical. However, they usually do not provide the same answer to the question "What is ethical?". As an overall evaluation, they provide necessary information to solve ethical concerns and dilemmas.

Medical Ethical Principles

Pharmacists are health professionals ensuring society health, prevention of diseases, delivery of medicine and support to patients to benefit from medicine. In the last two decades, the role of the pharmacy profession has inevitably changed, however the basic ethical principles are the same. These principles are being reviewed in order to define ethics in pharmacy. These principles are the guidelines for pharmacists, other health professionals, patients and society in order to ensure ethical behavior.

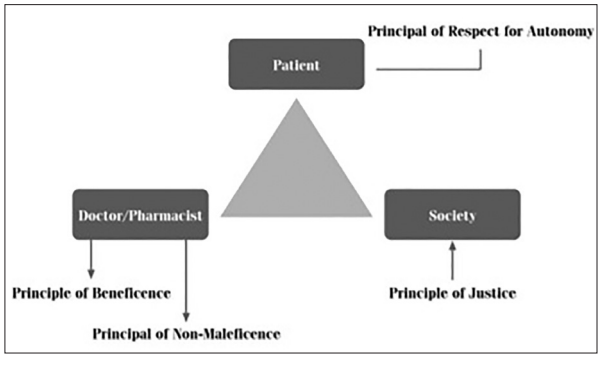

Figure 2. Relation between ethical principles (Arda and Şahinoğlu 1995).

According to Beauchamp and Childress (1979), the basic medical ethical principles are; respect for autonomy, non-maleficence, beneficence and justice (See Figure 2). First of all, the principle of respect for autonomy requires respectful treatment in disclosing information, ensuring understanding and voluntariness and fostering autonomous decision-making. In the health industry, it is the right of the patient to choose the treatment method, the medicine, the doctor and the pharmacy with free will after being provided with the necessary information. Additionally, according to the principle of non-maleficence, people ought not to hurt other people mentally or physically. Beneficence reveals people ought to do and promote good. Finally, the principle of justice emphasises equal access to the good in life that every rational person values (Beauchamp and Childress 2001). Of all the four principles, justice has a crucial role in ethics for the the right to access medicine in an equal way (Özçelikay et al. 1996).

Pharmacy Ethics: Ethical Concerns and Dilemmas

Due to that, pharmacy as a field, includes ethical considerations in nature and pharmacists have to ask themselves what to do and why they should do this. So it is clear that pharmacists are directly related to deontology and ethics (Weinstein 1993).

Pharmacy ethics deals with the discussions, explanations and solutions of value concerns which pharmacists face in practice. Therefore, pharmacy ethics aims to define attitudes and behaviors of pharmacists when they face ethical dilemmas and determine the right attitudes and behaviors in specific situations (Weinstein 1993).

Ethical concerns of the pharmacists can be summarized as follows (Kuçuradi 1993):

Providing information to the patient: This concern deals with not only telling the truth, but also providing useful and supportive information.

Privacy: This is about the limits pharmacists should express information regarding the patient's situation to third parties such as doctors, other pharmacists or to anyone. Secret information provided to third parties could result with ethical concerns.

Choosing the product: In pharmacy, it is possible to have several ethical problems due to the products chosen for sale. Those problems may include, providing generic drugs, medicine with insufficient information, hand-made medicine which is not approved by the Health Ministry and selling medicine with a close expiry date.

Objectivity: Nowadays price policies implemented by offical foundations conflict with the principal of objectivity in Turkey. Additionally, there is a serious gap between retail sales prices and offical foundation prices due to the fact that producers may allow a significant discount to offical foundations. These are all against objectivity and fairness principles.

Relations with the producers: The pharmaceutical companies or the distributers may use persuasive techniques in order to make their products preferred. If the pharmacists accept promotional gifts in an exchange of medical advice or recommendations, this is a serious ethical problem.

As an overall evaluation, pharmacists usually face ethical concerns and they have to decide what the right behavior is. So ethical dilemmas which occur when there is a conflict between ethical behavior and principles can be expressed from the side of the pharmacists as follow (Asil 1991):

Ethical dilemmas regarding recipes:

- Preparing a recipe which threathens health.
- Preparing a recipe involving a medicine which is of no real value, even though the pharmacist is aware of an alternative medicine which will help.
- Suggesting the patient an alternative drug treatment if the recipe has a problem.
- Providing drug treatment without a recipe.

Ethical dilemmas regarding beliefs and values: Selling or preparing medicine against the pharmacist's own beliefs and values.

Ethical dilemmas between the doctor and the pharmacist:

- Providing the patient information regarding a diagnosis if the patient has not been provided with this information before.
- Reviewing a doctor's degree of sufficiency.
- Keeping information from the patient's knowledge due to the doctor's request.

Ethical dilemmas regarding product sale:

- Selling products with insufficient information.
- Selling habit-forming drugs.
- Selling products with no use.
- Giving someone or a group a reduction.

Ethical dilemmas regarding research ethics: Participating in research which provides low use for patients.

Ethical dilemmas regarding euthanasia: Preparing medicine for a patient regarding euthanasia and participating in the process.

Considering the above-mentioned ethical problems and dilemmas which pharmacists face in practice, ethics in pharmacy education aims to provide pharmacist candidates the required skills in determining and solving ethical problems as well as using ethical principles in a proper way. In this way, a research intending to measure pharmacy students' ethical orientations has been designed.

MATERIALS AND METHODS

Objective and Scope of the Research

This study intends to determine the ethical orientations of pharmacy students and compare the ethical awareness of the students who participated in the pharmacy deontology course and of those who did not. Additionally, participants of the course are asked to evaluate the course in several dimensions. Therefore, the other objective of the research is to determine the course's effectiveness from the student's perspective.

The research universe consists of the pharmacy students in Turkey. Our sample is limited to the students at Istanbul University Faculty of Pharmacy. One of the reasons why Istanbul University Faculty of Pharmacy is chosen is because of the high number of students being educated there. Secondly, the Faculty is one of the oldest faculties in Turkey. Additionally, researchers could easily access the Faculty's students.

From the total number of 1.190 students in 2017, questionnaires were distributed to 250 students. As a result, 200 questionnaires were fully filled out and used for analysis, whereas the other 50 questionnaires were excluded from the research due to missing answers and sections. The grade distribution of the sample is presented in Table 1.

As mentioned before, the objective of the research involves determining the difference regarding ethical awareness between the participants who participate in the course "Pharmacy deontology" and those who did not. Therefore, it should be expressed that 110 participants participated in the course and the remaining 140 participants did not yet participate in the course. The questionnaire also involves a question asking the participants if they participated in the course or not.

Table 1. Grade distribution of the sample

Grades	Distributed	Collected and Analyzed
1	60	45
2	40	30
3	40	35
4	50	45
5	60	45
Total	250	200

Limitations

This research inevitably has several limitations. Firstly, the scope of the research is limited to the students of Istanbul University, Faculty of Pharmacy. Therefore, it is not possible to generalize the results to all pharmacy students in Turkey. Secondly, as mentioned before, the participants are divided into two groups with consideration to whether they participated in the pharmacy deontology course or not. Furthermore the ethical awareness of the students in the two groups is compared. Undoubtedly, there may be several other variables such as their personalities, their families' ethical orientations, and background, etc. affecting the ethical orientations of the students. However, it would be difficult to measure and control these variables' effects on their ethical orientations. Therefore, the differences between the two groups regarding ethical awareness are attached to whether they participated in the course or not.

Data Collection Method, Validity and Reliability

Research data has been collected through the questionnaire method. The questionnaire has been prepared by the researchers based on Deontology Charter by Turkish Pharmacists' Association and the Pharmacies and Pharmacists Law no. 6197 of 1953.

To assess content validity, 2 experts in the field of ethics and pharmacy reviewed and evaluated the items. The objective was to enhance readability, clarity and relevance of the items. Then the initial questionnaire was finally given to 10 pharmacy students who were different from the participants of the research sample to test the items. Depending on the feedback

Table 2. Ethical awareness of pharmacy students in general

Items	Strongly agree	Agree	Neither disagree nor agree	Disagree	Strongly disagree
(N= 200)			%		
1. I think pharmacy technicians can recommend drugs to the patients.	3.5	11	15.5	39	31
2. If it is urgent, I can deliver a prescription medicine without a recipe.	3	19	29	27.5	21.5
3. I think it is not necessary for a pharmacist to be always in the pharmacy.	8.5	13	9.5	39	30
4. I think it is unfavorable to share patients' information with demanding firms and individuals (except legal cases)	52.5	21	5.5	11	9
5. I can deliver the drugs to the patient without contacting the doctor even if I am in doubt about that there is a mistake in the recipe.	3.5	2.5	4.5	31.5	43
6. It is ok for me to recommend the alternative drugs because of that they are more expensive.	6	12	24	35	26
7. In today's competitive work environment, it is possible to have promotional activities in the pharmacy.	16.5	35.5	20.5	18.5	5
8. I can recommend the patients several non-medical health products other than medicine.	18.5	23	21.5	28	9
9. It is possible to use advertisements of non-medical products in the pharmacy.	13	45	18.5	15	8.5
10. I think it is right to sell non-medical health products in social media.	2	13,5	6	17	51.5

provided, the questionnaire had the final design which was applied to the participants between 01.04.2017 and 20.04.2017.

To estimate reliability, the Cronbach's alpha value has been calculated via SPSS 17. The Cronbach's alpha value is 0,868 revealing the scale exhibits good internal consistency.

RESULTS

The sample of the research consists of students from Istanbul University, Faculty of Pharmacy. Of the 200 pharmacy students participating in the research, 70 % are female and 20 % are younger than 20 years old. Nearly half of the participants (47 %) would like to own a pharmacy, whereas 20 % would like to work in pharmaceutical companies, 16 % would like to work in hospitals and 12 % would like to have an academic career in the future.

The findings of the research will be presented under three titles. These are the ethical awareness of the students in general, similarities and differences regarding ethical orientations between the two groups, and views and evaluations of the students who have the pharmacy deontology course.

Ethical awareness of the pharmacy students in general

First of all, findings regarding ethical awareness of the participants, regardless of whether they have done the pharmacy deontology course or not, are presented in Table 2.

Findings regarding several items (items 1, 3, 4, 5, 6, 10) reflect a great deal of ethical awareness whereas ethical awareness is generally low in providing prescription medicine and non-medical health products, promotional activities and the use of advertisements.

Table 3. T-test results indicating the similaritites and differences of the two groups' ethical awareness item by item

Pharmacy deontology course	N	$\bar{x}$	Ss	T	Sd	p
Item 1	*"I think pharmacy technicians can recommend drugs to the patients."*					
Yes	90	2.16	0.982	0.112	197.973	0. 911
No	110	2.17	1.188			No sig. difference
Item 2	*"If it is urgent, I can deliver a prescription medicine without a recipe."*					
Yes	90	2.46	1.172	1.195	198	0.234
No	110	2.65	1.072			No sig. difference
Item 3	*"I think it is not necessary for a pharmacist to be always in the pharmacy."*					
Yes	90	2.56	1.126	3.086	197.382	0.002
No	110	2.03	1.303			Sig. difference p< 0,05
Item 4	*"I think it is unfavorable to share patients' information with demanding firms and individuals (except legal cases)."*					
Yes	90	3.94	1.328	0.716	198	0.475
No	110	4.08	1.377			No sig. difference
Item 5	*"I can deliver the drugs to the patient without contacting the doctor even if I am in doubt about that there is a mistake in the recipe."*					
Yes	90	1.97	1.136	-3.088	198	0.002
No	110	1.54	0.831			Sig. difference p< 0,05
Item 6	*"It is ok for me to recommend the alternative drugs even if they are more expensive."*					
Yes	90	2.47	1.210	-0.388	198	0.698
No	110	2.40	1.205			No sig. difference
Item 7	*"In today's competitive work environment, it is possible to have promotional activities in the pharmacy."*					
Yes	90	3.61	1.154	2.871	198	0.005
No	110	3.17	1.154			Sig. difference p<0,05
Item 8	*"I can recommend the patients several non-medical health products other than medicine."*					
Yes	90	2.45	1.178	-5.408	191.149	0.00
No	110	3.34	1.163			Sig. difference p< 0,05
Item 9	*"It is possible to use advertisements of non-medical products in the pharmacy."*					
Yes	90	3.30	1.240	0.498	198	0.619
No	110	3.38	1.084			No sig. difference
Item 10	*"I think it is right to sell non-medical health products in social media."*					
Yes	90	1.78	1.139	-0.790	198	0.430
No	110	1.65	1.062			No sig. difference

Table 4. General t-test results indicating the difference between the two groups

Pharmacy deontology course	N	$\bar{x}$	Ss	T	Sd	p
Yes	90	2.55	0.440	0.104	198	0. 001
No	110	2.54	0.445			
Total	200					Sig. Difference p<0.05

Table 5. Ethical awareness of pharmacy students in general

Items	Strongly agree	Agree	Neither disagree nor agree	Disagree	Strongly disagree
(N= 90)			%		
Pharmacy deontology course is necessary in pharmacy education.	50	43.3	3.3	1.1	2.2
Ethical rules are crucial in pharmacy.	53.3	43.3	1	1.1	1.1
Time devoted for pharmacy deontology course is sufficient.	31.1	51.1	10	2.2	5.5
The lecturer of pharmacy deontology course ought to be an expert in this area.	53.3	33.3	4.4	1.1	4.4
Pharmacy deontology course is an effective course.	31.1	38.8	20	7.7	2.2
The information provided in pharmacy deontology course is useful in practice.	33.3	46.6	7.7	0	4.4
Information regarding ethics in pharmancy adds value to pharmacy profession.	41.1	48.8	5.5	2.2	2.2

Similarities and differences regarding ethical orientations between the two groups

Secondly, the findings on ethical awareness indicating the difference between the participants who have done the course and who have not got the course yet are presented item by item in Table 3.

Examining the findings in Table 3: First of all it is seen that there is a statistical significant difference between the two groups (the participants having the course or not) regarding the pharmacists' presence in the pharmacy (See item 3 in Table 3). According to Article 35 of the Pharmacies and Pharmacists Law no. 6197, the pharmacists ought to be always in the pharmacy. However, awareness regarding this article is low in the participants who have not done the course, whereas the participants of the course have a greater awareness regarding the issue indicating the effectiveness of the pharmacy deontology course.

Secondly, there is a significant difference between the two groups in what they ought to do when there seems to be a mistake in the recipe. According to Article 24 of the Pharmacies and Pharmacists Law no. 6197, the pharmacists ought to contact the doctor when they think that there is a mistake in the recipe (See item 5 in Table 3). On the other hand, awareness of the participants without having the course is lower than the other group.

Finally, the two groups' ethical awareness related to promotional activities and recommending the patients non-medical health products statistically diverge (See items 7 and 8 in Table 3). The participants of the course are more aware of the regulations in Deontology Charter by Turkish Pharmacists' Association (Articles 5 and 8) whereas awareness of the other group limited.

Overall, above mentioned findings reveal statistical differences between the two groups' ethical awareness when examined item by item. On the other hand, the research also investigated if there is a difference between the two groups' (participated in the course or did not) ethical awareness as a whole. The results can be seen in Table 4.

The findings in Table 4 indicate a statistical significant difference between the two groups' ethical awareness. Therefore we can conclude that the pharmacy deontology course adds value to pharmacy students' ethical orientations and awareness.

Views and evaluations of the students who have pharmacy deontology course

Finally, the views and evaluations of the pharmacy students who participated in the pharmacy deontology course are investigated in the research. The findings regarding this issue are presented in Table 5.

Depending on the findings, we can say that students who have participated in the pharmacy deontology course generally have positive views and evaluations regarding the course. On the other hand, 29 % of the participants can not express that the course is effective.

CONCLUSION

Today patients' rights are heavily regarded and valued and the nature of the relationship between health professionals and the patients has heavily changed. Due to these developments, there seems to be a problematic environment in terms of trust and peace between the parties. Therefore, ethical principles and rules are required to ensure a trustful and peaceful environment.

Pharmacists, just like the other health professionals, face ethical concerns in practice and they have to respond to these ethical concerns and dilemmas in a proper way. Undoubtedly, ethical orientations in pharmacy education will help and support pharmacist candidates to make ethical decisions.

This study intends to determine ethical awareness of pharmacy students and to compare the ethical orientations of the students who participated in and who did not participate in the pharmacy deontology course. Additionally, the course participants' views and evaluations regarding the course are presented. Depending on the research results, we can say that general ethical awareness of the pharmacy students is quite high. On the other hand, the most problematic issues are providing prescription medicine without a recipe, recommending non-medical health products, promotional activities and the use of advertisements in general.

The presence of the pharmacy deontology course seems to be benefical to make students aware that the pharmacists ought to be in the pharmacy consistently, contacting the doctor with any medical enquiries or suspicions. The course also helps to create awareness around promotional activities as well as recommending non-medical health products other than medicine that are deemed unethical.

As an overall evaluation, the pharmacy deontology course is benefical for pharmacist candidates and the findings in this study support the presence of this course in the cirriculum. In other words, universities strengthen their pharmacy cirriculum when using the pharmacy deontology course.

It was emphasized that this study inevitably has limitations. Considering the current limitations of this research, the scope of the study may be expanded to several other universities' with pharmacy courses. Thus, it will be possible to make comparisons between the ethical awareness of different universities' pharmacy students. Other limitations and variables involved in the current research, that may affect ethical orientations of students include their families' ethical orientations and their backrounds which can be controlled in future research.

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Original Article

Chemical characterization of *Glaucosciadium cordifolium* (Boiss.) B. L. Burtt & P. H. Davis essential oils and their antimicrobial, and antioxidant activities

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ABSTRACT

Chemical composition of volatile oils obtained from the roots, fruits and aerial parts of *Glaucosciadium cordifolium* (Boiss.) B.L. Burtt&P.H. Davis (Apiaceae) were analyzed using gas chromatography-flame ionization detector/mass spectrometry, simultaneously. Furthermore, antimicrobial and antioxidant activities of *G. cordifolium* volatile oils were investigated for possible utilization. Total of 62 volatile compounds were identified in *G. cordifolium* essential oils, where the main component was characterized as α-pinene in all parts, commonly. The other main components were β-pinene (15.7%), (*Z*)-β-ocimene (14%) and sabinene (7%) in the volatile oil of the aerial part; sabinene (10.1%), β-pinene (10.1%) and α-phellandrene (5.3%) in the essential oil of the fruits; hexadecane (12.2%), tetradecane (11.9%), octadecane (7.4%) in the essential oil obtained from the root, respectively. The *in vitro* microdilution method was used for the antimicrobial activity testing against *Salmonella typhi* ATCC 6539, *Acinetobacter baumanii* ATCC 19606, *Bacillus cereus* ATCC 14579, *Staphylococcus aereus* ATCC 6538, *Listeria monocytogenes* ATCC 19115, *Helicobacter pylori* ATCC 43504 and *Mycobacterium avium* ATCC 25291. The best antimicrobial activity of the volatile oils was against *L. monocytogenes* among the tested microorganisms. In addition, DPPH•-ABTS• scavenging activity was tested, none of the essential oils showed any significant antioxidant activity.

Keywords: Apiaceae, *Glaucosciadium cordifolium,* antimicrobial, antioxidant, gas chromatography, mass specrometry

INTRODUCTION

Glaucosciadium cordifolium (Boiss.) B. L. Burtt & P. H. Davis was used as an aphrodisiac in traditional medicine and known as "sakar otu" or "çakşır otu" in Turkey (Özhatay and Koçak 2011). According to the Flora of Turkey, the genus *Glaucosciadium* Burtt & Davis is represented by one taxon in Turkey and two taxa in the world (Davis 1982).

G. cordifolium has a charasteristic smell and grows in stony river banks, chalk screes and slopes (Davis 1982). This species is distributed in Central Anatolia, Mediterranean region and Northern Cyprus. Although the volatile oil composition of *G. cordifolium* aerial parts has been investigated previously (Baser et al. 2000), so far the volatile oil compositions of *G. cordifolium* fruits and roots have not been analyzed. Here, we report the comparative essential oil compositions of the aerial parts, fruits and roots of *G. cordifolium* by using gas chromatography-mass spectrometry (GC-MS) and flame ionization detector (FID) systems. In addition, antimicrobial-antioxidant activities of aforementioned volatile oils were studied by DPPH and ABTS radical scavenging and broth microdilution methods, respectively.

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To the best of our knowledge, this is the first report on the roots and fruits volatiles and antioxidant-antimicrobial activities of the volatile oils from different parts of *G. cordifolium*. The volatile oils were obtained by hydrodistillation method followed by the *in vitro* biological evaluation using various human pathogens and DPPH and ABTS as targets.

MATERIALS AND METHODS

Plant Material

The aerial parts, fruits and roots of *G. cordifolium* were collected in the vicinity of Karaman-Ermenek in September 2018. The voucher specimen has been deposited at the Herbarium of the Selcuk University (KNYA), Konya, Turkey (Voucher specimen no: 28001).

Distillation

Air dried aerial parts, fruits and roots were coarsely crushed and hydrodistilled using a Clevenger apparatus, separately. The aerial parts, fruit and root oils obtained in 0.5%, 0.4% and 0.2% yield were dried using anhydrous sodium sulfate and kept at 4°C until GC and GC-MS analyses as well as biological assays, respectively.

Chromatospectral Analyses

The Agilent 5975 GC-MSD system was used for GC-MS studies. Innowax FSC column with 60 m x 0.25 mm, 0.25 µm film dimensions and helium with 0.8 mL/min rate were used. GC oven conditions were set as follows; 60°C for 10 minutes, 220°C with 4°C/min ascending rate, 220°C for 10 minutes and 240°C with 1°C/min ascending rate along with split ratio of 40:1 and 250°C injector temperature. Mass spectra measurements were performed at 70 eV with *m/z* 35 to 450 range.

An Agilent 6890N GC system was used for the GC-FID analyses. The temperature of the FID detector was set to 300°C. Concurrent auto-injection was performed in two identical columns using the same conditions in the GC/MS system. Relative percentages (%) were calculated using FID chromatograms (see Table 1). Relative retention indices were used to characterize the essential oil components. This process was held either by authentic samples or analyzing relative retention index (RRI) of n-alkanes, along with GC/MS Library, MassFinder 3 Library, in-house "Başer Library of Essential Oil Constituents"(ESO 1999).

Antimicrobial Activity

The antimicrobial activity of the essential oil was determined using the broth microdilution assay (CLSI 2006).

Salmonella typhi ATCC 6539, *Acinetobacter baumanii* ATCC 19606, *Bacillus cereus* ATCC 14579, *Staphylococcus aereus* ATCC 6538, and *Listeria monocytogenes* ATCC 19115 strains were grown in Mueller Hinton Broth (MHB, Merck, Germany). All microorganisms were standardized to 1×10^8 CFU/mL using McFarland No: 0.5 in sterile saline (0.85%). Serial dilutions were prepared from the sample. Each strain along with the diluted samples were added to the wells and then allowed to incubate at 37°C for 24 hours.

Helicobacter pylori ATCC 43504 were grown for 24 hours in Brucella broth containing %5 (v/v) horse blood Colombia agar and containing %10 (h/h) fetal bovine serum (FBS) at 37°C in an anaerobic incubator (%5 CO_2). After the plates had been incubated at 37°C, 100 µL of 1:10 diluted and density modulated *H. pylori*'s strain was added to each microtitration petris (EUCAST 2011; Whitmire and Merrell 2012).

Mycobacterium strains were inoculated in Middlebrook 7H11 agar (Sigma Aldrich), and incubated in aerobic conditions at 37°C for 4-5 days. The microorganism was transferred to the cation doped MHB and incubated for a further five days. Growing cultures were vortexed and allowed to collapse for 30 min. Diluted bacterial suspensions (10^6 CFU/mL) were added to each well and then allowed to incubate at 37°C for 5 days (CLSI 2003; Chung et al. 1995; Lee et al. 2007).

The minimum inhibitory concentrations (MIC) were calculated as mean of three repetitions.

Antioxidant Activity

DPPH radical scavenging assay

The antioxidant capacity was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH·) scavenging activity (Blois 1958). The reaction mix contained 100 µM DPPH· and several concentrations of the crude extract. After 30 min, absorbance was read at 517 nm by using an UV–Vis spectrophotometer at 25±2°C and the radical scavenging activity (RSA) was determined as the percentage of radical reduction as follows:

$$DPPH^{\cdot}\ RSA\ \% = [(Absorbance_{control} - Absorbance_{test\ sample})/Absorbance_{control})] \times 100$$

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

ABTS radical scavenging assay

For the second method ABTS RSA is used to determine the antioxidant activity of the essential oils (Re et al. 1999). ABTS radicals were produced by reacting 7 mM aqueous ABTS radical and 2.45 mM potassium persulfate. The mixture was left at 25°C for 12 h in the dark. The colored ABTS· was diluted with ethanol. Absorbance was measured at 734 nm. The assay was performed in triplicate. Ethanol was used as the negative control. The assay was carried out on Trolox as a positive control, the water-soluble α-tocopherol analogue. The results were expressed as IC_{50} as follows:

$$ABTS^{\cdot}\ RSA\ \% = [(Absorbance_{control} - Absorbance_{test\ sample})/Absorbance_{control})] \times 100$$

RESULTS AND DISCUSSION

The air dried root material was hydrodistilled in a Clevenger-type apparatus for 6 hours to yield a dark yellow oil. Aerial part and fruit materials have light yellow oils and hydrodistilling procedures same as the roots. The *G. cordifolium aerial part, fruit and root* oils yield were 0.5% (v/w), 0.4% (v/w), 0.2% (v/w), respectively which were consquently analyzed both by GC-FID and GC-MS, simultaneously. Sixty-two compounds were identified in *G. cordifolium* essential oils obtained from different parts constituting

Table 1. Essential oil components of *G. cordifolium*

RRI	Compound	GcH %	GcF %	GcR %
1000	Decane	0.2	-	tr
1032	α-Pinene	27.7	60.8	18.4
1035	α-Thujene	1.1	0.3	tr
1076	Camphene	0.3	0.4	tr
1100	Undecane	-	-	0.3
1118	β-Pinene	15.7	6.8	0.4
1132	Sabinene	7.0	10.1	0.7
1174	Myrcene	3.0	2.8	1.2
1176	α-Phellandrene	6.0	5.3	0.3
1200	Dodecane	-	-	5.4
1203	Limonene	2.5	1.7	2.0
1218	β-Phellandrene	2.8	1.9	1.2
1246	(*Z*)-β-Ocimene	14.0	2.7	1.7
1255	γ-Terpinene	0.2	0.1	0.5
1266	(*E*)-β-Ocimene	2.9	0.3	0.5
1280	p-Cymene	1.8	1.5	2.3
1290	Terpinolene	0.2	0.1	1.6
1296	Octanal	-	-	0.3
1300	Tridecane	0.1	-	0.4
1400	Nonanal	-	-	0.3
1400	Tetradecane	-	-	11.9
1438	Tetradec-1-ene	-	-	0.5
1476	(*Z*)-β-Ocimene epoxide	0.2	-	-
1477	4,8-Epoxyterpinolene	-	-	0.3
1497	α-Copaene	-	0.3	-
1500	Pentadecane	-	-	1.0
1549	β-Cubebene	-	0.1	-
1600	β-Elemene	0.1	0.4	-
1600	Hexadecane	-	-	12.2
1611	Terpinen-4-ol	0.2	0.1	-
1612	β-Caryophyllene	2.1	0.3	-
1648	Myrtenal	0.2	-	-
1650	γ-Elemene	-	tr	-
1655	(*E*)-2-Decenal	-	-	0.9
1668	(*Z*)-β-Farnesene	-	0.2	-
1670	*trans*-Pinocarveol	0.2	0.1	-
1683	*trans*-Verbenol	-	0.3	1.0
1687	α-Humulene	-	0.2	-
1700	Heptadecane	-	-	0.1
1726	Germacrene D	-	0.6	-
1773	δ-Cadinene	0.2	0.7	-
1786	ar-Curcumene	-	0.2	-
1800	Octadecane	-	-	7.4
1804	Myrtenol	0.1	-	-
1823	*p*-Mentha-1(7), 5-dien-2-ol	0.1	0.2	-
1854	Germacrene-B	-	0.2	-
1864	*p*-Cymen-8-ol	-	-	0.8
1933	Tetradecanal	0.4	-	-
2000	Eicosane	-	-	3.4
2008	Caryophyllene oxide	0.5	-	-
2144	Spathulenol	0.1	0.2	-
2200	Docosane	-	-	1.3
2209	T-Muurolol	0.3	-	-
2273	Selin-11-en-4≈-ol	-	0.3	-
2296	Myristicine	-	-	0.3
2384	Dill apiole	-	-	0.5
2400	Tetracosane	-	-	0.4
2512	Benzophenone	-	-	2.2
2554	(*E*)-3-Butylidene phthalide	1.5	-	-
2609	(*Z*)-3-Butylidene-3, 4-dihydro phthalide (=(*Z*)-Ligustilide)	4.8	-	-
2655	Benzyl benzoate	-	-	0.7
2931	Hexadecanoic acid	-	-	1.8
	Monoterpene Hydrocarbones	85.2	94.8	30.8
	Oxygenated Monoterpenes	1.0	0.7	2.1
	Sesquiterpene Hydrocarbones	2.4	3.2	-
	Oxygenated Sesquiterpenes	0.9	0.5	-
	Fatty acid+esters	-	-	1.8
	Others	7.0	-	49.5
	Total	96.5	99.2	84.2

RRI: Relative retention indices calculated against *n*-alkanes
% calculated from FID data
GcH: *G. cordifolium* aerial part essential oil; GcF: G. *cordifolium* fruit essential oil; GcR: *G. cordifolium* root essential oil

Table 2. Antioxidant activity of *G. cordifolium* essential oils

	GcH	GcF	GcR	References
	IC50 ±SD (mg/mL)			
DPPH•	1.14±0.086	1.02±0.07	1.18±0.052	0.004±0.001 (Ascorbic acid)
ABTS•	0.94±0.075	1.01±0.069	1.09±0.075	0.015±0.008 (Trolox)

GcH: *G. cordifolium* aerial part essential oil; GcF: *G. cordifolium* fruit essential oil; GcR: *G. cordifolium* root essential oil

84.2-99.2% of the total oil. The essential oils were dominated by monoterpene hydrocarbons. These sixty-two volatile compounds are listed in Table 1 with their relative percentages. Main components were found as α-pinene (27.7%), β-pinene (15.7%), (*Z*)-β-ocimene (14%), sabinene (7%) for aerial part; α-pinene (60.8%), sabinene (10.1%), β-pinene (10.1%), α-phellandrene (5.3%) for

Table 3. Antimicrobial activity of *G. cordifolium* essential oils (MICs in mg/mL)

Bacteria / Sample	*St*	*Sa*	*Lm*	*Ab*	*Hp*	*Bc*	*Ma*
GcH	>10	>10	0.156	>10	>10	0.625	>10
GcF	>10	>10	0.078	>10	>10	0.312	>10
GcR	>10	>10	0.078	>10	>10	1.25	>10

(+ control) Antimicrobial: Chloramfenicol
(- control) DMSO.
GcH: *G. cordifolium* aerial part essential oil; GcF: *G. cordifolium* fruit essential oil; GcR: *G. cordifolium* root essential oil; *St: Salmonella typhii; Sa: Staphyllococcus aureus; Lm: Listeria monocytogenes; Ab: Acinetobacter baumanii; Hp: Helicobacter pylori; Bc: Bacillus cereus; Ma: Mycobacterium avium*

fruit; α-pinene (18.4%), hexadecane (12.2%), tetradecane (11.9%), octadecane (7.4%) for root essential oil, respectively. In a previous study, limonene (39.7%), α-pinene (12.3%) and β-pinene (10.3%) were found as main components of the oil (0.7%) obtained from the aerial part of *G. cordifolium* (Baser et al. 2000). It can be thought that this is due to the collection of plant materials from different locations. It can be seen from the results, location differences in plants can change the phytochemistry of plants and hence biological activities. However, the essential oil of the aerial part includes phthalides such as (*Z*)-ligustilide (1.5%) and (*E*)-3-butylidene phthalide (4.8%) which are the important volatiles of *Apium graviolens* and some other Apiaceae plants. These compounds provide the characteristic odor of the celery specific to the plant.

Results of DPPH-ABTS radical scavenging activities are shown in Table 2. In DPPH testing system, RSA IC_{50} value of *G. cordifolium* aerial part, fruit and root essential oils were determined as 1.14, 1.02, and 1.18 mg/mL, respectively. When checked, in the ascorbic acid results (0.004 mg/mL) the oils were less effective than those of the standard ascorbic acid. In addition, the ABTS radical scavenging activity was also found at moderate levels (0.94, 1.01, and 1.09 mg/mL) and the results were compared with the standard Trolox (0.015 mg/mL).

Some pathogenic Gram (+) and (–) bacteria are listed in Table 3, were challenged with *G. cordifolium* essential oils. Among the tested bacteria in this present study, *L. monocytogenes* was the more sensitive to the essential oils, while *H. pylori* and *M. smegmatis* appeared to be the most resistant. Growth of *L. monocytogenes* was remarkably inhibited by essential oil of *G. cordifolium* aerial part, root and fruit parts. This present study results indicated that these volatile oils can be natural, potential antimicrobial agents in the food industry and improve the microbial safety of foods. The results of this study were promising for the use of these oils as an antimicrobial ingredient for the safety foodborne microorganisms.

As a conclusion, to the best of our knowledge, this is the first comparative report on the volatiles and *in vitro* antioxidant-antmicrobial activities of *G. cordifolium* aerial part, root and fruit essential oils.

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Original Article

The correlation between botanical source and the biologically active compounds of propolis

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ABSTRACT

In this research, five propolis samples collected from Turkey were investigated to observe the correlation between botanical sources and chemical contents of the samples and in this way emphasize the influence of botanical sources of propolis on its chemical characterization. As a first step, to determine the botanical sources of the samples, microscopic analysis was performed. According to the microscopic analysis results; two samples that were collected from Rize (P1 and P2), were characterized as most probably being chesnut propolis; while one sample collected from Tekirdağ (P3) was evaluated as being a mixed type, in the other Tekirdağ sample (P4), the pollens belonging to the taxa of the Brassicaceae family were found as dominant. The sample collected from Sivas (P5); was also recognized as mixed type. The second step of the research was the chemical analyses of the propolis samples. According to the results; the balsamic contents of the propolis samples ranged between 59.97 and 83.31%, total phenolic contents were ranged between 27.56±0.05 and 171.93±0.28 mgGAE/g. The minimum flavone and flavonol content of 0.28±0.01% was found in the P1 sample described as chesnut propolis and colected from Rize. The maximum value 5.1±0.07% was found in the P4 sample as was total phenolic content. Flavanones and Dihydroflavonols contents varied between 6.58±0.009-12.94±0.007%. According to the GC-MS results the investigated samples contained compounds belonging to the various groups. With regard to the Excel correllation, the balsamic content showed a negative correlation with total phenolic content, flavone and flavonol content, flavanones and dihydroflavonols content.

Keywords: Propolis, chestnut, microscopic, total phenolic, UV-Vis, GC-MS

INTRODUCTION

The increasing attention on natural products and alternative medicines has elevated the interest in bee products such as honey, royal jelly, pollen and propolis (Daleprane et al., 2013).

Propolis is a natural material that is collected by honeybees from the buds and exudates of trees and plants. It has been used in folk medicines in many regions of the world since ancient times (Jun, 2006). Honeybees enrich this material with their saliva and secretions and it is used in their hives for various purposes such as construction, adaptation, and protection (Daleprane et al. 2013).

The physically, propolis is a sticky, dark-colored material. Its colour varies from yellow-green to dark brown depending on its botanical source and its freshness. It is hard and fragile when it is taken from the refrigirator, but becomes soft and very sticky when it is at room temperature (Ghisalberti 1979).

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The major plant sources of propolis are poplar, birches, willows, chesnut, elms, pine trees, oaks, spruces and ashes (Bonvehi et al. 1994).

The chemical composition of propolis depends on the botanical source. However, despite the differantion of the botanical sources, propolis samples generally share many similarities in their overall composition, (Daleprane and Abdalla 2013). Generally, it is composed of 50% resin, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% other substances, including organic remains (Burdock 1998). The chemical compounds in propolis resin (raw propolis) are sourced from: plant exudate collected by bees, from bee metabolism, and materials which are introduced during propolis elaboration (Marcucci 1995).

Propolis contains polyphenols, terpenoids, steroids and amino acids (Daleprane and Abdalla, 2013). Flavonoids are the major group identified in propolis extract and which are ever-present in the plant kingdom (Burdock 1998). The pharmacological and antioxidant activities of propolis may be caused by flavonoids (Bonvehi et al. 1994).

Owing to their geographical divergences, propolis samples from Europe, South America and Asia have different chemical compositions. While propolis from Europe and China contains mostly flavonoids and phenolic acid esters, the major components in Brazilian propolis are terpenoids and prenylated derivatives of p-coumaric acids (Kumazawa et al. 2004).

Propolis has therapeutic activities and may have uses in the pharmaceutical and food processing industries. It exhibits many biological activities; immunomodulatory, antibacterial, fungicidal, anti-inflammatory, healing, analgesic/anesthetic, and anticarcinogenic effects (Daleprane and Abdalla 2013). Although it has a wide range of biological activities, there are no standards for its extraction procedure or its composition (Cunha et al. 2004).

The efficiency of propolis in therapeutics is related with its collection conditions and other parameters like microscopical, chemical and microbiological. The broad variableness in the chemical content of propolis makes these controls more necessary (Woisky and Salatino 1998).

The quality of propolis is related with its chemical composition and botanical source. The botanical, geographical origin and climatic conditions mostly affect the phenollic contents of the propolis. So, the description and quantification of the phenolics of propolis are important for detecting its quality (Gomez-Carvaca et al. 2006).

In this research we determined botanical origin, the total phenolic content, flavone/flavonol and flavanones/dihydroflavonols contents of five propolis samples to reflect the correlation between the botanical source and chemical contents of the propolis. Also the samples were compared in terms of their volatile compound contents according to the GC-MS analysis results.

MATERIALS AND METHODS

Sample collection

Propolis samples were collected from Rize (Black Sea Region-European-Siberian Phytogeographical Region-P1, P2), Tekirdağ (Thracian Region of Turkey- European-Siberian Phytogeographical Region-sample P3, P4), Sivas (East Anatolia Region-Irano-Turanian Phytogeographical Region-P5) in the fall season of 2016 (Table 1). The samples were collected from the edges of beehives with a spatula by local beekepers.

Microscopic analysis of propolis samples

For microscopic analysis the samples were prepared according to the method of Warakomska and Maciejewicz (1992) with some modifications.

The propolis samples were ground to a powder and this was added to the mixture of ethanol-chloroform-acetone (1:1:1) and vortexed. After this process, it was filtered and centrifuged at 3500-4000 rpm for 20 min. Then, the supernatant was poured. The slides were prepared from the sediment using glycerin gelatin.

Propolis extraction

The extraction procedure was carried out following Popova et al. (2007).

Balsamic content

From each crude sample, three ethanolic extracts were prepared. Two ml of each extract were evaporated and the balsamic contents were calculated according to the weight of the dry residues (Popova et al. 2007).

Estimation of total polyphenol content by the Folin-Ciocalteu Colorimetric Method

The total polyphenol content of EEP was determined using the Folin-Ciocalteu colourimetric method (Slinkard and Singleton 1977). Gallic acid was used as standard compound and the results were given as mg gallic acid equivalents (GAE) in g^{-1} of propolis extract.

Determination of flavone and flavonol content by UV-Vis Spectrophotometer

Flavone and flavonol content were determined according to Popova et al (2007). Quercetin was used as a reference compound.

Determination of flavanone and dihydroflavonol content by UV-Vis Spectrophotometer

1mL of the the ethanolic propolis extract and 2 mL of DNP (2,4-dinitrophenylhydrazine) were mixed and then diluted in

Table 1. Symbols, locations and collection dates of propolis samples

Sample no	Location	Collection dates
P1	Rize	Fall-2016
P2	Rize	Fall-2016
P3	Tekirdağ	Fall-2016
P4	Tekirdağ	Fall-2016
P5	Sivas	Fall-2016

Table 2. Pollen types recorded from the propolis samples and their frequency

Sample No Plant family	Plant taxa	P1	P2	P3	P4	P5
Apiaceae		R	R	R	R	S
Asteraceae		R	R	M	M	M
	Centaurea spp.	R	R	R		
	Helianthus annus			M		
	Taraxacum spp.			R	R	
	Xanthium spp.		R	R	R	
Berberidaceae						M
Betulaceae		R	R	R	R	M
	Carpinus spp.			R		
	Corylus spp.			R		
Boraginaceae				R		
	Anchusa spp.			R		
	Echium spp.		R			
	Heliotropium spp.		R			
Brassicaceae		R	R	S	D	M
Caryophyllaceae				R		
Chenopodiaceae			R	R		
cistaceae		R			R	
Dipsecaeea	*Scabiosa* spp.				R	
Ericaceae		R	R	R	R	M
Fabaceae		R	R	M	M	M
	Lathyrus spp.				R	
	Lotus spp.				R	M
	Medicago spp.			R	R	
	Onobrychis spp.			R	R	M
	Trifolium spp.		R	R	R	
	Vicia spp.			R	R	
Fagaceae	*Castaneae sativa*	D	D	M		
	Quercus spp.				R	
Geraniaceae			R		R	
Lamiacaea		R	R		R	
	Thymus spp.			R		
	Teucrium spp.				R	
Liliaceae			R	R	R	
Pinaceae			R	R	R	M
Platanaceae	*Platanus* spp.				R	
Poaceae			R	R		M
Plantaginacae	*Plantago* spp.			R		
Rosaceae		R	R	R	M	
Salicaceae	*Populus* spp.			R	R	M
	Salix spp.	R		S	M	M
Solanaceae					R	

*Pollen types recorded from the propolis samples and their frequency (D: dominant: >=45%, S: secondary: 16-44%, M: minor: 3-15%: R: rare <3%).

100 mL of methanol. The solution was heated at 50 ^{0}C for 50 min. The solution was diluted with 10% KOH. 10 mL methanol was added to the solution and again diluted to 25 mL with methanol. Naringenin was used as a reference compound.

Chemical analysis of the propolis samples by GC-MS

A GC 6890N instrument coupled with a mass detector (MS5973; Agilent) was used for analysis of the volatile compounds in the propolis samples. A DB 5MS column (30 m x 0.25 mm, 0.25

Table 3. Balsamic contents (%) and concentration of polyphenols (total phenols, flavones and flavonols; flavanones and dihydroflavonols) in propolis extracts

Propolis sample	Balsamic content (%)	Total phenolic content (mgGAE/gEEP)	Flavone and Flavonol Content (%)	Flavanones and Dihydroflavonols (%)
P1	59.97	36.36±0,14	0.28±0.01	9.21±0.01
P2	83.31	27.56±0.05	0.31±0.01	6.58±0.009
P3	29.15	144.03±0.32	4.6±0.03	10.54±0.005
P4	41.15	171.93±0.28	5.1±0.07	12.82±0.01
P5	70.47	28.7±0.14	4.43±0.03	12.94±0.007

Table 4. The identified compound groups from the propolis samples by GC-MS analysis

Compounds	P1	P2	P3	P4	P5
Alcohols	9.45	11.27	3.64	9.49	6.42
Aldehydes	5.46	3.67	0.06	0.77	0.79
Aliphatic acids and their esters	2.03	3.04	2.38	7.89	8.69
Carboxylic acids and their esters	32.97	0.98	3.28	1.11	-
Flavonoids	8.66	5,78	34.87	32.38	47.03
Hydrocarbons	3.42	2.63	-	0.37	0.42
Ketones		0.36	0.54	-	-
Cinnamic acids and their esters	-	-	-	5.76	3.51
Terpenes	2.62	0.16	1.19	0.65	0.44

µm film thickness) was used and Helium for the mobile phase. The compounds were identified in Wiley's NIST Mass Spectral Library (Gençay and Salih 2005).

RESULTS

Microscopic analysis results

According to the microscopic analysis results, the P1 and P2 samples were characterized as chesnut propolis, P3 as a mixed type containing Brassicaceae and *Salix* spp. pollens in remarkable ratios and these pollens were defined as secondary. The P4 sample contained pollen belonging to the Brassicaceae family in a dominant ratio (Table 2). The P5 sample could also be characterized as a mixed type.

The microscopic analysis results show the possible botanical sources of the propolis samples and also reflects the geographical source of the areas where the propolis was collected.

Chemical analysis results

The balsamic contents of the propolis samples varied between 29.15-83.31% (Table 3). The maximum value belonged to the P2 sample collected from Rize and characterized as chesnut propolis.

The total phenolic contents of the samples ranged between 27.56±0.05 and 171.93±0.28 mgGAE/gEEP (Table 3). The minimum value was found in the chesnut sample (P2) collected from Rize and the maximum value (171.93±0.28 mgGAE/gEEP) was found in the P4 sample that was mostly sourced from plants belonging to the Brassicaceae family.

The minimum flavone and flavonol content of 0.28±0.01% was found in the P1 sample described as chesnut propolis and collected from Rize. The maximum value (5.1±0.07%) was found in P4 sample as was the total phenolic content (Table 3).

The *flavanones and dihydroflavonols* contents changed between 6.58±0.009-12.94±0.007% (Table 3). The minimum value was found in the P2 sample and the maximum value was found in the P5 sample.

According to the C-MS analysis results, the five propolis samples investigated contained compounds belonging to the alcohols, aldehydes, aliphatic acids and their esters, carboxylic acids and their esters, flavonoids, hydrocarbons, ketones, cinnamic acids and their esters, and terpens groups (Table 4).

With respect to the volatile compound analysis results it is appeared that the P5 sample had the highest flavonoid content (47.03%) and was characterized as a mixed type propolis. The P3 and P4 samples also had considerably high flavonoid contents (34.87%-32.38%) with Brassicaceae pollen in secondary ratios. Moreover, the P1 and P2 samples characterized as chesnut propolis had a lower flavonoid content.

DISCUSSION

The determination of the plant taxa of pollen occuring in propolis samples, gives information about the vegetation surrounding the beehive and also the geographical region where the propolis was gathered (Barth 1998).

The research related with Turkish propolis is generally about its chemical characterization or usage areas. The number of investigations into propolis pollen analysis is very limited not just in Turkey but also in the world. Gençay (2004) investigated the botanical sources of Erzincan propolis located in the Irano-

Turanian Phytogeographic Region. They found mostly the taxa belong to the Apiaceae, Asteraceae, Campanulaceae, Fabaceae, Fagaceae, Lamiaceae, Liliaceae, Pinaceae, Rhamnaceae, Rosaceae, Salicaeae, Scrophulariaceae families as souces of Erzincan propolis. They also found the *Salix* spp. pollen in their investigated propolis samples. We found *Salix* spp. pollen in four of the five samples (P1, P3, P4, P5) and of these, the Tekirdağ sample (P3) contained *Salix* spp. pollen in secondary ratios.

Çelemli and Sorkun (2012) determined the botanical choices made by honeybees when collecting propolis in Tekirdağ by microscopic analysis and the results show that plants which belonged to the Asteraceae, Boraginaceae, Brassicaceae, Fabaceae and Salicaceae families were the plants of choice. These results are similiar to our findings.

Also on a global level, the palynological research into propolis is very limited. Barth (1998), analysed Brazilian propolis samples according to their pollen contents and found *Eucalyptus* spp., *Eupatorium* spp. and *Mimosa caesalpiniaefolia* pollens with dominant ratios in some of the investigated samples.

The percentage balsamic content is extremely important for propolis because if the amount of balsam is high, the wax content will be low. A high balsam content causes a higher amount of biologically active components. Popova et al. (2007) investigated some poplar propolis. They found the minimum balsamic content value as 18%, maximum value 82% and mean value 57%.

We found the minimum balsamic content value in the P3 sample (29.15%) and the maximum in the P2 sample (83.31%). According to the Excel correllation; the balsamic content results had a negative correllation with total phenolic (mgGAE/gEEP), Flavone and Flavonol , Flavanones and Dihydroflavonols contents.

Many compounds that are isolated from propolis as phenolics have important protective effects against oxidation reactions. Flavones, coumarines and other phenolics have a reducing activity, hydrogen donors and metal chelating properties.(Gülçin et al. 2010).

Bankova (2005) proposed that the total phenolics amounts are related with biological activity and are more informative that the quantification of individual components. It means that calculating the amounts of active compound groups is more effective than determining individual components.

As given in Table 3, the total phenolic compound of the investigated samples varies between 27.56±0.05 and 171.93±0.28 mgGAE/gEEP. According to our results the P1, P2, and P5 samples have lower total phenolic contents compared to the other two samples. The P1 and P2 samples were identified as chesnut propolis and P5 as a mixed type. The highest total phenolic content (171.93±0.28 mgGAE/gEEP) belonged to the P4 sample. Its possible botanical origin is observed as the taxa belonging to the Brassicaceae family and *Salix* spp. in particular.

Popova et al. (2005) studied the total Phenolic contents of some propolis from Turkey (Yozgat, İzmir, Kayseri, Adana, Erzurum and Artvin). The Yozgat , İzmir ve Kayseri samples described as typical poplar samples displayed very similiar phenolic and flavonoid content. The Adana, Erzurum and Artvin samples were characterized by low phenolic and very low flavonoid concentrations. Total phenolic contents were found as 26.4%, 30.4%, 27.5%, 8.2%, 10.5% and 14.5% respectively. These results are lower than ours. In further research concerning Turkish propolis Gülçin et al., (2010) found the total phenolic content of lyophilized aqueous extract of propolis from the Erzurum province of Turkey to be 124.3 µg (GAE)/g (LAEP).

Moreira et al. (2008) investigated the total phenolic content of one propolis type that contains 45% *Castanea sativa* pollen and found it to be 329 mgGAE/g. Yet in our samples that contain *Castane sativa* pollen in dominant ratios, there was a lower total phenolic content (27.56±0.05 and 36.6±0.14 mgGAE/g).

According to previous global research, the total phenolic contents of propolis from different countries can be summarized as: Argentina (187-212±9.2 mg/g), Australia (269±16.3 mg/g), Brazil (8.8-299 mg/g), Bulgaria (220±2.5 mg/g), Chile (210±11.1 mg/g), China (23.20-302±4.3 mg/g), Cyprus (85.7±5.1-100.4±7.2 mgCAE/g), Greece (146.2±7.2-338.5±13.2 mgCAE/g), Greek islands (80.2±3.2-146.2±10.2 mgCAE/g), Hungary (242±0.2 mg/g), India (159.10±0.26 mg/g), Iran (3.08 and 36%.), Korea (160.6±2.4-307.2±5.3 mg/g), New Zealand (237±6 mg/g), Portugal (151±0.01-329mg/g), South Africa (99.5±4.4 mg/g), Taiwan (210±20-335mgCE/g), Thailand (31.2±0.7 mg/g), Ukraine (255±7.4 mg/g), United States (256±15.7 mg/g), Uruguay (18.7- 187±8.5) mg/g) and Uzbekistan (174±6.7 mg/g) (Ahn et al. 2007, Bonvehi et al.1994, Chen et al. 2004, Choi et al. 2006, Choi et al. 2013, Cottica e al. 2011, Daleprane and Abdalla 2013, Kalogeropoulos et al. 2009, Kumazawa et al. 2004, Mohammadzadeh et al. 2007, Moreira et al. 2008, Popova et al. 2004, Woisky and Salatino 1998, Yaghoubi et al. 2007).

Popova et al (2007). analysed 114 poplar propolis samples and they found a minimum phenolic value of 4.6% and maximum 46% with a mean value of 28%. They also they found an indicative negative correlation between the total phenolics and the MIC values.

Sarıkaya et al. (2009) found the total phenolic contents of two chesnut propolis samples to be 313±9.48 mg/g and 476±4.78mg/g. In comparison with these results, our total phenolic results are too low for chesnut propolis samples (P1, P2).

With regard to the Excel correlation, the total phenolic contents had a positive corellation with flavone and flavonol content, *flavanones and dihydroflavonols* contents.

Colorimetric analysis is used for quantitative identification of flavonoids in propolis. The aluminium chloride method is used to calculate the flavone and flavonol content in propolis (Caravaca et al. 2006).

With regard to the spectrophotometric results , the flavone and flavonol contents of the propolis samples varied between

0.28±0.0.01 and 5.1±0.07 %. The P4 sample shows the maximum content for flavone and flavonol content as matching total phenolic content. The P1 and P2 samples show similarity in their flavone and flavonol contents (0.28±0.01, 0.31±0.01%) as well as their total phenolic contents. Although the P5 sample had a lower total phenolic content compared to the P3 and P4 samples, its flavone and flavonol content (4.43±0.03%) was very similiar to the P3 and P4 samples.

Poplar-type propolis from numerous countries was analysed by Popova et al. (2007) and they found the minimum flavones and flavonols value to be 1.3% and the maxium value to be 17.9%.

Popova et al. (2007) couldn't find any significant correlation between total flavones/flavonols and MIC values. Popova et al. (2005) studied flavone and flavonol contents of Yozgat, İzmir, Kayseri, Adana, Erzurum and Artvin samples. They found the values to be 8.7%, 9.6%, 5.6%, 1.5%, 2.0% and 2.0% respectively.

Trusheva et al., (2007) determined the total amounts of extracted total flavones and flavonols according to the different extraction methods . By maceration 72 h and with ratio of propolis solvent (1:20) and (1:10) were analysed and total flavones and flavonols were found as 8.6±0.1% and 8.8±0.1% respectively. By ultrasound extraction the value of total phenolics for 1:20 propolis/solvent 10 min was 9.4±0.2%, 1:20 propolis/solvent 30 min 9.4±0.2 % and 1:20 propolis/solvent 30 min 8.6±0.1%. MAE (Microwave assayed extraction)results were 9.6±0.8% for 1:20 propolis/solvent 2x10s was 9.3±0.1%;1:10 propolis/solvent 2x10s was 8.7±0.1%; 1:10 propolis/solvent 3x10s was 10.7±1.7%.

With regard to the Excel correlation, flavone and flavonol content had a positive correllation with flavanones and dihydroflavonols content.

To quantify flavanones and dihydroflavonols the DNP method was used. This is based upon the interaction of these compounds with DNP in acidic media to form coloured phenylhydrazones. The sum of the flavone and flavonol- flavanones and dihydroflavonols methods closely represents the real content of total flavonoids (Gomez-Caravaca et al. 2006).

The flavanones and dihydroflavonols content of the investigated samples were found between 6.58±0.0009 and 12.94±0.007%. The P5 sample showed the highest content and the P2 sample had the lowest content.

Popova et al. (2005) studied the flavanone and dihydroflavonol contents of some Turkish propolis. They found the values in the Yozgat sample was 6.0%, İzmir 5.5%, Kayseri 4.8%, Adana 2.7%, Erzurum 1.5% and Artvin 3.0%. With respect to our results these values are a bit lower.

In other research, Popova et al. (2007) found the flavanones and dihydroflavonols content minimum value 1.5%, the maximum value 15.2% and the mean value 6% in poplar-type propolis samples. Furthermore, they researched six propolis samples (two from Bulgaria, Two from Italy and Two from Switzerland) and they found the Flavanones and dihydroflavonols values between 4.8 and 7.1 mg/mL (Popova et al. 2004). These results are similiar to our results.

Kalogeropoulos et al. (2009) analysed 12 propolis samples from Greece, the Greek islands and East Cyprus using GC-MS. They found compounds belonging to the alcohols, aliphatic acids, phenolic acids and esters, anthraquinones, flavonoids, sugars and terpenes groups. The hignest ratios were observed in flavonoids groups with a maximum value of 37.18%.

Popova et al., (2005) carried out qualitative analysis of some Turkish propolis using GC-MS and found that the Adana sample contained diterpenic acids and a high amount of cinnamyl cinnamate, the Erzurum sample had expressive amounts of hydroxy fatty acids and triterpenic alcohols and the Artvin sample had phenolic glycerides, indicative of the *Populus euphratica* Oliv. bud exudates

Flavonoid compounds are more effective in the biological activities of propolis (Maciejewicz et al. 2001). Of the investigated samples, P5 had the highest flavonoid content (47.03%). Acccording to the microscopic analysis results for the P5 sample, it was observed as a mixed type propolis . The P4 sample had a content of flavonoids with a ratio of 34.87% and was sourced mostly from plants belonging to the Brassicaceae family. The P3 sample had a 34.87 flavonoid content, that was mostly sourced from plants belong to the Brassicaceae family and *Salix* xpp.. The two chestnut propolis samples (P1 and P2) had lower flavonoid contents and total phenolic, flavone-flavonol and flavanones-dihydroflavonols contents.

Maciejewicz et al. (2001) investigated five propolis samples from Poland and identified pinostrobin chalcone, pinocembrin, tetrochrysin, chrysin, galangin, 5-Hydroxy-4',7-dimethoxyflavone, pilloin and apigenin using GC-MS.

According to the literature the most commonly identified flavonoids in different propolis samples from the various countries were pinocembrin, tetrochrysin, chrysin, and galangin. The occurence of pilloin in propolis was reported firstly by Maciejewicz et al., (2001). Similiar to previous research, we found chrysin, tetrochrysin, pinostrobin and chalcone in the investigated samples.

CONCLUSION

The results obtained in our work allow a preliminary pollen characterization of Turkish propolis and its correlation with the content of biologically active compounds. It is observed that the balsamic content has no positive correllation with total phenolic, flavone-flavonol and flavanones-dihydroflavonols contents, but total phenolic contents have a positive corellation with flavone and flavonol content, flavanones and dihydroflavonols contents.

Total phenolic and flavone-flavonol contents were found highest in the sample that sourced from the taxa belonging to the Brassicaceae family, this was contrary to common belief as the chesnut propolis has higher phenolic contents.

Through this research, we determined the possible botanical sources, geographical origins and their influence on the chemical characterization of propolis samples. These results can be helpful for further research. To reach certain botanical sources and characterize propolis on a regional base, more samples are necesary for investigation.

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Original Article

Determination of aflatoxin B1 in cosmetics containing botanical ingredients by enzyme linked immunosorbent assay

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ABSTRACT

Botanicals are widely used in cosmetic products due to their rich chemical compositions. There are numerous factors that affect the chemical profile of botanical content including growing, harvesting, drying and storage conditions. The reliability of a cosmetic product containing botanical ingredients is closely related to the quality control processes of the raw material. Aflatoxins can be transmitted to the botanicals at every stage of the production. The purpose of this study is to evaluate the ELISA method for the determination of aflatoxins in 20 personal care products containing plant extracts and/or oil. In this study, aflatoxin B1 (AFB1) was not detected in one of the samples containing chamomile extract, rose, shea & orange, and cocoa butter. Of the tested samples, 35% exceeded the legal limits of AFB1. The highest level was determined in sample containing coconut butter (15.13 ppb). These results show that raw materials of cosmetic products should be regularly and effectively controlled.

Keywords: *Aspergillus flavus,* aflatoxin B1, cosmetics, ELISA, mycotoxin

INTRODUCTION

Aflatoxins are considered one of the major mycotoxins because of their toxicological effects on human health. *Aspergillus* strains are aflatoxin producers (Stroka et al. 1999). These contain *A. flavus, A. parasiticus, A. nomius, A. pseudotamarii,* and *A. bombycis* (Wilson et al. 2002; Gnonlonfin et al. 2013). Although aflatoxins are soluble in methanol, chloroform and acetone, they are insoluble in water and petroleum ether. When dry, they are heat resistant to their melting point (Hacibekiroglu and Kolak 2013). Diseases caused by aflatoxin are called aflatoxicosis. Exposure to high doses of aflatoxin (over 6000 mg) may induce acute toxicity with fatal effects (Groopman and Kensler 1999). Prolonged exposure to aflatoxin has been shown to be associated with liver cancer.

Raw plant materials are frequently contaminated with aflatoxin produced by fungi generated from the soil. Contamination of plant materials can also occur pre- or post-harvest during processing, preparation or in storage (Trucksess and Scott 2008). Plant extracts are usually prepared to modify any possible benefits that the plant may have. Some of these extracts are standardized to protect a known concentration of the active compound and to ensure that the concentration of the compound will be stable (Draelos and Thaman 2006), and they are used for different purposes. These properties of cosmetics containing natural products were made more effective by increasing the reliability and biodiversity of raw materials. With the improvement of the basic methods and the development of new technologies, new sources for pure raw material have been found. Botanicals have been an important part of cosmetics. Botanical oils have been used to soften skin and mask body odour, and body sprays have been made from mixtures of essential oils such as chamomile, rose, and cedar, merged in oils of olive, sesame, or almond (Davis 1990; Wagemaker et al. 2013). Botanicals are not just for perfumes and colour; many natural product extracts also have been used in various pharmaceutical drugs (Buchbinder et al. 1999; Draelos and Thaman 2006; Boga et al. 2011).

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Biologically active compounds from natural sources may provide health benefits as well as cosmetic benefits (dal Belo et al. 2009). Today, botanical extracts can be found in everything from hand cream to lipstick (Frauen et al. 2002; Gianeti et al. 2013; Beiki et al. 2018). Botanical extracts/oils may come in many forms (Marks 1997). However, the addition of botanical extracts to various cosmetic products requires the analysis of mycotoxin toxicity, especially aflatoxin (Voss 2007). Maximum levels set by the European community for most food products are 2 ppb aflatoxin B1. However; maximum levels have not been specified for botanical oils or cosmetic oils. Aflatoxins are analyzed in major foods but they are not analysed in botanical raw materials (Mahoney and Molyneux 1998).

In this study, 20 hand lotions containing botanical extracts and/or oils were analyzed for aflatoxin B1 (AFB1) (Figure 1) contamination by using ELISA due to its simplicity, sensitivity and adaptability. Enzyme-linked immunosorbent assay is the most commonly applied test to detect aflatoxins.

MATERIAL AND METHODS

Reagents

ELISA was performed using Ridascreen Fast Aflatoxin (Art No: R5202) (R-Biopharm AG, Darmstadt, Germany). AFB1 standard came with a ready-to-use ELISA kit. All solvents were of analytical grade.

Samples

Twenty hand cream samples containing botanical ingredients were randomly purchased from different local markets and supermarkets in Istanbul, Turkey. All samples were kept in their containers and stored at room temperature until sample preparation, and then stored at +4 ºC in the dark until the analysis. Brands were not recorded.

Sample Preparation

A sample of one gram was placed into falcon tubes and 10 mL of methanol (70%) was added. After shaking vigorously for 2 minutes with a vortex, the extract was filtered through a syringe filter. A measured quantity of 1 mL of the filtrate was diluted to 1 mL with distilled water; 50 µL of the diluted filtrate was used per well in the test. All reagents and standards were brought to room temperature (20-25ºC) before use.

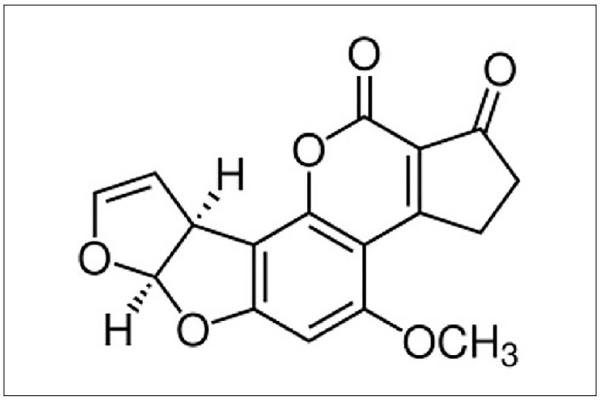

Figure 1. AFB1.

Procedure

50 µL of the standards (0, 1, 5, 10, 20, 50 µg/kg) were transferred into 5 wells of the first strip and 50 µL of the samples were transferred into wells of the second strip of the ELISA plate.

First, the enzyme conjugate solution (50 µL) and then the aflatoxin antibody solution (50 µL) were added to the plate. After mixing slowly by shaking the plate manually and incubating for 10 minutes at room temperature, the liquid was dumped out of the wells. The wells were filled with PBS-tween buffer (250 µL per well). The wells were emptied again removing all remaining liquid. The washing step was repeated two more times. 100 µL of substrate/chromogen was added to each well and the plate was gently mixed manually by shaking and then incubated for 5 minutes at room temperature in the dark. Finally, 100 µL of a stop solution (10% sulphuric acid) was added to each well and the plate was mixed gently by shaking manually The absorbance was measured at 450 nm with an ELISA Reader (Biotek® Synergy H1 Hybrid Reader) and evaluated with RIDA®SOFT Win (Art. No. Z9999) software, specialized to the Ridascreen® enzyme immunoassays.

Recovery

Analyses were performed with the R-Biopharm ELISA system (Darmstadt, Germany), which is accredited by DQS (the German Accreditation body) to ISO 9001, 13485 and 17025. For the recovery study, the AFB1 standard solution (100 µg/kg) was prepared in methanol-water (30:70) and each sample containing raw botanical cosmetics was spiked with three different concentrations of AFB1 (1.0, 2.5, 5.0 µg/kg). The recovery percentages were calculated by the following equation:

Recovery (%)=(Spiked amount of standard / Actual amount added) x 100

RESULTS AND DISCUSSION

Due to its simplicity, sensitivity and adaptability, the ELISA assay is the most commonly used method to detect aflatoxins (Biermann and Terplan 1980; Biermann and Terplan 1982; Danier et al. 1994). To detect the aflatoxins, the direct competitive ELISA method is used. This method detects and quantifies an antigen (aflatoxin) in a sample by using an enzyme labelled toxin and antibodies specific to the antigen. In this assay, the absorbance measurement of the blue product is made at 450 nm by the ELISA reader. The absorbance is inversely proportional to the aflatoxin concentration in the sample; the lower the absorbance shows, the higher the aflatoxin concentration (R-Biopharm 2006).

A cosmetic product made from plant material is a cosmetic product obtained from the entire plant or specific parts of the plant (roots, flowers, fruits, leaves or seeds). In considering the reliability of botanical ingredients used in personal care products, it is important to pay attention to the specific botanical material, the amount of the botanical in the raw material and final product, and the route of exposure. Personal care product manufacturers are required by law to sufficiently prove the reliability of the products containing botanical ingredients. Of the various botanical ingredients used in making cosmetics, aflatoxin is one of the most hazardous substances to controls.

Samples of each cosmetic containing botanical ingredients were obtained from different local markets. The botanics in hand creams were chamomile (five samples), green tea (three samples), coconut (three samples), rose (two samples), shea & orange (one sample), lavender (one sample), lemon & sage (one sample), avocado (one sample), lilly pilly fruit. (one sample), jojoba oil (one sample), and cocoa butter (one sample). In order to see the recovery, before the sample analysis, the purchased raw botanicals were triply spiked with AFB1 at low, middle, and high levels (1.0, 2.5, and 5.0 ppb, respectively). The spiked samples were then subjected to a clean-up procedure and analyzed for recovery by ELISA detection. The recovery rate of AFB1 is between 76.5% and 102.3% (Table 1), which shows good method performance. The sample containing raw botanical cosmetics was also spiked with AFB1 to ensure that the oils were not contaminated with aflatoxin prior to the procedure. Aflatoxin standards with different concentrations were read with the ELISA Reader at 450 nm (R-Biopharm 2006). Results were evaluated with RIDAWIN in Software and a calibration curve was generated. Standard, sample concentrations, and calculated ppb are shown in supplementary data, and the number of the samples with AFB1 in the range are shown in Table 2.

Table 1. Recovery percentage of spiked sample containing raw botanical cosmetics. LOD is 1.0 µg/kg for AFB1, as indicated by R-Biopharm test kit manuals

Sample group (containing botanical cosmetic)	AFB1 spike level (µg/kg)	Recovery (%)
Camomile	1.0	94.3
	2.5	82.8
	5.0	102.3
Green tea	1.0	100.2
	2.5	88.1
	5.0	102.2
Coconut	1.0	94.4
	2.5	89.9
	5.0	99.5
Rose	1.0	99.5
	2.5	93.2
	5.0	91.2
Shea butter & orange	1.0	100.2
	2.5	95.5
	5.0	89.1
Lavender	1.0	99.5
	2.5	97.0
	5.0	89.3
Lemon & Sage	1.0	98.5
	2.5	89.2
	5.0	76.5
Avocado	1.0	96.7
	2.5	91.0
	5.0	83.1
Lilly pilly fruit	1.0	97.2
	2.5	92.4
	5.0	81.9
Jojoba oil	1.0	98.5
	2.5	92.9
	5.0	80.6
Cocoa butter	1.0	99.9
	2.5	94.1
	5.0	78.8

*For each concentration n=3

Of the tested samples, 35% exceeded legal limits of AFB1. This study shows that the highest AFB1 levels were in three of the chamomile samples, all of the green tea samples and one of the coconut samples. According to the results, the highest AFB1 level was found to be in the sample containing coconut butter (15.13 ppb). Similar to our results, in a previous study, the chloroform extracts of the coconut samples showed that 5 out of 25 samples tested were contaminated with AFB1 (15-25 µg/kg) (Zohri and Saber 1993). The aflatoxin contamination in some edible oils, like coconut oil, has been reported in some regions of England and Sri Lanka, respectively (Bordin et al. 2014).

In one of the shea & orange concluding samples, and in one of each of the chamomile and rose concluding samples, no aflatoxin B1 was detected (< LOD). In another study, natural oil seeds including cocoa-butter substitutes (shea), were studied as substrates for aflatoxin production by two strains of *A. parasiticus*, and gave different levels of AFB1. AFB1 were found at low levels in that study, as well. (Kershaw 1982).

In the current study, a low AFB1 level was detected in the lavender, lemon & sage and lily pilly fruit concluding samples (2.42, 1.56, 4.48 ppb, respectively). In previous studies, no information about aflatoxin analysis in lavender and lilly pilly fruit was found. However, Gomori et al. tested the effect of clary

Table 2. AFB1 concentration ranges in the samples

	Number of samples with AFB1 in the range (µg/kg)		
Sample matrix	<LOD*	1.0-5.0	>5.0
Chamomile	1	1	3
Green tea	-	-	3
Coconut	-	2	1
Rose	1	1	-
Shea & orange	1	-	-
Lavender	-	1	-
Lemon & sage	-	1	-
Avocado	-	-	1
Lilly pilly fruit	-	1	-
Jojoba oil	-	1	-
Cocoa butter	1	-	-

Note: *LOD: <1.0 µg/kg for AFB1, indicated by R-Biopharm test kit manuals

sage and lemon essential oil vapours on growth, aflatoxin production and sporulation of *Aspergillus parasiticus*. They found that clary sage essential oils showed concentration-dependent growth inhibition. Antifungal index and aflatoxin production using the weak antifungals, lemon essential oils, increased in parallel. The same trend was found using clary sage essential oil vapours up to 0.11 mg/cm^3 concentration, while higher concentrations caused a sharp decrease in aflatoxin production. Only essential oil concentrations with strong growth and sporulation inhibitory effects were suitable to inhibit the aflatoxin production of *A. parasiticus* (Gomori et al. 2018). For this reason, in this study lemon & sage essential oils may inhibit the AFB1 molecule at certain concentrations. As Dimic et al reported, the results showed that the lemon essential oil exhibited a good inhibition of growth of the tested molds at >=1.25 mu L/mL in the agar medium and in the vapor atmosphere. (Dimic et al. 2015).

CONCLUSION

Cosmetics containing natural products can become contaminated with aflatoxins, and contaminated products may cause serious health problems. In this study, 20 hand cream samples containing natural extracts/oils were analysed. Of the tested samples, 35% exceeded legal limits of AFB1. These findings confirm the existing knowledge that raw materials of the cosmetic products should be regularly and effectively controlled.

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Review

Medicinal plants with reported anxiolytic and sedative activities in Nigeria: A systematic review

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ABSTRACT

Medicinal plants have been widely used in folklore medicine in the treatment of various diseases such as mental and neurological disorders. Mental disorders like anxiety and depression are very common among Nigerian populace. The purpose of this review was to access and evaluate several articles published on the anxiolytic and sedative properties of medicinal plants in Nigeria and to find out the gaps left for further research and drug development. Eighty-two publications available among Nigerian Universities and Research Institutes between 2008 and 2018 were selected. Seven electronic databases such as Nigerian Plant Database, HerbMed, AGRICOLA, MedlinePlus, PubMed, ScienceDirect, and Springer-Link were thoroughly explored from which 226 relevant articles were obtained using Google Scholar, Hotbot and FreeFullPdf as search engines. A number of studies conducted to test for the anxiolytic and sedative activity of medicinal plants in Nigeria were included. Articles published between 2008 and 2018 were selected. The studies were conducted in Nigeria. Research carried out before 2008 was not selected. All publications with authorship outside Nigeria were excluded. Several medicinal plants on which experiments were conducted were reported to have anxiolytic and or sedative properties in Nigeria. Several medicinal plants have shown promise as anxiolytic and sedative agents in laboratory animals' studies. The majority of these plants were used traditionally in the past to treat anxiety in Nigeria. Further research on the efficacy and safety of these medicinal plants could yield a more cost effective and perhaps safer alternative in the treatment of anxiety among Nigerians.

Keywords: Anxiolytic, sedative, medicinal-plants, phytotheraphy, Nigeria

INTRODUCTION

Herbal medicine has played a vital role in the phytotheraphy of various ailments including central nervous system disorders. Various part of the plants such as leaves, stems, roots, fruits, seeds, flowers etc. were used by both traditional and orthodox medicine practitioners as their source of medicaments (Magaji et al., 2008; Akindele and Adeyemi, 2010; Onasanwo et al., 2010; Egharevba et al., 2015; Adebiyi et al., 2016). The application of medicinal plants in the treatment of mental and neurological disorders has been documented over decades. Categorically, medicinal plants comprising secondary metabolites such as alkaloids, tannins, saponnins, flavonoids and sterols are highly associated with anxiolytic and sedative activities (Magaji et al., 2008; Asuquo et al., 2013; Edewor-Kuponiyi, 2013; Tijjani et al., 2014; Rungsung et al., 2015; Adebiyi et al., 2016). In addition, orthodox medicines available such as benzodiazepines are commonly associated with physical dependence, day time fatigue, tolerance and cognitive impairment. As such, there is need to search for medicinal plants that are capable of alleviating mental disorders without many side effects (Onasanwo et al., 2010; Edewor-Kuponiyi, 2013; Magaji et al., 2015).

Anxiety belongs to the group of mental disorders which are characterized by a sudden feeling of intense fear, panic, shortness of breath, chest pain, insomnia, fatigue, sweating, etc (Martinez et al. 2007; Keeton et al. 2009). The disorder occurs because of hyper

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responsiveness of amygdala and limbic system connected to the prefrontal cortex in the brain (Martinez et al. 2007; Keeton et al. 2009). Anxiety is mediated in the central nervous system via GABA, norepinephrine, serotonin, dopamine or peptide receptors (Martinez et al. 2007; Keeton et al. 2009). Globally, about 450 million people are affected by various mental and neurological disorders. Also, the prevalence of both mental and neurological disorders in Nigeria is increasing rapidly. Unfortunately, only small parts of the population are getting access to proper diagnosis and treatment (Wambebe 1998; WHO 2001; Danjuma et al., 2009). Reports have shown that 70% of patients in developing countries use phytotherapy as a means of treatment. Also, about 25% of orthodox medicines used worldwide were derived from medicinal plants (Wambebe 1998; WHO 2001; Danjuma et al., 2009). For these reasons, more research on the use of medicinal plants as a source of pharmaceutical treatment has become indispensable. This review aims to assess various research studies conducted on anxiolytic and sedative properties of medicinal plants in Nigeria and to find the gaps left for further improvement and drug development.

Aims of the Study

(i). To study several journals articles available on anxiolytic and sedative activities of medicinal plants within Nigeria.

(ii). To identify new areas that require further investigation and to make several recommendations

MATERIALS AND METHOD

Study Selection: Studies carried out and made available online between 2008 and 2018 were carefully selected. Consequently, this review will portray the picture of various medicinal plants tested for anxiolytic and sedative activity in Nigeria.

Data Sources: Popular academic search engines including Google Scholar, FreeFullPdf and HotBot were used to search for relevant publications using Nigerian Plant Database, HerbMed, AGRICOLA, MedlinePlus, PubMed, ScienceDirect, Springer-Link data bases. The research generated 226 relevant articles on anxiolytics and sedative properties of medicinal plants used in Nigeria.

Inclusion Criteria: Research studies carried out on medicinal plants between 2008 and 2018 were selected. Manuscript published within Nigeria that focusing primarily on the anxiolytic and sedative activity of medicinal plants were selected.

Exclusion Criteria: The review excludes all articles published before the year 2008.Studies conducted on medicinal plants outside Nigeria were not included. The study was carried out between April and September 2018.

Data Extraction: Several publications obtained from various universities and research institutions that fulfilled the inclusion criteria were considered. Finally, 82 articles were chosen and thoroughly scrutinized for eligibility. Based on the various outcomes obtained, the result was discussed, and several recommendations made. The reference sections of each article reviewed were used to search for more relevant publications and this has been included in this study.

RESULTS

Description of the Articles Included

The number of articles included were 82 from various universities and research institutes. The plant materials studied were obtained from various locations within the six geopolitical zones in Nigerian. These include Southwest, Southeast, South, Northwest, Northeast and central parts of Nigeria. In this review, about 82 publications met the inclusion criteria between 2008 and 2018 indicating the abundance of plants with anxiolytic and sedative activity as shown in Table 1.

DISCUSSION

(A). Anxiolytic Actions of Medicinal Plants

(i). Open Field Test (OFT): This method was employed to assess both anxiolytic and sedative activity of medicinal plants' extracts or isolated compounds as well as their effects on locomotor activity in laboratory animals (Acher 1973; Prut and Belzung 2003). Rodents placed in a new environment may experience signs of anxiety such as decreased mobility, exploration, grooming and rearing with concurrent increased micturition and defecation (Acher 1973; Prut and Belzung 2003). The anxiolytic action of medicinal plants reviewed was observed as follows: increase in central square crossing in *Allium ascalonicum* (Akindele et al., 2012) and *Citrus aurantium* (Yusuf et al., 2016). Similarly, increase in frequency of rearing was noticed in *Curcuma longa* (Ibironke and Alemonu 2013) and *Parkia biglobosa* (Tijjani et al., 2014). These medicinal plants have clearly shown anxiolytic potential although other tests are necessary to confirm this activity.

(ii). Elevated Plus Maze (EPM): This method was carried out specifically to test for the anxiolytic action of medicinal plants by observing their ability to alleviate fear of an open space in rodents tested (Pellow et al., 1985; Lister, 1987). In this experiment, mice displayed signs of anxiety by entering closed arm or avoiding open arm. Anxiolytic property is indicated by an increase in the frequency of entry into open arm and duration of stay (Handley and Mithani, 1984). Among the articles reviewed, anxiolytic action was demonstrated by the increase in the frequency of entry in to the open arm as observed in *Paulinia pinnata* (Aliyu et al., 2014) and *Telfairia occidentalis* (Ajao and Akindele, 2013). In addition, the increase in the time spent in an open arm was shown by *Cnidoscolous acontifolius* (Adebiyi et al., 2012) and *Maerua angolensis* (Malami et al., 2014b).

(iii). Y Maze Test (YMT): In this test anxiolytic action was established by the increase in the frequency of entry into the open arm and was noticed in *Asystesia gangetica* (Adeyemi et al., 2014) and *Zizyphus spina-christii* (Sadiq et al., 2010). Also, increase in the time spent in an open arm was shown by *Citrus aurantum* (Yusuf et al., 2016) and *Byrsocarpus coccineus* (Akindele and Adeyemi 2010).

(iv). T Maze Test (ETM): In this experiment anxiolytic action is revealed by an increase in the frequency of entry in to the open arm as well as time spent there as shown by *Vernonia amygdalina* (Oloruntobi et al., 2014).

Table 1. Showing the various plant families, methods of extraction and their pharmacological actions

S/N	Name of Plant	Family	Part of Plant	Solvents	Findings	References
1	*Adenopus breviflorus* (Roberty)	Cucurbitaceae	Fruit	Ethanol	Anxiolytic Sedative	Olusina and Aderibigbe, 2016
2	*Albizia glaberrima* (Schum. & Thonn.) Benth.	Leguminosae-Mimosodeae	Leaf	Distilled Water	Anxiolytic Sedative	Adebesin et al., 2015
3	*Alchornea cordifolia* (Schumach.&Thonn.)	Euphorbiaceae	Leaf	Ethanol	Anxiolytic CNS-depressant (Little)	Akanmu et al., 2011
4	*Allium ascalonicum* Linn.	Liliaceae	Aerial part	Hydroethanol	Anxiolytic	Akindele et al., 2012
5	*Alternanthera brasiliana* (L.) KUNTZE	Amaranthaceae	Leaf	Ethanol	Anxiolytic CNS stimulant	Oyemitan et al., 2015a
6	*Annona muricita* (L.)	Annonaceae	Leaf	Distilled Water	Anxiolytic	Okokon et al., 2018
7	*Annona senegalensis* Pers.	Annonaceae	Leaf	Methanol	Anxiolytic CNS-depressant	Okoli et al., 2010a
8	*Artocarpus altitis* (Parkinson) Fosberg.	Moraceae	Seed	Methanol	Anxiolytic	Onasanwo et al., 2017
9	*Asystasia gangetica* (Linn.)	Acanthaceae	Leaf, Stem	Water	Anxiolytic Sedative	Adeyemi et al., 2014
10	*Balanites aegyptiaca* (L.) Del.	Balanitaceae	Root bark	Water, Buthanol, Acetic acid	Anxiolytic Sedative	Ya'u et al., 2011
11	*Burkea Africana* Hook.	Fabaceae	Root bark	Methanol	Anxiolytic Sedative	Yaro et al., 2015a
12	*Byrsocarpus coccineus* (Schum & Thonn.)	Connaraceae	Leaf	Water	Anxiolytic Sedative	Akindele and Adeyemi, 2010
13	*Carissa edulis* (Forssk.) Vahl.	Apocynaceae	Root bark	Ethanol	Sedative	Ya'u et al., 2010
14	*Cissus cornifolia* (Baker.) Planch	Vitaceae	Leaf	Methanol,Butanol, Chloroform, Ethyl acetate	Anxiolytic CNS-depressant	Yaro et al., 2015b
15	*Cissus cornifolia* Baker. Planch	Vitaceae	Leaf Root	Methanol	Sedative	Yaro et al., 2009
16	*Cissus cornifolia* Baker. Planch	Vitaceae	Leaf	Methanol	Sedative	Musa et al., 2008
17	*Citrus aurantium* L.	Rutaceae	Root, Leaf, Fruit	Distilled water	Anxiolytic	Yusuf et al., 2016
18	*Cnestis ferruginea* Vahl. ex DC.	Conaraceae	Amentoflavone	Methanol,n-butanol Chloromethane,Ethylacetate	Anxiolytic Antidepressant	Ishola et al., 2012
19	*Cnidoscolous aconitifolius* (Miller)	Euphorbiaceae	Leaf	Water, Methanol	Anxiolytic	Adebiyi et al ., 2012
20	*Cola millenii* K. Schum	Sterculiaceae	Leaf	Methanol	CNS depressant	Oyemitan et al., 2016a
21	*Crinum glaucum* A. Chev.	Amaryllidaceae	Bulb	Distilled Water	Anxiolytic Hypnotic	Ishola et al., 2013
22	*Crinum zeylanicum* L.	Amaryllidaceae	Bulb	Distilled Water, Lime, Ammonia,Chloroform	Sedative	Tijani et al., 2012a
23	*Cucurma longa* L.	Zingiberaceae	Rhizome	Ethanol	Anxiolytic Antidepressant	Ibironke and Alemonu, 2013
24	*Cucurma longa* L.	Zingiberaceae	Rhizome	Distilled Water	Anxiolytic Sedative	Oyemitan et al., 2017
25	*Cymbopogon citrates* (DC.) Stapf	Poaceae	Root	Distilled Water	Anxiolytic	Arome et al., 2014
26	*Datura stramonium* L.	Solanaceae	Seed	Distilled Water	Sedative	Malami et al., 2014a
27	*Dennettia tripetala* G. Baker	Annonaceae	Leaf, Fruit Seed, Stem	Distilled Water	Hypnotic	Oyemitan et al., 2013
28	*Ficus ingens* Miquel.	Moraceae	Stem bark	Methanol	Anxiolytic Sedative	Offiah et al., 2015
29	*Ficus platyphylla* Del. Holl.	Moraceae	Stem bark	Methanol	Sedative	Chindo et al., 2015

30	*Ficus platyphylla* Del. Holl.	Moraceae	Stem bark	Methanol	Sedative	Chindo et al., 2014
31	*Grewia carpinifolia* Juss.	Tiliaceae	Leaf	Ethanol	CNS-depressant	Adebiyi et al., 2016
32	*Gymnema sylvestre* R. Br.	Asclepiadaceae	Leaf Stem Flower	Distilled Water	Sedative Hypnotic	James et al., 2014
33	*Hedranthera barteri* Hook. f.	Apocynaceae	Root	Hexane Dicloromethane	Anxiolytic Antidepressant	Onasanwo et al., 2010
34	*Hippocratea Africana* Loes.ex Engl	Celastraceae	Root	Distilled Water Ethano, Chloroform	CNS-depressant	Okokon et al., 2014
35	*Homalium letestui* Pellegr.	Flacourtiaceae	Stem	Ethanol	CNS-depressant	Okokon and Davies, 2014
36	*Hydrolea glabra* (Schum. & Thonn.)	Hydrophylaceae	Leaf	Methanol	Sedative Anxiolytic	Anyanwu-Ndulewe et al., 2018
37	*Indigofera pulchra* Willd (LIP).	Leguminosae	Aerial part	Distilled water Methanol	Anxiolytic	Tanko et al., 2009
38	*Laggeria aurita* Linn.	Asteraceae	Leaf	methanol	Anxiolytic	Guragi et al., 2018
39	*Leonotis nepetifolia* (Linn)	Lamiaceae	Whole Stem	Methanol	Anxiolytic Sedative	Ayanwuyi et al., 2016
40	*Leucas martinicensis* (jacq.) R.Br.	Lamiaceae	Leaf	Distilled Water Sedative	Ugwah-Oguejiofor et al., 2015	
41	*Lopira alata* (Banks ex Gaertn. f.)	Ochnaceae	Stem bark	Distilled water	Anxiolytic Sedative	Iniaghe et al., 2015
42	*Maerua angolensis* DC. subsp.	Capparaceae	Stem bark	Methanol	Anxiolytic Sedative	Malami et al., 2014b
43	*Mitracarpus villosus* (Sw.) DC.	Rubiaceae	Leaf	Ethylacetate	Sedative	John-Africa et al., 2014
44	*Mondia whitel* (Hook. f) Skeels	Periplocaceae	Para-pentyl benzoate	Ethanol	Sedative	Taiwo et al., 2017
45	*Moringa oleifera* (Lam.)	Moringaceae	Leaf	Ethanol	Sedative	Bakre et al., 2013
46	*Mucuna pruriens* (L.) DC.	Fabaceae	Seed	Distilled Water	CNS-depressant	Magaji et al., 2012
47	*Musa sapientum* Linn.	Musaceae	Leaf	Distilled Water	No Significant Anxiolytic	Salako et al., 2018
48	*Nymphaea lotus* L.	Nymphaeaceae	Leaf	Distillled Water	Anxiolytic Sedative	Fajemiroye et al., 2018
49	*Nymphaea lotus* L.	Nymphaeaceae	Leaf	Methanol	Anxiolytic	Aduema et al., 2018
50	*Ocimum gratissimum* L.	Lamiaceae	Leaf	Methanol	Anxiolytic	Okoli et al., 2010b
51	*Olax subscorpioidea* Oliv.	Olacaceae	Leaf	Ethanol	No anxiolytic Antidepressant	Adeoluwa et al., 2015
52	*Parkia biglobosa* (Jacq Benth)	Mimosoideae	Stem bark	Water	Anxiolytic Sedative	Tijjani etal., 2014
53	*Paullinia pinnata* L.	Sapindaceae	Leaf	Methanol	Anxiolytic	Aliyu et al., 2014
54	*Paullinia pinnata* L.	Sapindaceae	Leaf	Water	CNS-depressant	Dayom et al., 2014
55	*Persea Americana* Mill. var.	Lauraceae	Seed	Ethanol	Sedative	Oyemitan et al., 2016b
56	*Piliostigma thonningii* (Schum.) Milne-Rech	Caesalpiniaceae	Leaf	Ethanol	Anxiolytic	Adamu et al., 2013
57	*Piliostigma thonningii* (Schum.) Milne-Rech	Caesalpinioideaea	Leaf	Ethanol, Distilled Water	No Tranquilization	Ozolua and Alonge, 2008
58	*Piper guineense* (Schum. & Thonn.)	Piperraceae	Essential Oil	Water	CNS-depressant Antipsychotic	Oyemitan et al., 2015b
59	*Randia nilotica* Stapf.	Rubiaceae	Stem bark, Leaf, Root bark	Hydroethanol	Hypnotic Sedative	Danjuma et al., 2008
60	*Randia nilotica* Stapf.	Rubiaceae	Stem bark (Saponin)	Petroleum ether Ethanol	Sedative	Danjuma et al., 2014

61	*Securidaca longipedunculata* Fresen.	Polygalaceae	Root	Distilled water	Anxiolytic CNS-depressant	Adeyemi et al., 2010
62	*Securinega virosa* Roxb Baill.	Euphorbiaceae	Bargenin	Methanol, Chloroform Pet ether, n-butanol Ethyl acetate	Sedative	Magaji et al., 2015
63	*Securinega virosa* Roxb Baill.	Euphorbiaceae	Leaf	Methanol	Sedative Antipsychotic	Magaji et al., 2014
64	*Securinega virosa* Roxb Baill.	Euphorbiaceae	Root bark	Ethylacatate	Sedative	Garba et al., 2013
65	*Securinega virosa* Roxb Baill.	Euphorbiaceae	Leaf	Methanol	Sedative	Aiyelero et al., 2012
66	*Securinega virosa* Roxb Baill.	Euphorbiaceae	Root bark	Distilled Water	Anxiolytic Sedative	Magaji et al., 2011
67	*Securinega virosa* Roxb Baill.	Euphorbiaceae	Root bark	Methanol	Sedative	Magaji et al., 2008
68	*Senna occidentalis* L.	Fabaceae	Leaf	Ethanol	Sedative	Cletus et al., 2017
69	*Spondias mombin* L.	Anacardiaceae	Leaf	Ethanol	Anxiolytic	Asuquo et al., 2013
70	*Spondias mombin* L.	Anacardiaceae	Coumaroyl Quercetin Garlic acid	Ethanol, Buthanol Ethylacetate, Water	Anxiolytic	Ayoka et al., 2013
71	*Stachytarpheta cayennensis* (Rich) Vahl.	Verbanaceae	Leaf	Methanol, Buthanol Ethylacetate, Water	Anxiolytic Sedative	Olayiwola et al., 2013
72	*Struchium sparganophora* (Linn) Kuntze	Compositae	Leaf	Ethanol	CNS-depressant	Aderibigbe & Agboola, 2011
73	*Telfairia occidentalis* (Hook. f.)	Cucurbitaceae	Leaf	Hyroethanol	Anxiolytic Sedative	Ajao and Akindele, 2013 74
74	*Tetrapleura tetraptera* (Schum and Thonn) Taub	Mimosaceae	Fruit	Distilled water, Methanol, Chloroform, Buthanol	CNS depressant	Aderibigbe et al., 2010a
75	*Tetrapleura tetraptera* (Schum and Thonn) Taub	Mimosaceae	Aridanin (Bioactive compound)	Distilled water, Methanol, Chloroform, Buthanol	CNS-depressant	Aderibigbe et al., 2010b
76	*Treculia africana* Decne.	Moraceae	Stem bark	Ethanol	Sedative	Aderibigbe & Agboola, 2010
77	*Treculia africana* Decne.	Moraceae	Stem bark	Ethanol	Sedative	Aderibigbe et al., 2010c
78	*Vernonia amygdalina* Del.	Asteraceae	Leaf	Water	Anxiolytic Sedative	Oloruntobi et al., 2014
79	*Vitex doniana* L.	Verbenaceae	Stem bark	Ethanol	CNS-depressant	Tijjani et al., 2012b
80	*Xeromphis nilotica* Stapf.	Rubiaceae	Stem bark	Water, Methanol Buthanol, Diethylether	Sedative	Danjuma et al., 2009
81	*Ziziphus mauritiana* (L.)	Rhamnaceae	Seed	Water, Ethanol, Ethyl acetate	Sedative	Sadiq et al., 2009
82	Ziziphus *spina-christi* (L.)	Rhamnaceae	Root bark	Chloroform, Methanol, Ethylacetate, Hexane	CNS-depressant	Adzu et al., 2008

(v). Elevated Zero Maze Test (EZM): Anxiolytic action was indicated by increase in frequency of entry and time spent in an open arm as shown by *Parkia biglobosa* (Tijjani et al., 2014) and *Citrus aurantium* (Yusuf et al., 2016).

(vi). Light and Dark Exploration Test (LDE): This experiment was conducted to test for both anxiolytic and sedative action of a medicinal plant. In this test, a rodent is considered highly anxious if it spends less time in the lit compartment and more time in the dark compartment (Belzung et al., 1987). Increased time spent in the lit compartment is an indication of anxiolytic action. (Belzung et al., 1987). The parameters tested were latency in entry to the dark box and time spent in the dark box. Increase in the latency of entry into the dark box was noticed in *Allium ascalonicum* (Akindele et al., 2012) and *Tefairia occidentalis* (Ajao and Akindele 2013). Likewise, *Cnestis ferruginea* (Ishola et al., 2012) shows a decrease in the time spent in the dark box which revealed anxiolytic action.

(vii). Stress Induced Hyperthermia Test (SIH): Decrease in body temperature was observed following the administration of *Cymbopogon citratus* (Arome et al., 2014) and *Struchium sparganophora* (Aderibigbe and Agboola 2011) which is an indication of antianxiety activity.

(viii). Staircase Method Test (SMT): Parameters tested were frequency of rearing and number of upward steps climbed by the laboratory animals under study. Decrease in frequency of rearing indicates antianxiety while decrease in number of upward steps climbed implies CNS-depression (Simiand et al., 1984). Anxiolytic action is shown as decrease in rearing by *Securinega virosa* (Magaji et al., 2011) and *Ficus ingens* (Offiah et al., 2015). In addition, decrease in the number of upward steps climbed was observed following the administration of extract of *Balanites aegyptiaca* (Ya'u et al., 2011) and *Paulinia pinnata* (Aliyu et al., 2014) indicating sedative property.

(ix). Social Interaction Test (SIT): An increase in social activities such as grooming, sniffing, or following their partner by the laboratory animal without simultaneous rise in motor activity is an indication of anxiolytic action (File and Seth, 2003). Spontaneous increase in grooming, sniffing as well as following the partner was observed in *Allium ascalonicum* (Akindele et al., 2012) and *Telfairia occidentalis* (Ajao and Akindele, 2013) which revealed antianxiety potentials of the above medicinal plants.

(x). Hole-Board Test (HBT): In this test, an increase in head dipping by the experimental animal following the administration of a plant extract is an indication of anxiolytic action (Takeda et al., 1998) whereas decrease in head dipping signifies sedative action (File and Wardil, 1975; File and Pellow, 1985). The anxiolytic activity, which is an increase in the number of head dips by the experimental animals, was demonstrated by *Allium ascalonicum* (Akindele et al., 2012) and *Curcuma longa* (Oyemitan et al., 2017). Furthermore, the sedative action of a medicinal plant extract was shown by a decrease in the number of head dips by *Adenopus breviflorus* (Olusina and Aderibigbe, 2016) and *Persea americana* (Oyemitan et al., 2016b).

(B). Sedative Action of Medicinal Plants

(i). Open Field Test: In this test medicinal plants such as *Grewia carpinifolia* (Adebiyi et al., 2016) and *Hippocratea africana* (Okokon et al., 2014) showed sedative activity as indicated by the decrease in the number of squares crossed. Also, reduction in the frequency of rearing was noticed in *Securinega virosa* (Magaji et al., 2011) and *Spondias mombin* (Asuquo et al., 2013) indicating anxiolytic property.

(ii). Novelty Induced Behavior (NIB): This test was done to assess the CNS-depressant action of a medicinal plant. After injecting the plant extract, the parameters measured were the number of squares crossed with both limbs (locomotion), number of times the animal raised its pawns on air or placed them on a cage wall (rearing) and number of head dips (Takeda et al., 1998). Significant decrease in rearing was observed in *Persea americana* (Oyemitan et al., 2016b) and *Piper guineense* (Oyemitan et al., 2015b). Correspondingly, a decrease in locomotor activity was observed in *Spondias mombin* (Ayoka et al., 2013) and *Stachytarpheta cayennensis* (Olayiwola et al., 2013) signifying sedative activity.

(iii). Beam Walking Assay (BWA): This is another behavioral study carried out to induce minimal form of anxiety in a rodent. In this experiment, decrease in motor coordination after the administration of a plant extract suggests sedative property (Stanley et al., 2005). The parameters evaluated were the time taken to reach the goal box and the number of foot slips involving one or both hind limbs. A significant increase in the number of foot slips was noticed in *Cissus cornifolia* (Yaro et al., 2015b) demonstrating CNS-depressant.

(iv). Phenobarbitone Induced Sleeping Time (PIST): This test was conducted to assess the sedative nature of medicinal plants. The primary focus was onset of sleep which is loss of righting reflex. That is when mice were rolled sideways and could not stand upright using all four limbs (Miya et al., 1973). The first parameter was decrease in latency of sleep as shown by *Crinum zeylanicum* (Tijani et al., 2012a) and *Gymnema sylvestre* (James et al., 2014). The second parameter assessed was increase in the duration of sleep as seen in *Hippocratea Africana* (Okokon et al., 2014) and *Homalium letestui* (Okokon and Davies, 2014) all indicating sedative activity.

(v). Pentobarbitone Induced Sleeping Time: In this experiment *Zizyphus spina-christii* (John-Africa et al., 2014) significantly reduced the onset of pentobarbitone induced sleep while *Mondia whitel* (Taiwo et al., 2017) significantly increased the total sleeping time showing CNS depression. Potentiation of phenobarbitone and pentobarbitone sleeping time was believed to take place via augmentation of chloride ion linked GABA receptor inhibition (Tijani et al., 2012a; Dhawan et al., 2004). However, the tests described above only suggested the possibility of sedative activity of medicinal plants. This is because some medicinal plants only inhibit phenobarbitone metabolism via inhibition of cytochrome P450, prolonging its activity giving a false impression of CNS depression (Gobubkova et al., 1998).

(vi). Diazepam Induced Sleeping Time: This is another test for sedative activity of a medicinal plant. It involves evaluation

Table 2. Showing dose-dependent and none-dose dependent anxiolytic and sedative responses

		Low Dose Response		High Dose Response		
S/N	Name of Plant	Anxiolytic	Sedative	Anxiolytic	Sedative	References
1	*Adenopus breviflorus* (Roberty)	High	Low	Low	High	Olusina and Aderibigbe, 2016
2	*Albizia glaberrima* (Schumach.&Thonn.)	Low	Low	High	High	Adebesin et al., 2015
3	*Alchornea cordifolia* (Schumach.&Thonn.)	Low	Little	High	Little	Akanmu et al., 2011
4	*Allium ascalonicum* Linn.	High	---	Low	---	Akindele et al., 2012
5	*Alternanthera brasiliana* (L.) KUNTZE	Low	High	High	Low	Oyemitan et al., 2015a
6	*Annona muricita* (L.)	None-Dose Dependent	---	None-Dose Dependent	---	Okoronko et al., 2018
7	*Annona senegalensis* Pers.	High	Low	Low	High	Okoli et al., 2010a
8	*Artocarpus altitis* (Parkinson) Fosberg.	Low	---	High	---	Onasanwo et al., 2017
9	*Asystasia gangetica* (Linn.)	Low	Low	High	High	Adeyemi et al., 2014
10	*Balanites aegyptiaca* (L.) Del.	High	Low	Low	High	Ya'u et al., 2011
11	*Burkea Africana* Hook.	High	Low	Low	High	Yaro et al., 2015a
12	*Byrsocarpus coccineus* (Schum. & Thonn.)	High	Low	Low	High	Akindele and Adeyemi, 2010
13	*Carissa edulis* (Forssk.) Vahl.	---	Low	---	High	Ya'u et al., 2010
14	*Cissus cornifolia* (Baker.) Planch	High	Low	Low	High	Yaro et al., 2015b
15	*Cissus cornifolia* Baker. Planch	---	Low	---	High	Yaro et al., 2009
16	*Cissus cornifolia* Baker. Planch	---	None-Dose Dependent	---	None-Dose Dependent	Musa et al., 2008
17	*Citrus aurantium* L.	High	---	Low	---	Yusuf et al., 2016
18	*Cnestis ferruginea* Vahl. ex DC.	High	Low	Low	High	Ishola et al., 2012
19	*Cnidoscolous aconitifolius* (Miller)	High	---	Low	---	Adebiyi et al ., 2012
20	*Cola millenii* K. Schum	---	Low	---	High	Oyemitan et al., 2016a
21	*Crinum glaucum* A. Chev.	High	Low	Low	High	Ishola et al., 2013
22	*Crinum zeylanicum* L.	---	Low	---	High	Tijani et al., 2012a
23	*Cucurma longa* L.	High	---	Low	---	Ibironke and Alemonu, 2013
24	*Cucurma longa* L.	High	Low	Low	High	Oyemitan et al., 2017
25	*Cymbopogon citrates* (DC.) Stapf	High	---	Low	---	Arome et al., 2014
26	*Datura stramonium* L.	---	Low	---	High	Malami et al., 2014a
27	*Dennettia tripetala* G. Baker	High	Low	Low	High	Oyemitan et al., 2013
28	*Ficus ingens* Miquel.	High	Low	Low	High	Offiah et al., 2015
29	*Ficus platyphylla* Del. Holl.	High	Low	Low	High	Chindo et al., 2015
30	*Ficus platyphylla* Del. Holl.	High	Low	Low	High	Chindo et al., 2014
31	*Grewia carpinifolia* Juss.	---	None-Dose Dependent	---	None-Dose Dependent	Adebiyi et al., 2016

32	*Gymnema sylvestre* R. Br.	---	Low	---	High	James et al., 2014
33	*Hedranthera barteri* Hook. f.	High	High	Low	Low	Onasanwo et al., 2010
34	H*ippocratea Africana* Loes.ex Engl	Low	Low	High	High	Okokon et al., 2014
35	*Homalium letestui* Pellegr.	---	Low	---	High	Okokon and Davies, 2014
36	*Hydrolea glabra* (Schum. & Thonn.)	None-Dose Dependent	Low	None-Dose Dependent	High	Anyanwu-Ndulew et al., 2018
37	*Indigofera pulchra* Willd (LIP).	High	---	Low	---	Tanko et al., 2009
38	*Laggeria aurita* Linn.	High	Low	Low	High	Guragi et al., 2018
39	*Leonotis nepetifolia* (Linn)	Low	Low	High	High	Ayanwuyi et al., 2016
40	*Leucas martinicensis* (jacq.) R.Br.	---	Low	---	High	Ugwah-Oguejiofor et al., 2015
41	*Lopira alata* (Banks ex Gaertn. f.)	High	None-Dose Dependent	Low	None-Dose Dependent	Iniaghe et al., 2015
42	*Maerua angolensis* DC. subsp.	Low	Low	High	High	Malami et al., 2014b
43	*Mitracarpus villosus* (Sw.) DC.	---	Low	---	High	John-Africa et al., 2014
44	*Mondia whitel* (Hook. f) Skeels	High	Low	Low	High	Taiwo et al., 2017
45	*Moringa oleifera* (Lam.)	---	Low	---	High	Bakre et al., 2013
46	*Mucuna pruriens* (L.) DC.	---	Low	---	High	Magaji et al., 2012
47	*Musa sapientum* Linn.	High	High	Low	Low	Salako et al., 2018
48	*Nymphaea lotus* L.	High	Low	Low	High	Fajemiroye et al., 2018
49	*Nymphaea lotus* L.	High	---	Low	---	Aduema et al., 2018
50	*Ocimum gratissimum* L.	Low	---	High	---	Okoli et al., 2010b
51	*Olax subscorpioidea* Oliv.	None-Dose Dependent	---	None-Dose Dependent	---	Adeoluwa et al., 2015
52	*Parkia biglobosa* (Jacq Benth)	Low	---	High	---	Tijjani etal., 2014
53	*Paullinia pinnata* L.	High	---	Low	---	Aliyu et al., 2014
54	*Paullinia pinnata* L.	---	Low	---	High	Dayom et al., 2014
55	*Persea Americana* Mill. var.	---	Low	---	High	Oyemitan et al., 2016b
56	*Piliostigma thonningii* (Schum.) Milne-Rech	Low	---	High	---	Adamu et al., 2013
57	*Piliostigma thonningii* (Schum.) Milne-Rech	---	Low	---	High	Ozolua and Alonge, 2008
58	*Piper guineense* Schum & Thonn	---	Low	---	High	Oyemitan et al., 2015b
59	*Randia nilotica* Stapf.	---	Low	---	High	Danjuma et al., 2008
60	*Randia nilotica* Stapf.	---	Low	---	High	Danjuma et al., 2014
61	*Securidaca longipedunculata* Fresen.	High	Low	High	Low	Adeyemi et al., 2010
62	*Securinega virosa* Roxb Baill.	---	Low	---	High	Magaji et al., 2015
63	*Securinega virosa* Roxb Baill.	---	Low	---	High	Magaji et al., 2014
64	*Securinega virosa* Roxb Baill.	---	Low	---	High	Garba et al., 2013
65	*Securinega virosa* Roxb Baill.	---	Low	---	High	Aiyelero et al., 2012
66	*Securinega virosa* Roxb Baill.	High	Low	Low	High	Magaji et al., 2011

67	*Securinega virosa* Roxb Baill.	---	Low	---	High	Magaji et al., 2008	
68	*Senna occidentalis* L.	Low	Low	High	High	Cletus et al., 2017	
69	*Spondias mombin* L.	High	---	Low	---	Asuquo et al., 2013	
70	*Spondias mombin* L.	High	---	Low	---	Ayoka et al., 2013	
71	*Stachytarpheta cayennensis* (Rich) Vahl.	High	Low	Low	High	Olayiwola et al., 2013	
72	*Struchium sparganophora* (Linn) Kuntze	---	Low	---	High	Aderibigbe and Agboola, 2011	
73	*Telfairia occidentalis* (Hook. f.)	High	Low	Low	High	Ajao and Akindele, 2013 74	
74	*Tetrapleura tetraptera* (Schum and Thonn) Taub		---	Low	---	High Aderibigbe et al., 2010a	
75	*Tetrapleura tetraptera* (Schum and Thonn) Taub		---	Low	---	High Aderibigbe et al., 2010b	
76	*Treculia africana* Decne.	---	Low	---	High	Aderibigbe and Agboola, 2010	
77	*Treculia africana* Decne.	---	Low	---	High	Aderibigbe et al., 2010c	
78	*Vernonia amygdalina* Del.	High	Low	Low	High	Oloruntobi et al., 2014	
79	*Vitex doniana* L.	---	Low	---	High	Tijjani et al., 2012b	
80	*Xeromphis nilotica* Stapf.	---	Low	---	High	Danjuma et al., 2009	
81	*Ziziphus mauritiana* (L.)	---	Low		High	Sadiq et al., 2009	
82	*Ziziphus spina-christi* (L.)	---	Low	---	High	Adzu et al., 2008	

of loss of righting reflex (Miya et al., 1973). Significant decrease in the onset of sleep was observed in *Balanites aegyptiaca* (Ya'u et al., 2011) and *Cissus cornifolia* (Yaro et al., 2015b) which is an indication of sedative property. In addition, *Datura stramonium* (Malami et al., 2014a) and *Denettia tripetala* (Oyemitan et al., 2013) demonstrated an increased duration of sleep.

(vii). Ketamine Induced Hypnosis: Mice were used to test for the CNS-depressant property of medicinal plants. Studies involving *Securinega virosa* (Magaji et al., 2014) and *Piper guineense* (Oyemitan et al., 2015b) showed an increase in the duration of ketamine-induced sleeping time.

(C). Other Behavioral studies

(i). Apormorphine Induced Streotypy (AIS): This experiment was carried out to evaluate the CNS-depressant action of a medicinal plant. The parameters assessed were climbing behavior and frequency of rearing (Moore and Axton, 1998). The apormorphine induced streotypy was measured and scored as follows: 0=Absence of Stereotypy; 1=Occasional Sniffing; 2=Occasional Gnawing; 3=Frequent Gnawing; 4=Continuous Gnawing; 5=Gnawing Intensively and Staying on the Same Spot (Okoli et al., 2010a). A significant decrease in climbing scores was observed in *Ficus platyphylla* (Chindo et al., 2015) and *Securinega virosa* (Magaji et al., 2014). Likewise, a significance decrease in frequency of rearing was observed in *Piper guineense* (Oyemitan et al., 2015b) indicating sedative property. The plant extract or isolated bioactive compounds were believed to reverse the apomorphine induced hyperactivity via central blockade of D_2 dopaminergic receptor leading to CNS depression (Stolk and Rech, 1970). Other tests that give similar outcomes include Dexamphetamine Induced Stereotypy in which an increase in climbing score was shown by *Piliostigma thonningii* (Ozolua and Alonge, 2008).

(ii). Rotarod Performance Test: This method was used to assess the CNS-depressant action as well as muscle relaxant action of a medicinal plant. The experiment evaluates the duration of stay on a rotarod and loss of motor coordination by the laboratory animal (Dunhan and Miya, 1957). Notably, the decrease in the time spent on a rotarod as well as decrease in fatigue resistance is possibly due to blockade D_2 dopaminergic receptor (Stolk and Rech, 1970). Furthermore, a significant decrease in motor coordination was apparent in *Annona senegalensis* (Okoli et al., 2010a) and *Hedranthera barteri* (Onasanwo et al., 2010). Similarly, a significant decrease in fatigue resistance was observed in *Ficus platyphylla* (Chindo et al., 2014) and *Piper guineense* (Oyemital et al., 2015a) indicating CNS-depression.

(D). Dose-Dependence Response

Generally, a medicinal plant may show anxiolytic property at a lower dose and at the same time may demonstrate sedative ability when given at much higher doses (Treit et al., 1984). The majority of the articles reviewed showed a dose-dependent anxiolytic or sedative response. This implies that the plant extract of most plants revealed high anxiolytic activity at a lower dose and low anxiolytic activity at a higher dose. Correspondingly, the extracts showed low sedative property at a lower dose and high sedative property at a higher dose. This is shown in Table 2. However, only a few medicinal plant extracts deviate

from this analogy. As such, low anxiolytic activity was seen at a lower dose and high anxiolytic activity at a higher dose by *Albizia glaberrima* (Adebesin et al., 2015), *Alchornea cordifolia* (Akanmu et al., 2011), *Alternanthera brasiliana* (Oyemitan et al., 2015a), *Artocarpus altitis* (Onasanwo et al., 2017), *Asystesia gangetica* (Adeyemi et al., 2014), *Cissus cornifolia* (Yaro et al., 2015b), *Hippocratea africana* (Okokon et al., 2014), *Leonotis nepetifolia* (Ayanwuyi et al., 2016), *Maerua angolensis* (Malami et al., 2014b), *Ocimum gratissimum* (Okoli et al., 2010b), *Parkia biglobosa* (Tijjani et al., 2014), *Piliostigma thonningii* (Adamu et al., 2013), and *Senna occidentalis* (Cleatus et al., 2017). Similarly, high sedative property was seen at a lower dose with low sedative property at a higher dose by *Hedranthera barteri* (Onasanwo et al., 2010) and *Musa sapientum* (Salako et al., 2018).

CONCLUSION

Several research studies using laboratory animals were reviewed which tested the reported claims of anxiolytic or sedative activities of medicinal plants. About 82 different medicinal plants were tested between 2008 and 2018 indicating an abundance of plants with anxiolytic and sedative activity in Nigeria. The plants on which experiments were conducted showed dose-dependent anxiolytic and sedative activity with better anxiolytic response at lower doses and greater sedative at higher doses. Nonetheless, findings from this review suggested that there are still several medicinal plants with anxiolytic and or sedative activities claims in Nigeria yet to be scientifically tested. Techniques such as Open Field Test, Elevated Plus Maze, Y Maze Test, T Maze Test, Elevated Zero Maze Test, Light and Dark Exploration Test, Stress Induced Hyperthermia Test, Staircase Method Test, Social Interaction Test, and Hole-Board Test were commonly employed in testing the anxiolytic property of medicinal plants in Nigeria. In addition, Open Field Test, Novelty Induced Behavior, Beam Walking Assay, Phenobarbitone Induced Sleeping Time, Pentobarbitone Induced Sleeping Time, Diazepam Induced Sleeping Time, and Ketamine Induced Hypnosis methods were frequently used in testing the sedative activity of medicinal plants. Furthermore, other studies commonly employed in behavioral studies include Apormorphine Induced Streotypy and Rotarod Performance Test. Consequently, this has led to advancements made in the field of psychopharmacology in Nigeria. In order to obtain target specific activities of these medicinal plants, the bioactive molecules that are responsible for the identified pharmacological activity should be figure out and isolated. Also, due to the abundance of medicinal plants among the Nigerian flora with various claims in folklore medicine, delving into the advanced ethno medicinal research will yield several phytomedicines that could be used in the treatment of anxiety in Nigeria.

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Review

Check-list of additional taxa to the supplement flora of Turkey IX

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ABSTRACT

The ninth of the check-list series entitled "Check-list of additional taxa to the supplement flora of Turkey" includes 159 taxa based on 120 papers published the period between January 2017 and December 2018. These taxa have not been recorded in none of the 11 volumes of the Flora of Turkey nor in the eight previously published supplementary check-lists. With this paper the following are added to the Turkish flora: 117 taxa new to science and 42 taxa new records.

Keywords: Additional taxa, Turkish flora, new species, new records

INTRODUCTION

The diversity of vascular plants of Turkey has been documented in the "Flora of Turkey and the East Aegean Islands" and published in nine volumes between 1965 and 1985. After the publication of this Flora, subsequent studies have greatly increased and many new taxa have been added because of Turkey's rich flowering diversity. The identification of these additional taxa has necessitated the publication of the supplementary volumes to the Flora of Turkey vol. 10 in 1988 (Davis et al. 1988) and vol. 11 in 2000 (Güner et al. 2000) have been published.

After the 11th volume (2000) an additional 1353 taxa, either as a new species (1004 taxa) or new records for Turkish flora (349 taxa), have been recorded. These checklists have been published in a series paper by Özhatay et al. as Checklist III, IV, V, VI, VII and VIII (Özhatay and Kültür 2006; Özhatay et al. 2009; 2011; 2013; 2015; 2017) According to these data, for each year about 70 taxa is added to the Turkish flora. The all original publication of these taxa was subsequently drawn together by the authors as a collection housed in the library of the Department of Pharmaceutical Botany, Istanbul University (ISTE Herbarium Library). The aim of this paper is to present all published taxa added to the flora of Turkey during 2017 to 2018 with the missing records (Table 1).

In this paper 159 taxa are listed, 117 of them as new taxa to science (comprising 102 species, 10 subspecies, 2 varieties, 3 hybrids), and further 42 taxa as new record for Turkey (33 species, 7 subspecies and 2 varieties).

Cardiospermum L. (Sapindaceae), *Cyclospermum* Lag. (Umbelliferae), *Diptychocarpus* Trautv. (Cruciferae), *Proboscidea* Schmid. (Martyniaceae) and *Symphyotrichum* Nees (Compositae) are the new genera for Turkish flora.

The genus *Phelipanche* Pomel, *P. kelleri* (Novopokr.) Sojak (Orobanchaceae), seems to be a new genus, but it is recorded in the Plant List as syn. of *Orobanche kelleri* Novopokr.

Aethionema (with 13 taxa), *Allium* (with 8 taxa), *Taraxacum* (with 6 taxa) and *Dianthus* (with 5 taxa) are the four genera which represented with the most taxa in this study (Figure 1).The locality information is written in the same way as the original references. e= endemic species; * = new record for Turkish flora; **= new taxon for science; ■ = new genus for Turkish flora.

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Table 1. Statistical table of summary data of additional taxa for the checklists. (N: new taxa for science, R: new record for Turkey)

	Check-list I (1994)		Check-list II (1999)		Check-list III (2006)		Check-list IV (2009)		Check-list V (2011)		Check-list VI (2013)		Check-list VII (2015)		Check-list VIII (2017)		Check-list IX 2019	
	N	R	N	R	N	R	N	R	N	R	N	R	N	R	N	R	N	R
Sp.	96	36	77	42	154	75	85	40	158	53	141	32	76	19	110	21	102	33
Subsp	16	8	26	4	12	16	23	13	20	4	29	5	1	2	12	2	10	7
Var.	11	2	5	4	15	13	9	1	9	3	8	4	-	-	3	-	2	2
Hyb.	1	-	1	1	8	2	3	-	1	-	8	1	1		2	2	3	-
Total	124	46	109	51	189	106	120	54	188	60	186	42	78	21	127	25	117	42

Pinus L. (1:72)

e***P. sylvestris* L. var. ***elicinii*** Kandemir & Mataracı in Resimli Türkiye Florası 2: 338-342, 2018 [Kandemir & Mataracı 2018].

Type: Türkiye. A8 Trabzon: Sürmene, Sürmene-Of arası, Çamburnu-Sargona mevkii, 37T 4530669 K, 602442 D, 116 m, 26.xi.2016, A. Kandemir 10878 (holo: NGBB 5770; iso. ANK, GAZI, HUB, ISTE, ISTO).

DICOTYLEDONES

AMARANTHACEAE

Atriplex L. (2:305)

e***A. sukhorukovii* Başköse & Yaprak in Phytotaxa 369 (3): 227-235, 2018 [Başköse & Yaprak 2018].

Type: Turkey. B9 Erzurum: Karaçoban district, Kırmızı saltworks (Çoban saltworks), salt pools around, 1570 m, 17 August 2013, N 039º17.377' E 042º06.333', Yaprak and Başköse 2236 (holotype and isotype ANK).

Corispermum L. (2:318)

******C. nitidum* Kit. ex Schult. Oestr. Fl., ed. 2 1: 7 (1814). [Keskin et al. 2017].

Examined specimens: Türkiye, İstanbul: Sarıyer, Uskumruköy, Kıztaşı deresi, Arıburnu altı, Boğaziçi Üniversitesi kampüs kumulları, 01.viii.2015, 0 m, M.Keskin 5836a; Sarıyer, Boğaziçi üniversitesi, Sarıtepe kampüsü, dere kenarı, orman sınırı, 06.x.2015, M.Koçyiğit Avcı ve T.Avcı (ISTE 115039); Sarıyer, Boğaziçi Üniversitesi, Sarıtepe Kampüsü, iki plaj arası, dere çevresi yeşil alan sınırı, kumullar, 18.ix.2016, 0 m, N 41°14›41,4" ve E 029°00›24,8", M.Keskin 6527, N.Özhatay ve E. Özhatay. Samsun: Kızılırmak deltası, Karaboğaz gölü'nün kuzeyine bitişik kıyı kumulları, kurak kıyı kumulları, 14.x.1994, yaklaşık 5 m, A.J. Byfield B 1373 (ISTE 67614); Terme, Akçay nehrinin ağzı, nemli nehir kenarları, 12.x.1994, yaklaşık 2 m, A.J.Byfield B 1351 (ISTE 67592).

ANACARDIACEAE

Rhus L. (2:543)

****R. chinensis*** Mill. var. ***chinensis***, in Gard. Dict., ed. 8, Rhus no. 7. 1768. [Terzioğlu & Coşkunçelebi 2017].

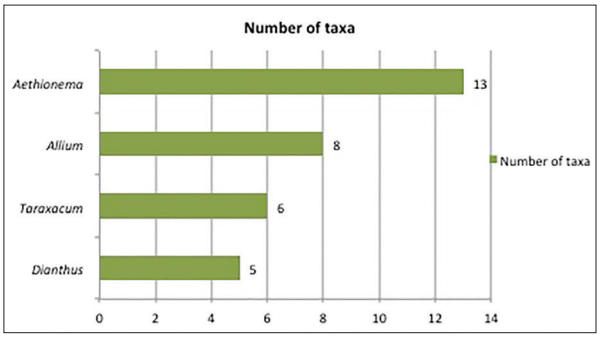

Figure 1. The most species have been added in Check-list IX.

Examined specimen: A8 Artvin: Hopa-Kemalpaşa, in a mixed broadleaved forest, UTM: 0711597, 4598023; 10 m, 26.ix.2014, S. Terzioğlu and K. Coşkunçelebi 1209, (KATO: 9857, KTUB); Rize: Hazar Mahallesi, in thickets amongst tea plantations, UTM 0613517, 4539580; 86 m, 14.ix.2014.

ARISTOLOCHIACEAE

Aristolochia L. (7:552)

e**A. altanii İlçim & Behçet in Turk J Bot 39: 835-840, 2015 [İlçim et al. 2015].

Type: Turkey. C6 Kahramanmaraş: upper parts of Dereboğazı and Hartlap Village, 680–700 m, 03.v.2012, (fl), rocky places, *A.İlçim* 1819 (Holotype: Mustafa Kemal Univ. Herb., isotype: Bingöl Univ. Herb.).

BORAGINACEAE

Alkanna Tausch (6:414)

****A. strigosa*** Boiss. & Hohen., Diagn. Pl. Orient. ser. 1, 4: 46. (1844) [Yıldırım et al. 2017].

Examined specimens: Türkiye, Gaziantep: iv.1907, Haradjian 1469 (W); iii.1907, Haradjian 831 (W); Dülükbaba, ca. 1330 m, Haradjian 1200 (W). Hatay: Kırıkhan, İncirli Köyü civarı, Perişan mevkii, ceylan koruma sahası yanı, taşlık yamaçlar, 230 m, 18.iv.2016, H. Yıldırım 3776 (EGE). Malatya: 1000-2500 m, v.1932, Jul. v. Ajtai-Kovach (W); in alpibes Akdagh, Aucher-Elloy 2358 (syntype of A. syriaca: G). Şanlıurfa: Karaköprü, Maşuk Köyü civarı, step, 37°12′ 52″ K, 38°47 06″ D, 648 m, 24.iv.2015, H. Yıldırım 3194 (EGE); Şanlıurfa-Diyarbakır yolu, Yenice Köyü, step, 37°19′ 48.73″ K, 38°59′ 34.81″ D, 619 m, 19.iv.2015, M.M. Balos 4000 (EGE); Diyarbakır yolu sol tarafı, Maşuk Köyü civarı, step, 37°13′ 48.12″ K, 38°47′ 03.68″ D, 643 m, 16.v.2015, M.M. Balos 4001 (EGE); Karaköprü Atatürk Ormanı civarı, step, 37°14.404 N, 38°48.519′ E, 727 m, 19.v.2016, M.M. Balos 4002 (EGE); Germuş Köyü (Dağ eteği köyü), step, 37°11′ 54.89″ K, 38°50′ 36.23″ D, 621 m, 20.v.2016, M.M. Balos 4003 (EGE); Karaköprü Badıllı Köyü tepelikleri, step, 37°12′ 49.04″ K, 38°48′ 58.16″ D, 673 m, 15.vi.2016, M.M. Balos 4004 (EGE); Şanlıurfa-Mardin yolu sol tarafı, Göbeklitepe civarı, step, 37°11′ 43.52″ K, 38°53′ 48.77″ D, 620 m, 16.vi.2016, M.M. Balos 4005 (EGE).

Onosma L. (6:326)

e***O. erzincanica*** Binzet & Eren in Phytotaxa 356(2): 117-130, 2018 [Binzet & Eren 2018].

Type: Turkey. B7 Erzincan: Between Kemah and İliç, 25 km after Kemah, on limestone screes, 39°36′44□ N, 38°41′15□ E, 1570 m, 02.vi.2014, Binzet 201417 (holotype: ANK, isotypes: AYDN, GAZI, Herbarium of Mersin University).

Paracaryum (DC.) Boiss. (6:282)

e** ***P. leventshikii*** Yıld., in Ot Sistematik Botanik Dergisi, 25(2): 1-22 (2018). [Yıldırımlı, 2018b].

Type: Turkey. A3 Ankara: Nallıhan, Davutlar köyü berisi, kuş cennetine varmadan, jipizli yamaç ve tepe, 600-625 m, 07.vi.2018, Ş. Yıldırımlı 44657 (holo.Yıldırımlı Otluk'u; iso. HUB).

Solenanthus Ledeb. (6:303)

e** ***S. abayi*** Yıld. & G. Tuttu, in Ot Sistematik Botanik Dergisi, 24(2): 47-57 (2017) [Yıldırımlı et al. 2017].

Type: Turkey. A5 Kastamonu: Tosya, Yeşilgöl, DSİ Göleti çevresi, yol kıyısı, sarıçam (*Pinus sylvestris*) açıklıkları, 1550 m, 40°51□26 81□ N, 34°00□05 75□ E, 13.vi.2015, G. Tuttu 2312, fruiting specimen (holo. Hb. Yıldırımlı; iso. HUB).

CAMPANULACEAE

Campanula L. (6:2)

e***C. baskilensis*** Behçet in Nordic J Bot 36(10): 1-5 (2018) [Behçet & İlçim 2018].

Holotype: Turkey. B7 Elazığ: 15 km south of Baskil Town, 38°27□18□N, 38°49□41□E, southeast of Topalkem Village, rocky area, 900–950 m a.s.l., 31.vii.2016, L. Behçet 11860 (holotype: ANK, isotypes: Bingöl Univ. Herb. and Mustafa Kemal Univ. Herb.).

e***C. leblebicii*** Yıldırım in Phytotaxa 376 (2): 114-122 (2018). [Yıldırım, 2018].

Type: Turkey. B1 İzmir: Kemalpaşa district, Ovacık Village, top of Çaldede Mountain, phyllite-schist rocks crack, 1385 m, 38 20 N. 27 38 E, 09.vii.2014, H. Yıldırım 3013 (holotype: EGE42445, ANK, HUB, NGBB).

CAPRIFOLIACEAE

Cephalaria Schrad. ex Roem. & Schult. (4:585).

e***C. cilodaghensis*** Ranjbar & Z. Ranjbar in Feddes Repertorium 129: 13–24 (2018) [Ranjbar & Ranjbar 2018].

Holotype: Turkey. C9 Hakkari: Cilo Mountain, above Diz deresi, 37°29□55□ N 43°57□35□ E, 2438.4 m, 7.viii.1954, Davis 23,941,0. Polunin s.n. (E, photo BASU).

CARYOPHYLLACEAE

Bolanthus (Ser.) Reichb. (2:171)

e***B. sandrasicus*** Hamzaoğlu & Koç in Nordic Journal of Botany 35: 563–568 (2017) [Hamzaoğlu et al. 2017a].

Type: Turkey. C2 Muğla: Köyceğiz, south of Kartal Lake, 1960 m a.s.l., subalpine serpentine flats, 17.vii.2016, Koç 2343 and Hamzaoğlu (holotype: GAZI, isotypes: GAZI, ANK, Bozok Univ. Herb.).

Cerastium L. (2:73)

****C. brachypetalum*** Pers. subsp. ***pindigenum*** (Lonsing) P.D.Sell & Whitehead, Feddes Repert. 69:18 (1964) [Keskin, 2018].

Examined specimen: İzmir: Kemalpaşa, Nif dağı alpinik zon, 1400 m, 21.v.1980, Ö. Seçmen 2161, L. Bekat (EGE 17534).

Dianthus L. (2:99)

e***D. halisdemirii* Hamzaoğlu & Koç in KSÜ Tarım ve Doğa Derg 21(4): 545-55 (2018) [Hamzaoğlu et al. 2018].

Type: Turkey. B2 Kütahya: Gediz, Murat Mountain, road of ski resort, towards summit, serpentine slopes, 2050 m, 02.viii.2015, Hamzaoğlu & Koç 2127 (holo. GAZI, iso. GAZI, ANK)

e***D. sancarii* Hamzaoğlu & Koç, in Biodicon 11(1): 30-34, (2018) [Hamzaoğlu & Koç, 2018].

Type: Turkey. B9 Bitlis: Tatvan, above Koruklu Village, 1990 m a.s.l., *Quercus* openings, damp places, 12.vii.2015, Hamzaoğlu 7038 & Koç (holotype: GAZI; isotypes: GAZI, ANK, HUB).

e***D. somanus* Oskay in Phytotaxa 347 (4): 263-271 (2018) [Oskay, 2018].

Type: Turkey. B1 Soma district, Manisa province, Kocasivri Hill, 39°15′41.4□N, 27°43′20.2□E, 950 m, July 2009, (holotype EGE-42705, isotypes EGE).

e***D. ucarii* Hamzaoğlu & Koç in Turk J Bot 41: 486-492 (2017) [Hamzaoğlu et al. 2017b].

Type: Turkey. A1(E) Kırklareli: Vize to Kıyıköy, ca. 5–6 km, 430 m, 15.vi.2013, *Quercus* forest clearings, Hamzaoğlu 6719, Aksoy & Koç (holotype: GAZI; isotypes: GAZI, ANK).

e***D. varankii* Hamzaoğlu & Koç in KSÜ Tarım ve Doğa Derg 21(4): 545-55 (2018) [Hamzaoğlu et al. 2018].

Type: Turkey. A5 Kastamonu: Hanönü, Küreçayı Village turnout, Pinus brutia Ten. and Quercus pubescens Willd. openings, 480 m, 13.viii.2016, Hamzaoğlu 7241 & Koç (holo. GAZI, iso. GAZI, ANK).

Paronychia Miller (2:250)

e***P. aksoyii* Budak in Phytotaxa 291 (3): 224–230 (2017) [Budak et al. 2017].

Type: Turkey. A8 Erzurum: Tortum, near Tortumkale Village, 1580–1620 m, sandy steppes, 40°20′46□N, 041°29′36□E, 05.vii.2014, Budak & Hamzaoğlu 3060 (holotype GAZI, isotypes KTUB, ANK, Bozok Univ. Biology Dept. Herbarium).

e***P. davrazensis* Budak in Phytotaxa 291 (3): 224–230 (2017) [Budak et al. 2017].

Type: Turkey. C3 Isparta: above Davraz sky center, 1920–1960 m, calcareous rocky places, 37°46′22□N, 030°44′35□E, 10.vii.2014, Budak 3193 & Hamzaoğlu (holotype GAZI, isotypes KTUB, ANK, Bozok Univ. Biology Dept. Herbarium).

e***P. kocii* Budak in Phytotaxa 291 (3): 224–230 (2017) [Budak et al. 2017].

Type: Turkey. B8 Erzincan: between Pülümür and Erzincan, near pass, 1820–1880 m, steppes, 39°11′19□N, 039°52′27□E, 12.vii.2012, Budak & Hamzaoğlu 2685 (holotype GAZI, isotypes KTUB, ANK, Bozok Univ. Biology Dept. Herbarium).

Silene L. (2:179)

e***S. eminentis* Özçelik, in Bio-Science Research Bulletin, 18: 121-129 (2002) [Özçelik, 2002].

Type: Turkey. B8 Erzurum: Palandöken Mountains, 30 km to Tekman, alpinic steppe, 2200-2400 m, 19.vii.1993, Özçelik 6114 (holo. Hb. S. Demirel University, Isparta; ıso. Hb. Yüzüncü Yıl University, Van).

e***S. guerbuezii* Özçelik, in Bio-Science Research Bulletin, 18: 121-129 (2002) [Özçelik, 2002].

Type: Turkey. C3 Isparta: Aksu, Dedegül Mountain, 8 km to town Yenişarbademli, around Pınargözü excursion spot, Pinus nigra forest glades, 1700 m, 2.ıx.1996, Özçelik 7681 (Holo: Hb. S. Demirel University, Isparta; ıso: Hb. GAZI, Ankara).

e***S. magenta* Yıld. & Kılıç, in Ot Sistematik Botanik Dergisi, 24 (2): 1-8 (2017) [Yıldırımlı & Kılıç 2017a].

Type: Turkey. B8 Bingöl: Kiğı, Karakoçan-Sancak yol ayrımından sonra y. 6. Km, yol kıyısı, yamaç, 1450 m, 24.v.2017, Ş. Yıldırımlı 43367 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. ANK, EGE, GAZİ, HUB, PAMUK, Yıldırımlı Otluk'u).

e***S. nemrutensis* K. Yıldız in Phytotaxa 292 (2): 189-195 (2017) [Yıldız et al. 2017].

Type: Turkey. C7 Adıyaman: Nemrut Mountain, around Commagene Kingdom monuments, rocky, stony areas, southern slopes, 2050-2070 m a.s.l., 37°58′583□N, 38°44′260□E, 3.viii.2005, Yıldız, Dodandı et Fırat 073 (holotype CBAH, isotypes MUFE, ERCH, VANF)

Phryna Pax & Hoff. (2:148)

e***P. hamzaoglui* Koç & Budak in PhytoKeys 109: 27–32 (2018) [Koç & Budak, 2018].

Type: Turkey. B6 Malatya: Between Hekimhan and Hasançelebi Town, 3 km from Hekimhan, 1100 m a.s.l., hillside, 10.x.2015, Koç 2353, Hamzaoğlu & Budak (holotype ANK; isotypes ANK, GAZI, Bozok Univ. Biology Dept. Herbarium).

COMPOSITAE

Artemisia L. (5:311)

e***A. taurica* Willd. var. ***vanensis*** Kursat & Civelek in Biodicon 11(3): 106-114 (2018) [Kursat et al. 2018].

Type: Turkey. B9 Van: 30th km of the highway from Van to Hakkari, slopes around Zernek irrigation dam lake, mountain steppe, 1960 m., 38º 20.872N, 43º 41.867E, 20.ix.2007, S. Civelek & M. Kursat 1056 (holotype FUH).

Centaurea L. (5:465)

e***C. bingoelensis* Behçet & İlçim in Turk J Bot 41: 180-188 (2017) [Behçet et al. 2017].

Holotype: Turkey. B8 Bingöl: 25 km west of city of Bingöl, north of Yelesen Village, southern rocky slopes 1900–2050

m, 26.vi.2014, L. Behçet 9648. (Holotype: Mustafa Kemal Univ. Herb., Isotype: ANK, Bingöl Univ. Herb.).

e***C. goerkii* Yıld., in Ot Sistematik Botanik Dergisi, 25(2): 1-22 (2018) [Yıldırımlı, 2018b].

Type: Turkey. B4 Ankara: Sivrihisar'a doğru, Acıkır-Sivrihisar arası, Demirci köyü berisi, jipizli yerler, 880 m, 07.vi.2018, Ş. Yıldırımlı, 44613 (holo. Yıldırımlı Otluk'u; iso. HUB).

e***C. kirmacii* Uysal & Armağan in Phytotaxa 362 (2): 233-238 (2018) [Armağan & Uysal 2018].

Type: Turkey. C2 Muğla: Kavaklıdere, Menteşe viillage, 3.5 km southern of Gökçukur plateu, above limestones, 1620 m, 37°19'1.6N, 28°22'27.4E, 27.vii.2017, Armağan 7797 (holotype KNYA)

e***C. mersinensis* Uysal & Hamzaoğlu in Plant Biosystems 151(5): 813-821 (2017) [Uysal & Hamzaoğlu 2017].

Type: Turkey. C4 Mersin, Aydıncık-Yenikaş köyü üstü, Pinus brutia orman açıklıkları, kireçli yamaçlar, 36° 08′ 38″ N, 33° 15′ 18″ E, 520 m, 3.vi.2014, Hamzaoğlu 7009 (Holotype: GAZI, isotypes; KNYA, ANK, HUB, GAZI).

Cirsium Miller (5:370)

e***C. bozkirensis* H.Duman, Dirmenci & Tugay in Turk J Bot 41: 375-382 (2017). [Duman et al. 2017a].

Type: Turkey. C4 Konya; Bozkır, between Sorkun District and Dikilitaş Yaylası, 1780 m a.s.l., 28.vii.2002, O.Tugay 3060 & Ertuğrul (Holotype: KNYA; Isotypes: GAZI, KNYA).

Crepis L. (5:814)

e***C. palaestina* subsp. *babcockii* Inceer & Aksu Kalmuk, in Bangladesh J. Plant Taxon, 25: 45-49 (2018) [Inceer & Kalmuk, 2018].

Type: Turkey. C3 Antalya: Manavgat, 10 m, 24.iv.2015, Inceer 1142 (Holotype: KTUB; Isotype: ANK).

Gundelia L. (5:325)

******G. armeniaca* Nersesian in Ann. Naturhist. Mus. Wien, B 116: 192 (2014) [Fırat, 2018a].

Examined specimen: Turkey. Ağrı, the specimen grows in *Astragalus* sp. steppe and near lowland at c. 1600-1900 m.

e***G. mesopotamica* Fırat in Acta Biologica Turcica 30 (3): 64-69 (2017) [Fırat, 2017b].

Type: Turkey. C8 Mardin: 2-3 km from Mardin to Nusaybin (Nisêbîn), eroded slopes, aride steppe, 807 m, 37°17′36″N, 40°46′20″E, coll. 8.v.2017, M. Fırat 33725 (holotype VANF, isotypes ANK, Herb. M. Fırat).

****G. rosea*** Al-Taey & Hossain in Notes Roy. Bot. Gard. Edinburgh 42(1): 41 (1984) [Fırat, 2017c].

Examined specimens: Turkey. C10 Hakkari: Şemdinli Province, Sad Mountain, Derîyê Kera region, meadows and stony slopes, 1662 m, 37°16′17″ N, 44°20′09″ E, coll. 25.v.2011, M. Fırat 27393 (Herb. M. Fırat); C10 Hakkari: Şemdinli Province, Sad Mountain, Derîyê Kera region, meadows and stony slopes, 1662 m, 37°16′17″ N, 44°20′09″, coll. 27.vii.2011, M. Fırat 27620 (Herb. M. Fırat) (in fruit).

Jurinea Cass. (5:439)

e***J. efea* N. Aksoy, in Phytol. Balcan. 24(3): 351-359 (2018) [Aksoy et al. 2018].

Turkey. A3 Düzce, Yığılca, on in the road between Yoğunpelit and Yaylatepe Villages, 1175 m, in a *Fagus-Quercus* mixed forest and in open limestone rock areas, on southeastern slopes, 40°50′03″ N, 031°39′37″ E, 19.vi.2016, N. Aksoy 7926 (Holotype: DUOF 7020; isotypes: GAZI, ISTO).

LactucaL. (5:776)

****L. leucoclada***Rech.f. & Tuisl in Anz. Österr. Akad. Wiss., Math.-Naturwiss. Kl. 101: 399. 1964 [Güzel et al. 2018].

Examined specimens: Turkey. B7 Erzincan, from Erzincan to Refahiye, rocky slopes, 1549 m, 39°51′N, 39°16′E, 17.vii.2014, Coşkunçelebi & Güzel 253 (KTUB); Erzincan, from Erzincan to Refahiye, 08.viii.2014 Kandemir 10629 (KTUB).

OnopordumL. (5:356)

****O. cinereum*** Grossh. in Trudy Tiflissk. Bot. Sada Ser. 2, 1: 38 (1920) [Pınar et al. 2018a].

Examined specimens: Turkey. B8 Bingöl: Kığı, Kığı-Yedisu arası, kayalık-taşlık yamaçlar, yol kenarı, 39°21′46 K, 40°20′20 D, 1650 m, 19.vii.2017 M.Pınar 6850, M. Fidan & H.Eroğlu.

RhaponticumHill. (5: 460)

****R. pulchrum*** Fisch & C.A. Mey in Linnaea 10(Lit.): 101 (1835) [Özbek et al. 2017].

Examined specimens: Turkey. A5 Kastamonu: Hanönü, above yukarı Küreçayı Village, *Pinus brutia* openings, rocky slopes, 500-600 m, 11.vi.2016, Koç 2305 & Hamzaoğlu.

Senecio L. (5:145)

e***S. oflasii* Yıld. & Kılıç, in Ot Sistematik Botanik Dergisi, 25(1): 1-10 (2018) [Yıldırımlı & Kılıç, 2018b].

Type: Turkey. B7 Tunceli: Ovacık, Yılan Dağı, Cevizlidere köyünden İtyokuşu-Eşek meydanı-Devboğazı-Tapiktepe-Deveboynu-Barasor deresiizleğiyle Karataş köyüne, bozkır, bozuk meşe ormanı, 1550-2250 m, 19.vi.2015, Ş. Yıldırımlı 41532 & Ö. Kılıç (holo. YO).

SolidagoL. (5:116)

****S. gigantea*** Aiton in Hort. Kew. 3: 211 (1789) [Yılmaz, 2017].

Examined specimens: TurkeyA2 Bursa: Uludağ; Soğukpınar, Soğukpınar-Aras vadisi, 1160 m, 4003 K-29 08 D, 28.vii.2015, Ö. Yılmaz 118/15-1, S. Karabulut (BULU).

■ ***Symphyotrichum*** Nees

S. pilosum (Willd.) G.L. Nesom var. ***pilosum*** in Phytologia 77: 289 (1995) [Tunçkol et al. 2017].

Examined specimens: Turkey. A3 Zonguldak: Ereğli district at elevation of 10-150 (200) m, at a roadside, field side and abandoned fields, 29.vii.2016, B. Tunçkol 4400 & H. Yaşayacak (DUOF 7023).

Taraxacum Wiggers (5:788)

T. caudatuliforme Soest in Proc. Kon. Ned. Akad. Wetensch., Ser. C, Biol. Med. Sci. 69: 467 (1966) [Gürdal et al. 2018].

Examined specimen: Turkey. A1(E) Edirne: Pazarkule yolu, 32 m, 23.iv.2015, B. Gürdal, BG-854–22, H. Gürdal, (ISTE 107350, no. det. 31329).

T. darbandense Soest in Koninkl. Nederl. Akad. Wetensch., ser. C, 69: 378 [Gürdal et al. 2018].

Examined specimen: Turkey. A5 Çorum: travnate meze mezi policky u Kiziltepe, 15 km Z od Osmancık [grass ridge between fields near Kiziltepe, 15 km W. of Osmancık], 430 m, 28.iv.1997, Z. Kaplan, 4356 (ISTE, PRA, No. det. 31355).

T. erythrospermum Andrz. in Enum. Pl.: 75 (1821) [Gürdal et al. 2018].

Examined specimen: Turkey. A1(E) Kırklareli: Pınarhisar, İslambeyli-Evciler, 1.5 km from Evciler, wet meadows, 371 m, 23.iv.2014, B. Gürdal, BG-621–39, M. Koçyiğit, Y. Yeşil, (ISTE 102441, No. det. 31317).

T. fascinans Kirschner, Mikoláš and Štěpánek in Preslia 69: 45 (1997) [Gürdal et al. 2018].

Examined specimen: Turkey. A3 Bolu: Abant Silsilesi Mts, Abant lake, SW of Bolu, ca 1100 m, v.1997, J. Sadlo, 4756 (ISTE, PRA, no. det. 31325).

T. oblongatum Dahlst. in Rep. Bot. Soc. Exch. Club Brit. Isles 9: 27 (1930) [Gürdal et al. 2018].

Examined specimens: Turkey. A1(E) Kocaeli: Kartepe, Kardelen mesire alanı çevresi, 651 m, 17.iv.2015, B. Gürdal, BG-846-41, H. Gürdal, (ISTE 107342, no. det. 31341); A1(E) Kocaeli: Sapanca, İ.Ü. Su Ürünleri tesisi bahçesi, 58 m, 17.iv.2015, B. Gürdal, BG-844-441, H. Gürdal, (ISTE 107340, no. det. 31347).

e***T. pseudobithynicum* B.Gürdal, Štěpánek, Zeisek, Kirschner & N. Özhatay in Phytotaxa 373 (3): 197–210 (2018) [Gürdal et al 2018].

Type: Turkey. A2(A) Bursa: Uludağ, Volfram çevresi, 2176 m, 11.viii.2014, B. Gürdal 835-16, M. Koçyiğit (holotype: ISTE 102911).

CONVOLVULACEAE

Ipomoea L. (6:221)

I. hederifolia L. in Syst. Nat., ed. 10. 2: 925 (1759) [Hançerli et al. 2018].

Examined specimen: Turkey. C5 Adana: Sarıçam, maize fields, 37°1'26.17 N and 35°23'12.81 E, 2016.

I. triloba L. in Sp. Pl. 1: 161 (1753) [Yazlık et al. 2018].

Examined specimen: Turkey. C3 Antaya: Serik, 36°55032.6″ N 31°03024.7″ E and Aksu, 36°55026.6″ N 30°52042.3″ E, vii.2014, Ege University Botanical Garden and Herbarium Research and Application Centre as herbarium accession number EGE42414.

CRUCIFERAE

Aethionema W.T. Aiton (1:314)

e***A. adiyamanense* Yıld. & Kılıç, in Ot Sistematik Botanik Dergisi, 25(1): 25-30 (2018) [Yıldırımlı & Kılıç, 2018a].

Type: Turkey. C7 Adıyaman: Koçali-Çelikhan arası, sulu vadi, meşe (*Quercus brandii*) ormanı, 1061-1120 m, 29.iv.2018, Ş. Yıldırımlı 44198 & Ö. Kılıç (holo.Yıldırımlı Otluk'u; iso.ANK, EGE, GAZİ, HUB, PAMUK, Yıldırımlı Otluk'u).

e***A. alidaghenicum* Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. B5 Kayseri: Talas, Alidağ eteğinden doruğa, kuzey ağaçlandırmalı ve doğu meşelik yamaçlar, bozkır, y. 1300-2000 m, 24.vi.1995, Ş. Yıldırımlı 18672 (holo. Yıldırımlı Otluk'u (YO), iso. HUB, MUH, Yıldırımlı Otluk'u).

e***A. annuum* Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. B2 Manisa: Soma, HAcımusa-Bayat-Işıklar köyleri ilerisi, maden yolu yakını, maki-orman, çakıllı yerler, 650 m, 27.v.2009, Ş. Yıldırımlı35747 & Görkem Yıldırımlı (holo. Yıldırımlı Otluk'u, iso. HUB)

e***A. apetalum* Yıld. et Kılıç in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. B7 Tunceli: Merkez, Rabat (Çemçeli) köyü alt yolu, kırmızı kayrak taşlık yer, meşe ormanı, 1300 m, 30.v.2015, Ş. Yıldırımlı 41056 & Ö. Kılıç (holo. Yıldırımlı Otluk'u (YO), iso. GAZI, HUB, Nezahat Gökyiğit Botanik Bahçesi Otluk'u, Yıldırımlı Otluk'u).

e***A. bingoelicum* Yıld. et Kılıç in Ot 23 (1-2): 1-66, 2016 [Yıldırımlı & Kılıç 2016a].

Type: Turkey. B8 Bingöl: Merkez, Alatepe (Arçuk, Armani) köyü üstü, Fırın deresi çevresi, yayla, meşe ormanı ve açıklığı, yamaç, taşlık, 1900-1950 m, 11.vi.2015, Ş. Yıldırımlı 41402 & Ö. Kılıç (holo. Yıldırımlı Otluk'u, iso. HUB).

e***A. dincii* Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. C6 Osmaniye: Amanos dağları, Zorkun yaylası üstü, alpin step, 2000 m, 27.viii.2000, M. Dinç 949 (holo. Yıldırımlı Otluk'u).

e***A. dincii* Yıld. subsp. ***baytopiae*** Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. C5 Adana: Karsantı, Şamadan bölgesi, Karapınar mevkisi, ağaçlandırma sahası çevresi, 1700 m, 26.vii.1979, E. Tuzlacı & M. Saraçoğlu (ISTE 43295) (holo. Yıldırımlı Otluk'u).

e***A. dumelicum* Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. A4 Çankırı: Eldivan (Dümeli), Karadere boyunca, karışık orman kıyısı, 900-1200 m, 27.v.1983, Ş. Yıldırımlı 5005 (holo. Yıldırımlı Otluk'u, iso. HUB, Yıldırımlı Otluk'u).

e***A. ertughrulli* Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. B5 Kırşehir: Mucur, Ayrı dağları etekleri, bozkır, 1300-1450 m, 21.v.1989, Ş.Yıldırımlı 12202 (holo. Yıldırımlı Otluk'u, iso. HUB).

e***A. kilicii* Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. B6 Sivas: Zara, Halkalı ve Korkut köyleri karşısı, bozkır, jipsli dere, tepe, bayır, karışık orman ve açıklığı, 1385-1500 m, 19.vi.2014, Ş. Yıldırımlı 40044 & Ö. Kılıç (holo. Yıldırımlı Otluk'u, iso. ANK, HUB, Yıldırımlı Otluk'u).

e***A. ozbekii* Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. B7 Tunceli: Ovacık, Karagöl kepezi (vadisi), kayalık ve taşlık alanlar, 135 m, 30.vi.1983, Ş. Yıldırımlı 5406 (holo. Yıldırımlı Otluk'u).

e***A.turanicum* Yıld. in Ot 23 (1-2): 1-66 (2016) [Yıldırımlı & Kılıç 2016a].

Type: Turkey. A7 Gümüşhane: Şiran, karaçam (*Pinus nigra*)-tüylümeşe (*Quercus pubescens*)- dağardıcı (*Juniperus excelsa*) ve dikenliardıç (*Junipeus oxycedrus*) karışık ormanı, 1400-1550 m, 19.vii.1984, Ş. Yıldırımlı 6732 (holo. Yıldırımlı Otluk'u, iso. EGE, HUB, YO).

e***A. yildirimlii* Kılıç in Ot 23 (1-2): 1-66, 2016 [Yıldırımlı & Kılıç 2016a].

Type: Turkey. C3 Konya: Beyşehir, Çamlık kasabası, Kızıldağ, doğu yamaç, karaçam (*Pinus nigra*) ormanı, kayalık ve taşlık, 1470-1705 m, 01.vi.2008, Ş. Yıldırımlı 34914 & Görkem Yıldırımlı (holo. Yıldırımlı Otluk'u, iso. HUB, PAMUH, Yıldırımlı Otluk'u).

Aubrieta Adanson (1:444)

e***A. alshehbazii* Dönmez, Uğurlu & M.A.Koch in Phytotaxa 209(1): 13-110 (2017) [Dönmez et al. 2017].

Type: Turkey. Afyon: Çay, Karakuş Mountain, between Aydoğmuş-Armutlu Villages, limestone, 38°23'11N, 030°46'55E, 1100 m, 31.iii.2001, A.A. Dönmez 8282 (holotype HUB, isotypes HEID, HUB).

Berteroa DC. (1:360)

e***B. physocarpa* Yüzb. & Al-Shehbaz in Phytotaxa 305(2): 87-96 (2017) [Yüzbaşıoğlu et al. 2017].

Turkey. A2(A) Kocaeli: KArtepe, zirve çevresi, 1600 m, 24.vii.1994, A.J. Byfield 1058 (holotype ISTE 67180, isotype ISTE).

Erysimum L. (1:466)

e***E. erolii* Yıld., in in Ot Sistematik Botanik Dergisi, 25(2): 1-22 (2018) [Yıldırımlı, 2018b].

Type: Turkey. A3 Ankara: Nallıhan, Davutlar köyü berisi, kuş cennetine varmadan, jipizli yamaç ve tepe, 600-625 m, 07.vi.2018, Ş. Yıldırımlı 44660 (holo.Yıldırımlı Otluk'u; iso. HUB).

e***E. nemrutdaghense* Mutlu, in Phytotaxa 336 (3): 239-251 (2018) [Mutlu 2018].

Type: Turkey. C7 Adıyaman: Kahta Village, The Northern side of Nemrut Mountain, 37 58 54.48 N-38 44 27 82 E, 2138 m, alpine steppe, 06.vi.2009, B. Mutlu 10839 (in flower) (holotype: INU; isotype: HUB, GAZI); ibid., 28.vi.2009, B. Mutlu 10958- Fatma and Can Mutlu (in fruit); ibid., 22.vii.2010, B. Mutlu 11232 & İ. Yıldırım (in mature fruit).

■ ***Diptychocarpus*** Trautv.

****D. strictus*** (Fisch. Ex M. Bieb.) Trautv in Bull Soc. Imp. Naturalistes Moscou 33(1): 108 (1860) [Fidan et al. 2017].

Turkey, B9 Van: Edremit, Kadembas district, behind the green valley picnic area, slopes, 28.iii.2010, 38°24.48′ N, 43°13.58′ E, 1683 m, M. Fidan 551; 18.iv.2014, M. Pınar 4063 & H. Eroğlu (in fruit).

Fibigia Medik. (1:356)

F. yildirimlii Kılıç, in Ot Sistematik Botanik Dergisi, 24(2): 27-35 (2017) [Yıldırımlı & Kılıç 2017c].

Type: Turkey. B7 Tunceli: Merkez, Rabat (Çemçeli) köyü alt yolu, kırmızı kayrak taşlık yer, meşe ormanı, 1300 m, 30.v.2015, Ş. Yıldırımlı 41058 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. HUB, Yıldırımlı Otluk'u).

Iberis L. (1:309)

e***I. gypsicola* Yıld., in Ot Sistematik Botanik Dergisi, 25(2): 1-22 (2018) [Yıldırımlı, 2018b].

Type: Turkey. B4 Ankara: Sivrihisar'a doğru, Acıkır-Sivrihisar arası, Demirci köyü berisi, jipizli yerler, 880 m, 07.vi.2018, Ş. Yıldırımlı, 44620 (holo. Yıldırımlı Otluk'u; iso. HUB).

e***I. saxatilis* subsp. ***magnesiana*** Oskay in Phytotaxa 306 (2): 153–158 (2017) [Oksay 2017].

Type: Turkey. B1 Manisa: Soma district, Kocasivri Hill, 850 m, April 2009, (holotype EGE 42417, isotypes EGE, CBÜ- Department of Biology).

Noccaea Moench

e***N. ali-atahanii* Güzel, Özüdoğru & Kayıkçı, in Turk J Bot 42: 780-789 (2018) [Güzel et al. 2018].

Type: Turkey. C6 Hatay: Defne, Subaşı, 36°11′8.74″ N, 36°7′33.71″ E, 120 m, 25.iv.2017, Güzel 902 (holotype HUB, isotypes ANK, GAZI).

Thlaspi L. (1:330)

****T. kilicii*** Yıld., in Ot Sistematik Botanik Dergisi, 24(2): 27-35 (2017) [Yıldırımlı & Kılıç 2017c].

Type: Turkey. B7 Tunceli: Nazimiye, Büyükyurt (Hakis) köyü üstü, Şirince ve Sinekli dere çevresi, titrekkavak (*Populus tremula*) ormanı, kuru dere boyunca, 1850-2000 m, 31.v.2015, Ş. Yıldırımlı 41211 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. HUB, Yıldırımlı Otluk'u).

GROSSULARIACEAE

Ribes L. (4:261)

****R. aureum*** Pursh in Fl. Amer. Sept. (Pursh) 1: 164 (1813) [Yıldırımlı et al., 2004; Fırat 2018c].

Examined specimen: Turkey. A9 Kars: Kağızman Province, at the natural garden, 1294 m, 40°10′03″ N, 43°08′11″ E, coll. 25.iv.2014, M. Fırat 30535 (VANF, Herb. M. Fırat), A9 Kars: Kağızman Province, at the natural garden, 1294 m, 40°10′03″ N, 43°08′11″ E, coll. 9.v.2016, M. Fırat 32659 (VANF, Herb. M. Fırat); B1 İzmir: Karşıyaka, Bostanlı, cultivated areas, 1 m, 13.ix.2003, Ş. Yıldırımlı 29271.

HYPERICACEAE

Hypericum L. (2:355)

e***H. bilgehan-bilgilii* Başköse & Savran, in Phytotaxa 374 (2): 110-118 (2018) [Başköse & Savran, 2018].

Type: Turkey. Konya: Beyşehir district, Dumanlı Village-Antalya road, 8 km S-SW of Dumanlı Village, Çürük Mountain, Düden cave environments, on calcareous rock, chasmophyte, 1650 m, 02.vii.2016, N 37°29′21.66″ E 31°19′35.81″, Başköse and Savran 4600, (holotype ANK, ısotypes ANK and Herbarium of Niğde Ömer Halisdemir University).

e***H. ekerii* E. Yüce & Aytaç in Pak. J. Bot., 49(5): 1763-1768 (2017) [Yüce Babacan et al. 2017].

Type: Turkey. B7 Tunceli, Mazgirt, c. 21 km E of Tunceli, c. 3 km SE of Çevrecik, Düzgün Baba Dağı (Munzur Mountain ranges), 1800–2060 m, 11.vi.2015, E.Yüce 3000 (holo. GAZI, iso. ANK)

LABIATAE

Lamium L. (7:126)

e***L. bilgili* Celep in Phytotaxa 312 (2): 263-270 (2017) [Celep 2017].

Type: Turkey. C2 Burdur-Muğla: Altınyayla (Dirmil), Yayla Karaçulha village, Karaağaç plateau, 1870-2100 m, 36°55′39.63″ N, 29°35′ 23.98″ E, 5.vi.2015, F. Celep 3860 (holotype GAZI, isotype ANK).

Marrubium L. (7:165)

e***M. amasiensis* Akgül & Ketenoğlu, in Ot Sistematik Botanik Dergisi, 24(2): 37-46 (2017) [Akgül et al. 2017].

Type: Turkey. A5 Amasya: Direkli Village, S of Sakarat dağı, location of Karanlıkönü, 1100-1400 m, 30.vi.2002, on glades in mixed and *Quercus* forest slopes, G. Akgül 2556 (holo. ANK.), GPS. 37°24′65″ E, 45°06′39″ N.

e***M. lanatum* Akgül, in Ot Sistematik Botanik Dergisi, 25(2): 23-30 (2018) [Akgül, 2018].

Type: Turkey. C5 Niğde: Centrum, near Azatlı Village, rocky slopes, 1600-1650 m, 15.vii.2012, G. Akgül 2418 (holo. ANK; iso. Yıldırımlı Otluk'u).

Micromeria Bentham (7:335)

e***M. aybalae* H. Duman & T. Dirmenci in Turk J Bot 41: 383-391 (2017) [Duman & Dirmenci, 2017].

Type: Turkey. C2 Muğla: Köyceğiz, Sandras Mountain, west of Çayhisar Village, calcareous rocks, 980–1000 m a.s.l., 4.xi.2015, H.Duman 10395 (holotype GAZI, isotypes ANK, HUB, ISTE).

Origanum L. (7:297)

e***O. ayliniae* Dirmenci & Yazıcı, in Turk J Bot 42: 73-90 (2018) [Dirmenci et al. 2018b].

Type: Turkey. C1 Aydın: Kuşadası, Dilek Peninsula National Park, rocky slopes, 1195 m, 22.vii.2016, Dirmenci 4584 (holotype GAZI, isotypes ANK, EGE, HUB, ISTE).

e***O.* × *adae* Dirmenci & Yazıcı, in Turk J Bot 42: 73-90 (2018) [Dirmenci et al. 2018b].

(*Origanum ayliniae* Dirmenci & Yazıcı x *Origanum sipyleum* L.)

Type: Turkey. C1 Aydın: Kuşadası, Dilek Peninsula National Park, rocky slopes, 1195 m, 22.vii.2016, Dirmenci 4583 (holotype GAZI, isotypes ANK, EGE, HUB, ISTE).

e***O.* × *malyeri* Dirmenci & Yazıcı, in Phytotaxa 371 (3): 145-167 (2018) [Dirmenci et al. 2018a].

(*Origanum vulgare* L. subsp. hirtum (Link) A. Terracc. × *Origanum boissieri* letsw.)

Type: Turkey. C5 İçel: between Çamlıyayla and Saydibi, 13. km, calcareous in open *Abies cilicia* and *Cedrus libani* forest, 6050 ft., 37.219730 N, 34,563330 E, 01.x.2015, Dirmenci 4502 & Yazıcı (Holotype GAZI, isotypes ANK, ISTE, HUB, EGE).

e***O.* × *sevcaniae* Dirmenci, Arabacı & Yazıcı, in Phytotaxa 371 (3): 145-167 (2018) [Dirmenci et al. 2018a].

(*Origanum vulgare* L. subsp. hirtum (Link) A. Terracc. × *Origanum vogelii* Greuter & Burdet)

Type: Turkey. C5 Niğde: Ulukışla, Horoz Village, Fenk throat, 4800 ft, 37.455940 N, 34,751250 E, 02.x.2015, Dirmenci 4508 & Yazıcı (Holotype GAZI, isotypes ISTE, ANK, HUB, EGE).

Ziziphora L. (7:395)

Z. clinopodioides Lam. subsp. ***elbursensis*** (Rech.f.) Rech.f. in Fl. Iranica 150: 487 (1982) [Fırat 2017e].

Examined specimen: Turkey. B9 Van: Nebırnaf plateau, steppe, 2900 m, 37°54′44″ N, 43°43′10″ E, coll. 27.viii.2012, M. Fırat 28914 (Herb. M. Fırat).

Z. clinopodioides Lam. subsp. ***filicaulis*** (Rech.f.) Rech.f. in Fl. Iranica 150: 486 (1982) [Fırat 2017e].

Examined specimen: Turkey. C9 Hakkâri: Kandil plateau, steppe, 2956 m, 37°37′56″ N, 43°57′05″ E, coll. 21.vii.2011, M. Fırat 27775 (Herb. M. Fırat).

Z. clinopodioides Lam. subsp. ***kurdica*** (Rech.f.) Rech.f. in Fl. Iranica 150: 487 (1982) [Fırat 2017e].

Examined specimen: Turkey. B9 Van: Bahçesaray, Sampaz, helin stream, steppe, 1760 m, 37°58′25″ N, 42°47′05″ E, coll. 28.vii.2016, M. Fırat 28862 (Herb. M. Fırat).

Z. clinopodioides Lam. subsp. ***rigida*** (Boiss.) Rech.f. in Fl. Iranica 150: 483 (1982) [Fırat 2017e].

Examined specimen: Turkey. B9 Van: from Hoşap to Karadağ, steppe, 2100 m, 30.vii.2012, M. Fırat 28862 (Herb. M. Fırat).

LINACEAE

Linum L. (2:425)

e***L. ayliniae* Yılmaz in A Journal for Botanical Nomenclature, 26(2):174-179 (2018) [Yılmaz 2018].

Type: Turkey. B3 Afyon: Akdağ, 30 km from Işıklı to Sandıklı, 30 km, 1077 m, 8.v.2017, Ö. Yılmaz 2151 (holotype, BULU; isotypes, ANK, NGBB).

e**L. platyphyllum (P.H.Davis) Yıld., subsp. ***guvensenii***, in Ot Sistematik Botanik Dergisi, 25(2): 1-22 (2018) [Yıldırımlı, 2018b].

Type: Turkey. A3 Ankara: Nallıhan, Davutlar köyü berisi, kuş cennetine varmadan, jipizli yamaç ve tepe, 600-625 m, 07.vi.2018, Ş. Yıldırımlı 44671 (holo. Yıldırımlı Otluk'u; iso. HUB).

LythrumL. (4:174)

e**L. anatolicum Leblebici & Seçmen subsp. ***vanense*** Pınar in Acta Biologica Turcica 30(1) 16-19 (2017) [Pınar, 2017].

Type: Turkey. B9 Van: Çatak, North of Çatak Valley, surroundings of Bilgi Village, stream side, wet and sandy places, 1723 m, 25.viii.2002, 38°06′184″N, 43°16□213″E, M. Pınar 1229 (holotype: VANF, isotypes: GAZI, EGE)

■ ***Proboscidea***Schmid.

P. louisianica (Miller) Thell. in Mém. Soc. Sci. Nat. Math. Cherbourg 38: 480 (1912) [Sevgi et al. 2017].

Examined specimens: Turkey. A1 Çanakkale: Karabiga-Biga. Between Güleçköy and Yeniçiftlik on roadsides, dried Ece lake around, 7.ix.2017, 20m, Sevgi E., ISTO Number: 37325.

OrobancheL. (7:3)

O. inulae Novopokr. & Abramov in Bot. Mater. Gerb. Bot. Inst. Komarova Akad. Nauk S.S.S.R. 13: 323 (1950) [Raab-Straube & Raus, 2017].

Examined specimens: Turkey. Trabzon, Maçka, Altındere Valley National Park, july 1866, B. Balansa (P02968068)

O. reticulata subsp. ***agigensis*** Rätzel & Uhlich in Willdenowia, 46(3):430-432. [Raab-Straube & Raus, 2018].

Examined specimens: Turkey. B5 Kayseri, Mountain Erciyes, 15 km from Hisarcık to Develi, 38°30′42″N, 35°29′58″E, 2100 – 2200 m, steppe, 14.vii.2009, Zare 490 & al. (HUB); ibid., 2 km S of Tekir lake, 38°29′52″N, 35°30′59″E, steppe, 27.vi.2010, Zare 600 & Bayrak (HUB); ibid., Develi to Kayseri, 8 km before (S of) Tekir lake, 27.vi.2010, Zare 606 & Bayrak (HUB).

■ ***Phelipanche*** Pomel

P. kelleri (Novopokr.) Sojak in Čas. Nár. Mus., Odd. Přír. 140(3-4): 130 (1972) [Raab-Straube & Raus, 2018].

Examined specimens: Turkey. Ankara: Şereflikochisar, 12.v.2013, *Tonsun*, as *Orobanche* sp. (photo [http://dogal hayat.org/property/Orobanche-10/]).

PAPILIONACEAE

Astragalus L. (3:49)

e***A. ihsancalisii* Dönmez & Uğurlu in Willdenowia 48: 399–404 (2018) [Dönmez & Uğurlu-Aydın, 2018a].

Holotype: Turkey. A8 Erzurum: Horasan, 1 km from road junction of İğdeli Village to Eleşkirt, 1795 m, 39°56′31″N, 42°19′49″E, 26.vi.2015, A. A. Dönmez 19559 (HUB; isotypes: B, GAZI, HUB).

Ebenus L. (3:590)

e***E. zekiyeae* Aytaç & Yıldırım in Ann Bot Fennici 55: 25-29 (2017) [Aytaç & Yıldırım 2017].

Type: Turkey. A8 Erzurum: Hınıs-Pasinler road, 33rd km, between Erduran and Dibekli Villages, 2040 m a.s.l., limestone rocks, slopes, 30.vi.2016, H. Yıldırım 3967 (holotype GAZI, isotypes EGE, ANK).

Genista L. (3:24)

e***G. unalii* M. Dinç & Y. Bağcı, in Phytotaxa 371 (1): 49-54 (2018). [Dinç & Bağcı, 2018].

Type: Turkey. C5 Karaman: Ereğli, foot of Bolkar Mountains, above Çatköy, steppe, 1880 m, 23.v.2007, M. Dinç 2979 & Y. Bağcı (holotype KNYA; isotypes GAZI, HUB and personal herbarium-Yıldırımlı Herb).

Hedysarum L. (3:549)

e***H. ketenoglui* Başköse, Yaprak & Akyıldırım in Phytotaxa 357(4): 291-297 (2018) [Başköse et al. 2018].

Type: Turkey. C4 Konya: Hadim district, Taşkent to Alanya road, Gevne valley, northeast side of Yalnızardıç Dam Lake, Küçüklü Village, roadside, 1600 m, 5.ix. 2014, A.E. Yaprak, B. Akyıldırım and İ. Başköse 2656 (holotype ANK, isotypes ISTF, ANK).

H. singarense Boiss. & Hausskn. Fl. Orient. 2: 522 (1872) [Hoşgören & Ertekin, 2018].

Examined specimen: Turkey. B8 Diyarbakır, Silvan, Silvan to Sason, 3-4 km, around of Hassuni Mağaraları, stony and calcareous slopes, 970-980 m, 17.v.2014, A. Selçuk Ertekin, DUF 10128. ibid. 08.vi.2014, A. Selçuk Ertekin, DUF 10165. 37683071 E 4222262 N.

Lathyrus L. (3:328)

L. pannonicus (Jacq.) Garcke subsp. ***collinus*** (J. Ortmann) Soo, Scripta Bot. Mus. Transs.1:46 (1942) [Güneş, 2018].

Examined specimen: Türkiye. Edirne: Lalapaşa, Vaysal köyü Develiağaç köyü yolu, Ömeroba köyü yol ayrımı çevresi, meşe ormanı altı ve açıklıkları, 04.v.2016, F. Güneş 4765.

Trigonella L. (3:452)

e****T. coerulescens*** (M.Bieb.) Halacsy subsp. ***ayvalikensis*** Erdoğan, Selvi & Tümen in Phytotaxa 319 (2): 167-174 (2017) [Erdoğan et al. 2017].

Type: Turkey. B1 Balıkesir: Ayvalik, Küçükköy, west of Badavut beach, sandy coast, 1 m, 12.III. 2016, 39°16'23"N, 26°37□43"E, Selami Selvi 1616 (holotype: The herbarium of Balikesir University, Altınoluk Vocational School, Programme of Medicinal and Aromatic Plants, isotypes: ANK, ISTE)

Onobrychis Mill. (3:560)

e**O. mehmetchiquii in Phytotaxa 298(1): 96-100 (2017) [Aybeke & Dane 2017].

Type: Turkey. A1(E) Edirne: Centre, between Budakdoganca and Ahiköy Villages, first degree military forbidden zone, II. Mehmetcik station environs, 41°47'24□N, 26°21'34□E, 110 m, 06.vi.2015, M. Aybeke (holotype EDTU 15050).

PLUMBAGINACEAE

Acantholimon Boiss. (7:478)

e**A. gemicianum Kaptaner İğci, Körüklü & Aytaç in Ann. Bot. Fennici 54: 83-88 (2017) [Kaptaner İğci et al. 2017].

Type: Turkey. B3 Eskişehir: Ahiler-Kurtşeyh Village, 830-850 m, steppe, 23.vii.2015 Körüklü 20113 (holotype GAZI; isotype ANK).

e**A. ibrahimii Akaydın in Phytotaxa 340(1): 48-54 (2018) [Akaydın, 2018].

Type: Turkey. C5 Niğde: Çamardı, Demirkazık Village, on calcareous slopes, 1650 m, 08.vii.2008, Akaydın 11971 (holotype: ANK, isotype: HUB)

Limonium Miller (7:465)

e**L. didimense Doğan & Akaydın, in Ot Sistematik Botanik Dergisi, 24(2): 9-26 (2017) [Doğan & Akaydın 2017].

Type: Turkey. C1 Muğla: Mils, on the coast of Cennet bay, Bozbük, 2-3 m, 29.x.2002, G. Akaydın 7690 & Doğan (holo. ANK.).

e**L. marmarisense Doğan & Akaydın, in Ot Sistematik Botanik Dergisi, 24(2): 9-26 (2017) [Doğan & Akaydın 2017].

Type: Turkey. C1 Muğla: Marmaris, Bozburun, on the coast of Müskebi island, 2-3 m, 28.vii.2004, G. Akaydın 9910 & M. Doğan (holo. ANK).

POLYGALACEAE

Polygala L. (1:533)

e**P. azizsancarii Dönmez, in Phytotaxa 340(3): 255-262 (2018) [Dönmez & Uğurlu-Aydın 2018b].

Type: Turkey. C8 Mardin: 8 km from Mardin to Mazıdağı, Akresta Pass, steppe, limestone, 1075m, 37°23'14□N, 040°39'14□E, 27.v.2016, A.A.Dönmez 19755 (holotype HUB; isotypes: E, EGE, HUB).

ROSACEAE

Pyrus L. (4:160)

P. demetrii Kuth., Zametki Sist. Geogr. Rast. 13: 25 (1947) [Uğurlu Aydın & Dönmez 2017].

Examined specimen: Turkey. Sivas: 3.3 km from Sincan to Zara, steppe, among deciduous scrub, 39°29'31" N, 037°55'07" E, 1275 m a.s.l., 2.x.2012, AAD 19237 (HUB)

Spiraea L. (4:4)

e**S. cudidaghense Fırat & N. Aksoy in J For Res 29(9): 1751–1756 (2018) [Fırat & Aksoy 2018].

Type: Turkey. C9 Şırnak: Cudi Mountains, Bılgan region, rocky slopes, 969 m, 37°25'11□N, 42°38'57□E, 15.x.2013, M. Fırat 30467 (holotype DUOF, isotypes ISTO, VANF).

RUBIACEAE

Galium L. (7:767)

e**G. cariense Daşkın & Bağçıvan in Phytotaxa 308 (2): 267-274 (2017) [Daşkın & Bağçıvan 2017].

Type: Turkey. C2 Muğla: Çameli Fethiye road, between Karabayır and Üzümlü, 36°53'37□N, 29°10'02□E, serpentine screes under *Pinus brutia* forests, 1207 m, 19.v.2011(fl), R. Daşkın 1085 (holotype: BULU, isotypes: ANK, GAZI).

e**G. karliovaense Yıld. & Kılıç, in Ot Sistematik Botanik Dergisi, 25: 31-39 (2018) [Yıldırımlı & Kılıç, 2018c].

Type: Turkey. B8 Bingöl: Karlıova, Göynük köyü içinden geçerek sulu dere boyunca, tepeye, taşlık, bozkır, 1860-2060 m, 06.ix.2018, Ş. Yıldırımlı 44756 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. PAMUK, Yıldırımlı Otluk'u).

e***G. ovitdaghense* Daşkın in Nordic Journal of Botany 036 (1-2): 01-06 (2018) [Daşkın, 2018].

Type: Turkey. A8 Rize, İkizdere, around Ovit Dağı Pass, rocky slopes, 2640 m a.s.l., 28.vi.2012, fl. & fr., R. Daşkın 1383 (holotype: BULU, isotypes: ANK, BULU, GAZI).

e***G. sancakense* Yıld. & Kılıç, in Ot Sistematik Botanik Dergisi, 25: 31-39 (2018) [Yıldırımlı & Kılıç, 2018c].

Type: Turkey. B8 Bingöl: Sancak, Yazgülü köyü çevresi, Uzunsavat baraj gölü batı kesimleri, taşlı yamaç, nemli yerler, 1500-1600 m, 04.vi.2018, Ö. Kılıç 5988 & Ş. Yıldırımlı (holo. Yıldırımlı Otluk'u; iso. PAMUK, Yıldırımlı Otluk'u).

RUTACEAE

Haplophyllum Jussieu (2:496)

e***H. ermenekense* Ulukuş & Tugay, in PhytoKeys 111:119-131 (2018) [Ulukuş & Tugay, 2018].

Type: Turkey. C4 Karaman: Ermenek, limestone slopes, steppe, 1200 m, alt., 36°37.356 N, 32°51.543 E, 21.vi.2014, O. Tugay 9641 & Ulukuş (holotype: KNYA; isotype: ANK, GAZI).

e**H. sahiniiTugay & UlukuşinPhytotaxa 297 (3): 265-272 (2017) [Tugay & Ulukuş, 2017].

Type: Turkey. C4Konya: Çumra, between Apasaraycık-Apa Village, rocky area, 1090 m, 37°22.482'N, 32°28.765'E, 18.vi.214, O. Tugay 9266 & Ulukuş, KNYA Herb. No: 26908 (holotype: KNYA; isotype: ANK, GAZI).

■ ***Cardiospermum***L.

****C. halicacabum*** L. in Sp. Pl. 1:366 (-367) (1753) [Yıldırımlı, 2018a].

Examined specimen: Turkey. C1 Aydın: Kuşadası, Davutlar, Çağay sitesi, ev harlası (garden), çite tırmanıcı, deniz kıyısına yakın (near sea shore), 1 m, 16.viii.2018, Ş. Yıldırımlı 44715 (Yıldırımlı Otluk'u, HUB).

PedicularisL. (6:768)

e** P. munzurdaghensisArmağan in Phytotaxa 333 (1): 124-130 (2018) [Armağan, 2018].

Type: Turkey. Tunceli: Pülümür, 11 km N of Ardıçlı (Gersunut) Village, Munzur Mountain (near to Erzincan border), ca. 2510 m, 18.vi.2014, Armağan 4874 (holotype HUB; isotype AYDN).

VerbascumL. (6:461)

e**V. faik-karavelioguallarii Çıngay & Cabi in Phytotaxa 372 (4): 263–272 (2018) [Çıngay et al. 2018].

Type: Turkey. C9 Hâkkari: 11 km north of Hâkkari, Berçelan Yaylası, riverside, limestone rocks, 2779 m elevation, 17.vii.2015, B. Çıngay 5317, F.A. Karavelioğlu, H. Tek & A. Akbaba (holotype: NGBB 4801, isotypes: ANK, CBB).

e***V. gimgimense* Fırat in Phytotaxa 291 (3): 209-216 (2017) [Fırat, 2017f].

Type: Turkey. B8 Muş: Gimgim (Varto) province, 6-7 km from Gimgim to Gulik, Suya Gonene region, stepe, 2043 m a.s.l., 39°12'08□N, 41°30'19□E, 4.vi.2014, M.Fırat 31026 (holotype VANF, isotypes ANK, Herb. M.Fırat).

e***V. golawanense* Fırat in Phytotaxa 305 (1): 21-28 (2017) [Fırat, 2017g].

Type: Turkey. B9 Van: from Van to Muradiye, Bendi mahi region, fallow fields, edge of fields, 1659 m, 38°57'42□N, 43°37'50□E, 1.vii.2011, M.Fırat 27749 (holotype ANK, isotypes E, GAZI, HUB, VANF).

e**V. mughlaeum H.Duman, Uzunh. & Kit Tan in Phytotaxa 291 (3): 231-236 (2017) [Duman et al. 2017b].

Type: Turkey. C2 Muğla: Fethiye, Saklıkent, on rocky limestone slopes, 110 m, 17.iv.1998, H. Duman 6630 (holotype GAZI, isotypes ANK, C, HUB).

CymbocarpumDC. (4:438)

e**C. alinihatii Menemen & Çıngay, in Turk J Bot 42: 91-99 (2018) [Menemen et al. 2018].

Type: Turkey Ankara: Beypazarı, near Sekli Village, roadside, scree rocks, 619 m a.s.l., 30.vi.2014, 40.194850N, 31.707329E, B. Çıngay, A. Ö. Çimen, R. Anşin, R. M. Aydınkal, Ö Demir (OBKA) 947 (holotype: NGBB 005901; isotypes: ADO, NGBB).

Ekimia H.Duman & M.F.Watson (11:142)

e**E. ozcan-secmeniiŞenol & Eroğlu inTurk J Bot 42: 510-517 (2018) [Şenol et al. 2018].

Type: Turkey. C2 Denizli: Between Karaismailler-Suçatı Village, Acıpayam, serpentine scree slopes, P. brutia open forests, around Dalaman stream; 37°06□62.2□N, 29°05□49.824□E, 650 m, 03.vi.2013, S.G. Şenol & V. Eroğlu 3560 (holotype: EGE42490).

▪ ***Cyclospermum***Lag.

****C. leptophyllum*** (Pers.) Sprague, Bot. Porto Rico 6: 52. (1925) [Yıldırım et al., 2018].

Examined specimen: Turkey. A8 Trabzon: in a forest nursery, amongst green-houses, 10 m, 03.ix.2014, UTM: 0611904, 4538117, KATO 9856.

OenantheL. (4:372)

****O. millefolia*** Janka Oesterr. in Bot. Z. 22: 177-178 (1872) [Doğan-Güner et al. 2016].

Examined specimen: Turkey. Kırklareli: Demirköy-İğneada, to 5-10 km Iğneada, under Pinus sylvestris forest, 273 m, 18.vii.2014, E. Doğan Güner 2044 & B. Bani (GAZI); ibid., 03.viii.2014, E. Doğan Güner 2075 & B. Bani (GAZI); Kırklareli: Sarpdere-Armutveren, under *Pinus sylvestris* forest, 33 m, 19.vi.2014, E. Doğan Güner 2046 & B. Bani (GAZI); Tekirdağ: Saray, Kıyıköy district, under *Quercus* forest, 247 m, 16.vi.2015, E. Doğan Güner 2101 & B. Bani (GAZI).

PeucedanumL. (4:473)

e**P. guvenianumYıldırım & H.Duman in Turk J Bot 41: 600-608 (2017) [Yıldırım & Duman 2017].

Type: Turkey. İzmir: Menderes-Gümüldür yolu, Tahtalı Baraj gölü karşısı, Gümüldür'e 40 km kala, maki içi, 237 m, 07.xi.2016, H.Yıldırım 4112 (holotype: EGE 42440, isotypes: GAZI, HUB, NGBB).

Rhabdosciadium Boiss. (4:311)

e**R. anatolyi Lyskov & Kljuykov in Phytotaxa 331(2): 253-262 (2017) [Lyskov et al. 2017].

Type: Turkey. Hakkari: 20 km E from Hakkari, wet rocky northern slope near the snowfield, alt. 2620-2630 m, 37°28'N, 43°49'E, 3.vii.2014, Lyskov & Krupitsky 14-TR-135 (holotype MW0595616; isotypes GAZI, W)

ParietariaL. (7:636)

****P. rechingeri*** Chrtek in Fl. Iranica 105: 12 (1974) [Fırat, 2018b].

Examined specimen: Turkey. C9 Şırnak: Cudi Mountains, Bılgan region, rocky slopes, 969m, 37°25'11" N, 42°38'57" E, 15.vii.2014 M. Fırat 31054, VANF.

VIOLACEAE

Viola L. (1:524)

eV. denizliensis** O.D.Düşen, Göktürk, U.Sarpkaya & B.Gürcan in Phytotaxa 369 (1): 37-46 (2018) [Düşen et al. 2018].

Type: Turkey. C2 Denizli: Nikfer, Bozdağ Mountain, serpentinite stony slopes, 1670 m, 29.iv.2017, O.D.Düşen 1512 & R.S.Göktürk (holotype: PAMUH, isotypes: Akdeniz University Herbarium).

MONOCOTYLEDONS

CYPERACEAE

Rhynchospora Vahl (9:70)

****R. caucasica*** Palla, Vestn. Tiflissk. Bot. Sada 30: 26-27 (1913) [Leblebici, 2017].

Examined specimen: Türkiye, Rize, Ardeşen, Işıklı, Afyon Baba tepesi çevresi, 19.viii.1985, 300-400 m, M. Vural 3981, A. Güner (HUB).

GRAMINAE

Aegilops L. (9:233)

eA. triuncialis** L. susbp. ***bozdagensis*** Cabi & Dogan in Acta Bıologıca Turcica 31(2): 56-61, (2018) [Cabi et al. 2018].

Type: Turkey. Denizli: Bozdağ, Geyran yaylası, roadsides, open forest, 1400-1650 m, 30.vi.2009, E. Cabi 4050 (holotype GAZI, isotype ANK).

Alopecurus L. (9:373)

eA. goekyigitiana** Cabi & Soreng in Turk J Bot 41: 189-199 (2017) [Cabi et al. 2017].

Type: Turkey. C4 Konya, Bozkır, Karacahisar köyü, Palaz yaylası, steep slopes on northwest side of pass to Hacıobası yaylası, 2015 m, 37.04410°N, 32.09117°E, 25.vii.2014, R.J. Soreng 8856, E.Cabi & B.Çıngay (holotype US, isotypes ANK, CAN, E, G, HAOC, ISTE, K, KNYA, LE, NAKU, W).

Cyperus L. (9:34)

****C. eragrostis*** Lam., Tabl. Encycl. Méth. Bot 1:146 (1791) [Şapcı & Vural 2018].

Examined specimen: Turkey. Samsun: Çarşamba, Yeşilırmak river side, 1.6 m, 41°12'245"K, 36°43'648"D, 25.viii.2014, HS1365, ERCH.

****C. microiria*** Steud., Syn. Pl. Glumac. 2: 23 (1854) [Şapcı & Vural 2018].

Examined specimen: Turkey. Rize: Çayeli, Büyükdere under the bridge, 15 m, 41°04'948"K, 40°42'778"D, 27.viii.2014, HS1390.

IRIDACEAE

Crocus L. (8:413)

eC. hatayensis** Rukšāns in Int Rock Gardener 108: 20-54 (2018) [Rukšāns, 2018].

Type: Ex culturae in horto Jānis Rukšāns. Plants originally collected in dry leaves, on the 29th of May, 2003 in SE Turkey, Hatay Province, Ziyaret Mts., along rd. from Antakya to Yayladağı Yolu, 36° 2.230' N and 36° 7.195' E, at alt. 960 m, RUDA-117. Holotype: GB (Gothenburg), isotype GAT.

eC. heilbronniorum** Erol in Phytotaxa 298(2): 173-180 (2017) [Erol et al. 2017].

Type: Turkey. Muğla: Fethiye, near Arpacık, 1200 m a.s.l., 01.iii.2016, O.Erol & L. Can (holotype ISTF 41115).

eC. tuna-ekimii** Yüzb. in Phytotaxa 314 (1): 110-116 (2017) [Yüzbaşıoğlu 2017].

Type: Turkey. Balıkesir: Dursunbey, Durabeyler köyü, *Pinus nigra* orman altı, kayalık arazi, 1000 m, 27.ii.2016. S. Yüzbaşıoğlu 4248 & F. Canız (holotype ISTE, isotypes ISTE, NGBB).

eC. youngiorum** Rukšāns & Zetterlund in Int Rock Gardener 102: 14-24 (2018) [Rukšāns & Zetterlund 2018].

Type: Ex culturae in hortus Jānis Rukšāns, 11-03-2018. Plants collected in Turkey, Tunceli Province, 27 km from Tunceli along the Munzur River towards Ovacik, along a roadside S of the river in a narrow ravine on a south-facing slope at 1000 m alt., leg. H. Zetterlund, 11-04-1990 (KPPZ 90-209). Holotype: GB (Gothenburg).

Iris L. (8:382)

e***I. zagrica* subsp. ***hakkariensis*** Fırat in Phytotaxa 305 (3): 209–216 (2017) [Fırat, 2017d].

Type: Turkey C10 Hakkari: Şemdinli district, from Ciyaye Govende (Güven Dağı) to Oremar (Dağlıca), in loose soil, 1543 m, 37°12′58″N 44°16′55″E, 24.iv.2013, M.Fırat 30011(holotype: VANF; isotypes: NGBB, Herb. M. Fırat).

LILIACEAE

Allium L. (8:98)

e***A. ankarense* Yıld., in Ot Sistematik Botanik Dergisi, 25(2): 1-22 (2018) [Yıldırımlı, 2018b].

Type: Turkey. A3 Ankara: Nallıhan, Davutlar köyü berisi, kuş cennetine varmadan, jipizli yamaç ve tepe, 600-625 m, 07.vi.2018, Ş. Yıldırımlı 44670 (holo. Yıldırımlı Otluk'u; iso. HUB).

e***A. gemiciana* Yıld. & Kılıç in Ot 22 (1-2): 1-24 (2016) [Yıldırımlı & Kılıç 2016b].

Type: Turkey. B7 Tunceli: Ovacık, at junction of Ovacık-Çemişgezek-Hozat-Kemaliye districts, Yılan Mountain, from Cevizlidere Village to İtyokuşu-Eşekmeydanı-Devboğazı-Tapiktepe-Deveboynu-Barasorderesi-Karataş Village, by path, stepe, 1550-2250 m, 13.vi.2015, Ş. Yıldırımlı 41590 & Ö. Kılıç (holo. Yıldırımlı Otluk'u).

e***A. guttatum* Steven subsp. ***kartalkayaense*** Yıld. in Ot 22 (1-2): 1-24 (2016) [Yıldırımlı & Kılıç 2016b].

Type: Turkey. A3 Bolu: Aladağlar, Kartalkaya yolu üzeri, üç yol ayrımı, sarıçam (*Pinus sylvestris*) altı ve açıklığı, volkanik kayalıklar, 1400-1410 m, 07.vii.2016, Ş. Yıldırımlı 42604 & H. Işıl Yıldırımlı (holo. Yıldırımlı Otluk'u; iso. HUB, PAMUH, Yıldırımlı Otluk'u).

e***A. hoshabicum* Fırat in Phytotaxa 312 (1): 129-134 (2017) [Fırat 2017a].

Type: Turkey. B9 Van: Hoşab (Güzeldere) province, Güzeldere passage, stepe and meadow, 38°10′28″ N, 43°55′44″ E, 2791 m, 21.vii. 2012, M. Fırat 28979 (holotype: VANF, isotypes: ANK, HUB, Herb. M. Fırat).

e***A. istanbulense* Özhatay, Koçyiğit, Brullo & Salmeri, in Phytotaxa 334 (2): 152-166 (2018) [Özhatay et al. 2018].

Type: Turkey. A2 (E) Istanbul Arnavutköy, Terkos (Durusu) Lake, NW end of the sea shore among *Quercus* shrubs 20-30 m, 12 August 2017, N. Özhatay, E. Özhatay & M. Keskin (7654) Ist. Bio. 3068 (ISTE 114975, holotype; isotypes NGBB, CAT).

e***A. pervariensis* Fırat & Koyuncu in Plant Biosystems, 152 (3): 305–310 (2018) [Fırat et al. 2018].

Holotype: Turkey. C9: Siirt, Pervari, Botan River, opposite to Bêdar (Beğendik) Village, fields converted from the *Quercus* forests, 1500–1700 m, 4.vii.2012. M. Fırat 29712 (AEF 26722).

e***A. tchihatschewii* Boiss. subsp. ***armani*** Yıld. & Kılıç in Ot 22 (1-2): 1-24 (2016) [Yıldırımlı & Kılıç 2016b].

Type: Turkey. B8 Bingöl: Merkez, Alatepe (Arçuk, Armani) köyü üstü, Kale yaylası, bulak başı çevresi, sulu dere, tepe, bayır, taşlık, 1900-2050 m, 11.vi.2015, Ş. Yıldırımlı 41482 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. GAZI, HUB, Yıldırımlı Otluk'u).

e***A. yilandaghense* Yıld. & Kılıç in Ot 22 (1-2): 1-24 (2016) [Yıldırımlı & Kılıç 2016b].

Type: Turkey. B7 Tunceli: Ovacık, at junction of Ovacık-Çemişgezek-Hozat-Kemaliye districts, Yılan Mountain, from Cevizlidere Village along patway İtyokuşu-Eşekmeydanı-Devboğazı-Tapiktepe-Deveboynu-Barasorderesi to Karataş Village, stepe, 1550-2250 m, 13.vi.2015, Ş. Yıldırımlı 41592 & Ö. Kılıç (holo. Yıldırımlı Otluk'u, iso. HUB, Yıldırımlı Otluk'u).

Fritillaria L. (8:284)

e***F. ozdemir-elmasii* Yıldırım & Tekşen in Resimli Türkiye Florası 2: 872-885 (2018) [Tekşen 2018].

Type: Muğla: Fethiye, Akdağlar silsilesi, Karamuar mevkii, Kastabara (Deliktaş) Antik kenti yukarıları, Darıözü Yaylası, açık yamaçlar ve tarla kenarları, 1655 m, 23.iv.2017, H. Yıldırım 4604 (holotype: EGE, isotype: NGBB).

Gagea Salisb. (8:312)

******G. chomutovae* (Pascher) Pascher in Bull. Soc. Imp. Naturalistes Moscou, n.s., 19: 372 (1907) [Tekşen & Karaman Erkul 2015].

Examined specimens: B9 Van: Bahçesaray, Kızılköprü to Hizan, 57 km to Hizan, 2255 m, stony slopes, 11.v.2010, M. Tekşen 2411 & S. Karaman (GAZI); Güzelsu, Hoşap to Günbaşı Village, 13. km, 1950 m, 15.v.2012, stepe, M. Tekşen 2878 & S. Karaman (GAZI); Güzelsu, Hoşap to Gürpınar, 7 km from Hoşap, 1971 m, 16.v.2012, stepe, M. Tekşen 2880 & S. Karaman (GAZI); ibidem, 18.v.2013, M. Tekşen 2914 & S. Karaman (GAZI).

e***G. goekyigitii* Eker & Tekşen in Bağbahçe Bilim Dergisi 4(1): 22-30 (2017) [Tekşen & Eker, 2017].

Type: C4 Karaman: Sarıveliler, Göktepe'den Göktepe Dağına 20 km, Çamurluk Beleni mevkii, Yüksek dağ çayırı, 1906 m, 05.vi.2012, İ.Eker 2845 (holotip: NGBB, izotip: AIBU, paratipler: NGBB, GAZI).

e***G. vanensis* Tekşen & Karaman in Phytotaxa 188 (5): 251-260 (2015) [Tekşen & Karaman Erkul 2015].

Type: Turkey. B9 Van: Gürpınar, Işıkpınar to Hacıköy, around the pond, N 38°18′872□, E 43°37′032□, 2154m, stepe, 13.v.2010, M. Tekşen 2460 & S. Karaman (holotype GAZI, isotypes ANK, HUB).

Muscari Mill. (8:245)

******M. botryoides* (L.) Mill. Gard. Dict., ed. 8. N. 1 (1768) [Pınar et al., 2018b].

Examined specimen: Turkey. B9 Van: Gürpınar, Van to Başkale road, 5 km before Güzelsu, 38 15 45 N, 43 52 09 E, meadows, 2140 m, 26.v.2015, M. Pınar 6485, M. Fidan & H. Eroğlu.

Scilla L. (8:214)

e***S. bilgineri* Yıldırım in Turk J Bot 41:88-95 (2017) [Yıldırım & Altıoğlu 2017].

Type: Turkey. Adıyaman: Gölbaşı, Akçabel köyü, Say Mevkii, 1152 m, Kırık kalker kayalık yamaçlar *Quercus* sp. açıkları 15.iii.2014, H Yıldırım 2788 (holotype: EGE 42435, isotypes: EGE 42436, ANK, NGBB).

Ornithogalum L. (8:227)

e***O. alatepense* Yıld. & Kılıç in Ot 22 (1-2): 1-24 (2016) [Yıldırımlı & Kılıç 2016b].

Type: Turkey. B8 Bingöl: Merkez, Alatepe (Arçuk, Armani) köyü üstü, Fırın deresi çevresi, yayla, meşe orman ve açıklığı, bayır, taşlık, 1900-1950 m, 11.vi.2015, Ş. Yıldırımlı 41429 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. HUB, PAMUH, Yıldırımlı Otluk'u).

e***O. yildirimlii* Kılıç, in Ot Sistematik Botanik Dergisi, 24 (2): 73-83 (2017) [Yıldırımlı & Kılıç 2017b].

Type: Turkey. B7 Elazığ: Keban, baraj aşağısı, Fırat alabalık tesisleri ilerisi, tekedilmiş yol boyunca, bozkır, 780-900 m, 19.v.2017, Ş. Yıldırımlı 43060 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. HUB).

e***O. kilicii* Yıld, in Ot Sistematik Botanik Dergisi, 24 (2): 73-83 (2017) [Yıldırımlı & Kılıç 2017b].

Type: Turkey. B8 Bingöl: Solhan, Hazarşah köyü, Aksakal göl mezrası, Yüzenada çevresi, yamaçlar, çayırlık, 1300-1350 m, 25.v.2017, Ş. Yıldırımlı 43429 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. HUB).

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