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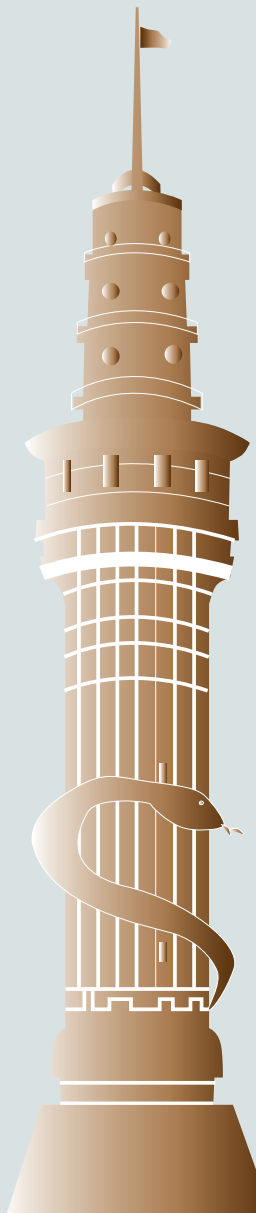

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The manuscript should be prepared in MS Word format by using Times New Roman font (12 pt) and double-spaced on one side of the paper with adequate margins (2.5 cm).

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Title page: A separate title page should be submitted with all submissions and this page should include:

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Units should be prepared in accordance with the International System of Units (SI).

Editorial Comments: Editorial comments aim to provide a brief critical commentary by reviewers with expertise or with high reputation in the topic of the research article published in the journal. Authors are selected and invited by the journal to provide such comments. Abstract, Keywords, and Tables, Figures, Images, and other media are not included.

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Short Papers: Please check Table 1 for the limitations for Short Papers.

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

Tables

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

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

All acronyms, abbreviations, and symbols used in the manuscript must follow international rules and should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

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

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

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.

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

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

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

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

Editor(s) as Author: Huizing EH, de Groot JAM, editors (2003) Functional reconstructive nasal surgery. Stuttgart-New York: Thieme.

Conference Proceedings: Bengisson S, Sothemin BG, (1992) Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; pp.1561-5.

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

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

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

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

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

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

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Accepted manuscripts are copy-edited for grammar, punctuation, and format. Once the publication process of a manuscript is completed, it is published online on the journal's webpage as an ahead-of-print publication before it is included in its scheduled issue. A PDF proof of the accepted manuscript is sent to the corresponding author and their publication approval is requested within 2 days of their receipt of the proof.

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CONTENTS

ORIGINAL ARTICLES

REVIEW

Original Article

Istanbul J Pharm 47 (3): 77-83
DOI: 10.5152/IstanbulJPharm.2017.0013

Evaluation of the anticancer effects of *Aloe vera* and aloe emodin on B16F10 murine melanoma and NIH3T3 mouse embryogenic fibroblast cells

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ABSTRACT

Aloe vera (L.) Burm. f. is well known for its beneficial effects on the skin. Moreover, the antioxidant, immunostimulant, and anticancer effects of the plant leaf extracts have been reported in scientific research. This study was conducted to demonstrate the cytotoxic effects of several leaf extracts and aloe emodin (AE) on a type of skin cancer. *A. vera* aqueous and methanolic extracts of fresh leaves, methanolic extract of dried leaves, and leaf gel extract (AVG) were prepared separately. Cytotoxicity was assessed using the MTT test. Apoptosis and necrosis were detected by flow cytometry using Annexin V/PI. All the extracts exhibited a selective cytotoxic effect on the cells. The mechanism of AVG cytotoxicity on B16F10 murine melanoma cells was found to be apoptosis, whereas that of AE was necrosis. The observation that treatment with AVG delayed the apoptosis in NIH3T3 cells, while it exerted an apoptotic activity on B16F10 cells, provides some scientific evidence for the folkloric and alternative uses of A. vera gel as a protective and skin healer. Therefore, *A. vera* gel and aloe emodin can be used as potential targets for anticancer drug research.

Keywords: *Aloe vera*, aloe gel, aloe emodin, melanoma, cytotoxicity

INTRODUCTION

Known for centuries as a "wonder" plant, *Aloe vera* (L.) Burm. f. has many biological and pharmacological activities. These effects are due to the variety of the chemical compounds including anthraquinones, glycoproteins, polysaccharides, vitamins and enzymes contained (Choi and Chung 2003; Du Plessis and Hamman 2014; Akev et al. 2015; Shrestha et al. 2015). Many of the medicinal effects have also been attributed to the immunomodulatory properties of the inner gel (Im et al. 2010), but it is also believed that synergistic action of the compounds contained in the whole leaf extracts is responsible for the multiple and diverse beneficial properties of the plant (Eshun and He 2004).

The mucilageneous gel part of *A. vera* is used commercially as a softener in various cosmetic preparations, soaps and shampoos due to its glycoprotein content. The first known effects of *A. vera* after its cathartic activity, is the wound and burn healing effect of the leaves gel portion, widely supported in scientific literature (Capasso et al. 1998; Chithra et al. 1998; Heggers et al. 1995). Because of this effect, the gel is also added to many preparations used for skin treatment.

Antitumor and cytotoxic potential of *A. vera* extracts continues to be the interest of scientific research from 1980's (Winters et al. 1981; Tsuda et al. 1993; Corsi et al. 1998) until recent years (Naveena Bharath and Selvasubramanian 2011; Du Plessis and Hamman 2014). In a research undertaken in our laboratory, *A. vera* leaf skin aqueous extract was proved to be effective as prophylactic against *Ehrlich ascites* tumours *in vivo* (Akev et al. 2007). The Food and Drug Administration of the USA has approved the developmental study of *A. vera* for the treatment of cancer and AIDS (Nandal and Bhardwaj 2012).

Antitumor effect of aloe emodin (AE), the major anthraquinone derivative of *A. vera*, was reported in recent years (Cárdenas et al. 2006; Lee et al. 2006; Lin et al. 2006) and attention has been given recently to the possibility of utilizing AE as a chemothera-

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peutic drug (Chiu et al. 2009; Tabolacci et al. 2010; Mahbub et al. 2013).

The aim of this study was to determine the cytotoxic and apoptotic/necrotic activity of various types of *A. vera* leaf extracts and AE in a type of skin cancer, B16F10 murine melanoma relative to normal fibroblast cell line (NIH3T3 mouse embryogenic fibroblast cells) and to investigate the underlying mechanisms. Cell viability was assessed by trypan blue and cytotoxicity experiments were done using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. *A. vera* extracts and AE and as positive control Imatinib (IM) were tested at different concentrations. Apoptosis and necrosis induction was monitored by flow cytometry using the Annexin V-FITC/ propidium iodide (PI) kit.

MATERIALS AND METHODS

Cell lines and cell culture

B16F10 murine melanoma cells (Department of Pharmacology, Akdeniz University School of Medicine, Antalya) and NIH3T3 mouse embryonic fibroblast cells (Department of Physiology, İstanbul Faculty of Medicine İstanbul University, İstanbul) were cultured in IMDM (Iscove's Modified Dulbecco Medium, Sigma-Aldrich) containing 10 % heated-inactivated fetal bovine serum (Capricorn FBS-12A) with 100,000 U/L penicillin and 100,000 µg/L streptomycin (Gibco 15140-122), at 37°C, in a humidified atmosphere of 95 % O_2/ 5 % CO_2. In order to reach the sufficient cell number for tests, cells were passaged after reaching 80% monolayer confluency. Cells were harvested gently by 0.25% trypsin (Merck)/EDTA (Chem Cruz) solution. Cells were sub-cultured every 2 or 3 days.

Plant material

Specimens of *A. vera* (L.) Burm. f. (Xanthorraceae; in Turkish Sarisabir) were collected from Kale (Demre) in Antalya (May 1993), identified by Prof. Dr. Nurhayat Sütlüpınar and cultivated since this date in the greenhouses of the Faculty of Pharmacy and further in İstanbul University Alfred Heilbronn Botanical Garden. A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy, ISTE No. 65118. The fresh leaves of this cultivated plant were used in the study.

Preparation of extracts

Four types of extract were prepared separately from *A. vera* leaves: Aqueous extract of fresh leaves, gel extract (AVG), methanol extract of fresh leaves and methanol extract of dried leaves.

Preparation of aqueous extract of fresh leaves and gel extracts

Freshly chopped *A. vera* leaves, were washed carefully with water and dried with filter paper (Whatmann 41) to remove dust and foreign materials. Then the washed leaves were longitudinally split in two, the gel (198.74 g) was separated by scraping with a spoon and homogenized in a Waring blendor. The remaining leaves (leaf skins 171 g) were cut in small pieces, homogenized in a Waring blender with 855 ml distilled water, filtered through cloth and then the filtrate was centrifuged (Thermo) at +4°C, 12 000 rpm, 15 min. The supernatant was lyophylized (11.57 g**)** and considered to be *A. vera* aqueous extract of fresh leaves. The gel was filtered through cloth and then filtrate was centrifuged at +4°C, 10 000 rpm, for 30 min. The supernatant was lyophylized (4.63 g) and considered to be *A. vera* gel extract (AVG).

Preparation of methanolic extracts of fresh leaves and dried leaves

Fresh leaf skins (33.67 g) were dried in ventilated oven at 60°C, 2 h. The dried samples were then ground to obtain powder which was stored at room temperature in the dark until extraction. The obtained dried leaf skin (4.25 g) and fresh leaf skin (73.4 g) were extracted separately with methanol for 3 days using Soxhlet extractor until complete extraction. After extraction, the samples were filtered with filter paper (fresh leaves filtrate 260 ml, dried leaves filtrate 143 mL). The methanol solvent was evaporated using a rotary evaporator under pressure for 30 min resulting in a semi solid crude extract and considered to be (3.32 g) *A. vera* methanol extract of fresh leaves. The same procedure was performed after drying leaf skins at 60°C for 2 h in an oven and considered to be (1.17 g) *A. vera* methanol extract of dried leaves.

These four different extracts prepared as described above were conserved at -20°C until further use.

Preparation of test materials and reference drugs

AE (1,8-dihydroxy-3-[hydroximethyl]-anthraquinone) was purchased from Sigma-Aldrich (St Louis, MO, cat no. A7687). *A. vera* extracts (10 mg/mL) and AE (20 mM) stock solutions were prepared in dimethyl sulfoxide (DMSO was spurchased from Sigma-Aldrich, D2650), aliquoted and stored in the dark at -20°C till use, then diluted with medium. The reference chemotherapeutic drug Imatinib (IM) was purchased from Santa Cruz, 10 mM stock solution was prepared in DMSO; aliquoted and stored in the dark at -20°C till use, then diluted with medium (see below); the final concentration of DMSO in medium was less than 1 % (v/v).

Trypan blue exclusion assay

The total number of viable cells was determined at each time point by the trypan blue exclusion test (Strober 2001). Exactly 10 µl of cell suspensions were stained with an equal volume of trypan blue [0.4 % in 10 mM in phosphate buffer saline (PBS)] for 1 min. Then the numbers of viable cells were counted with Neubauer Chamber by light microscopy (Olympus). Cells that retained a blue colour were considered as dead cells.

MTT colorimetric assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to screen for cytotoxic activity (Mosmann 1983). For this purpose 96-well plates were used and the assay was done in a total volume of 100 µl. Briefly, 10 µl/well of varying concentrations of *A. vera* extracts and as positive controls: IM (0.5 – 100 µM) and AE (10 – 100 µM) were added and subsequently the cells (90 µl/well; 10^5 cells/mL culture medium) were seeded to treat for 72 h. In addition, 90 µl cell suspension and 10 µl medium were added to control wells. After the aspiration of the supernatant (50 µl/well), and subsequent incubation with MTT (Sigma-Aldrich) solution (10 µl of 5 mg/ mL PBS) at 37°C for 3 h, cells were lyzed with 100

µl DMSO. Absorbance was measured at 570 nm using a ELISA microplate reader (Rayto RT-2100C). The percentage of viable cells (VI) determined with the equation (1):

VI = (Absorbance of the treated cells ÷ Absorbance of the control cells) ×100 (1)

The cytotoxic concentrations of extracts that provides 50% inhibition of cell growth (IC_{50}) were calculated from a dose-response curve. The cytotoxic effect of *A. vera* extracts and controls were evaluated by comparing the IC_{50} values of cell lines.

Flow cytometry analysis

Normal, apoptotic, and necrotic cells were distinguished using an Annexin V-FITC/PI assay kit (Millipore) according to the manufacturer's instructions. For this purpose a 6-well plate was used and the assay was done in a total volume of 2 mL. The three groups of cells (two untreated control cells group: to apply and unapplied Annexin V-FITC/PI for one test group; 1800 µl/well; 10^5 cells/mL culture medium) were seeded in a final concentration of IC_{50} (200 µl/well) of AVG and AE. Subsequent to culture at 37°C with 5% CO_2 for 72 h, the cells were harvested by trypsinization. Trypsinized and loose cells were then combined and pelleted by centrifuging at 2 000 rpm for 10 min. The pellets were resuspended and washed with PBS, then resuspended in 100 µl of Annexin Binding Buffer (4X) and stained with 3 µl Annexin V-FITC, 2 µl PI. The cell suspension was incubated for 45 min at room temperature in the dark. The cell suspension was then immediately analyzed by flow cytometry. Cell Quest software was used to analyze 10^4 cells. Acquisition of samples were determined with a FACS Calibur flow cytometer and analyzed with CELLQUEST software (BD Biosciences).

Statistical Analysis

The results were statistically analyzed using the independent Student's t-test. Data were represented as means ± standard deviation (S.D.) and at least in triplicate. Results were considered significant with $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

RESULTS

Among the four extracts studied, aqueous extract of fresh leaves and AVG has been shown to have selective cytotoxic effect on B16F10 cells in comparison to NIH3T3 cells with a higher IC_{50} level in the same concentrations. Cytotoxic effect of both methanol-extracts was lower than that of aqueous extract and AVG (Table 1).

In vitro cytotoxic effects of *A. vera* extracts (62.5, 125, 250 µg / mL) on B16F10 murine melanoma cells *in vitro* was as follows in decreasing order: aqueous extract of fresh leaves > AVG > methanol extract of dried leaves > methanol extract of fresh leaves (Figure 1a). No data on the IC_{50} value of IM for the B16F10 murine melanoma cells was found in the literature. In our study, IM was more cytotoxic to NIH3T3 cells in comparison to B16F10 cells (Table 1, Figure 1b). The IC_{50} for AE for B16F10 murine melanoma cells was reported to be 60 µM (Tabolacci et al. 2010). In our study, similarly, the IC_{50} value for B16F10 murine melanoma cells of AE was found to be 68.48±9.85 µM (Figure 1c). AE was found to be more effective in B16F10 cells than IM (120±7.41) (Table 1).

In vitro cytotoxic effects of *A. vera* extracts (100, 200, 300 µg /mL) on NIH3T3 mouse embryonic fibroblast cells *in vitro* was as follows in decreasing order: methanol extract of dried leaves > AVG > methanol extract of fresh leaves > aqueous extract of fresh leaves (Figure 2a). No data on the IC_{50} value of IM for NIH3T3 mouse embryonic fibroblast cells was found in the literature. In our study, the IC_{50} value of IM for NIH3T3 cells was found to be 100±2.11 µM (Table 1, Figure 2b). Either, no work on the *in vitro* cytotoxic effect of AE on NIH3T3 cells was found. In our study, the IC_{50} value for AE on NIH3T3 mouse embryonic fibroblast cells was 36.68±1.83 µM (Figure 2c).

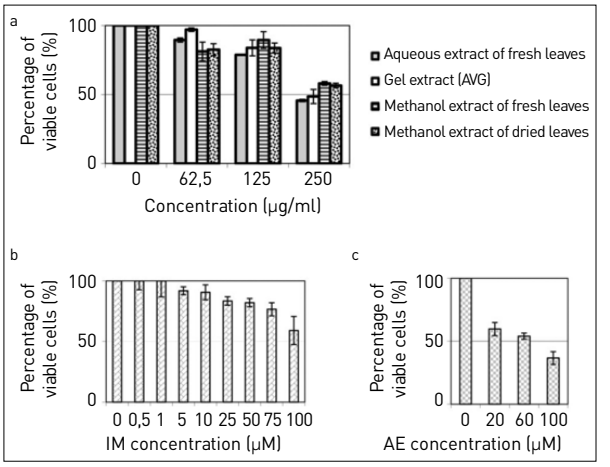

Figure 1. a-c. The cytotoxic effects of (a) *A. vera* extracts, (b) Imatinib and (c) Aloe emodin on B16F10 cell line. Data are presented as the mean of three replicates ± standard deviation (S.D.). IM: Imatinib; AE: aloe emodin

Table 1. IC_{50} values of *A. vera* extracts, IM and AE for cell lines.

	IC50* ± S.D.					
Cells	Aqueous extract of fresh leaves (µg/mL)	(AVG) (µg/mL)	Methanol extract of fresh leaves (µg/mL)	Methanol extract of dried leaves (µg/mL)	IM (µM)	AE (µM)
B16F10	239.26±3.68	259.79±25.65	321.04±9.57	297.98±11.33	120±7.41	68.48±9.85
NIH3T3	313.13±15	285.50±15.51	300.07±17.26	274.27±19.75	100±2.11	36.68±1.83

*IC_{50} is the effective concentration for which the cell alive was 50% and was obtained from dose-response curve. Data are presented as the mean of three replicates ± standard deviation (S.D.); AVG: A. vera gel extract; IM: Imatinib; AE: Aloe emodin

Apoptosis/necrosis studies with flow cytometry were undertaken with AVG and AE in order to elucidate the mechanism of the cytotoxic activity.

As seen in Figure 3a, the control group B16F10 murine melanoma cells showed 7.59% early apoptosis, 1.55% late apoptosis and 5.04% necrosis. Early and late apoptosis rates in B16F10 murine melanoma cells treated with AVG were 13.98% and 22.40%, respectively, while necrosis rate was 2.22%.

As shown in Figure 3b, early and late apoptosis rates in B16F10 murine melanoma cells treated with AE were 2.81% and 0.39% respectively, while necrosis rate was 21.25%.

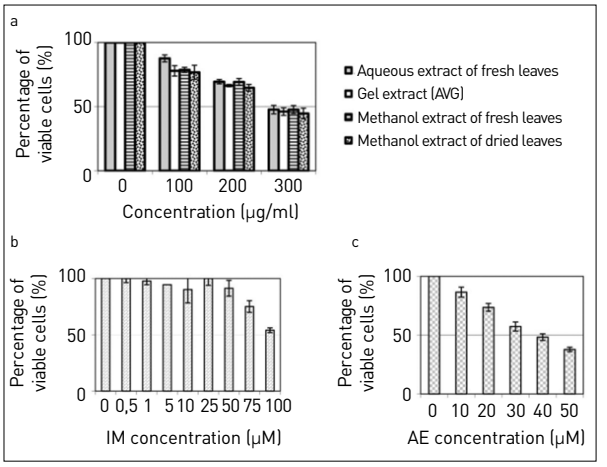

Figure 2. a-c. The cytotoxic effects of (a) *A. vera* extracts, (b) Imatinib and (c) Aloe emodin on NIH3T3 cell line. IM: Imatinib; AE: aloe emodin

For NIH3T3 mouse embryonic fibroblast cells treated with AVG, the control group showed 26.30% early apoptotic and 21.63% late apoptotic cell ratio. It is an important finding that, treatement of NIH3T3 mouse embryonic fibroblast cells with AVG retract the dead cell ratio to 8.49% and 19.66% (Figure 4a, b).

The dose dependent and selective cytotoxic effect of AVG treatment on B16F10 murine melanoma cells compared to NIH3T3 mouse embryonic fibroblast cells were also proved by the microscopic visualization (Figure 5a, b).

DISCUSSION

A. vera is often called the "Natural healer". Aloe gel is excellent for healing burns, releives inflammation and accelerates healing (Nandal and Bhardwaj 2012). It has also been demonstrated that the plant has a prophylactic effect if used before, during and after skin damaging events. The polysaccharides, mannose-6-phosphate and complex anthroquinones all contribute synergistically to the benefits of the plant (Dweck 2002).

Skin cancer is an increasing threat to humans because exposure to chemical carcinogens as well as UV irradiation increases day by day in the modern world. Studies about the anticancer effect of *A. vera* gel and leaf extracts have been undertaken on different cancer types *in vivo* (Corsi et al. 1998; Akev et al. 2007; Naveena Bharath and Selvasubramanian 2011) and cell lines *in vitro* (Al-Oqail et al. 2016). In another study, it was stated that *A. vera* protects mice against DMBA/croton oil induced skin papillomagenesis (Saini et al. 2010). Literature on the effect of aloe extracts on skin cancer are scarce. Only one study was found on the effect of *A. vera* extract on B16F10 melanoma

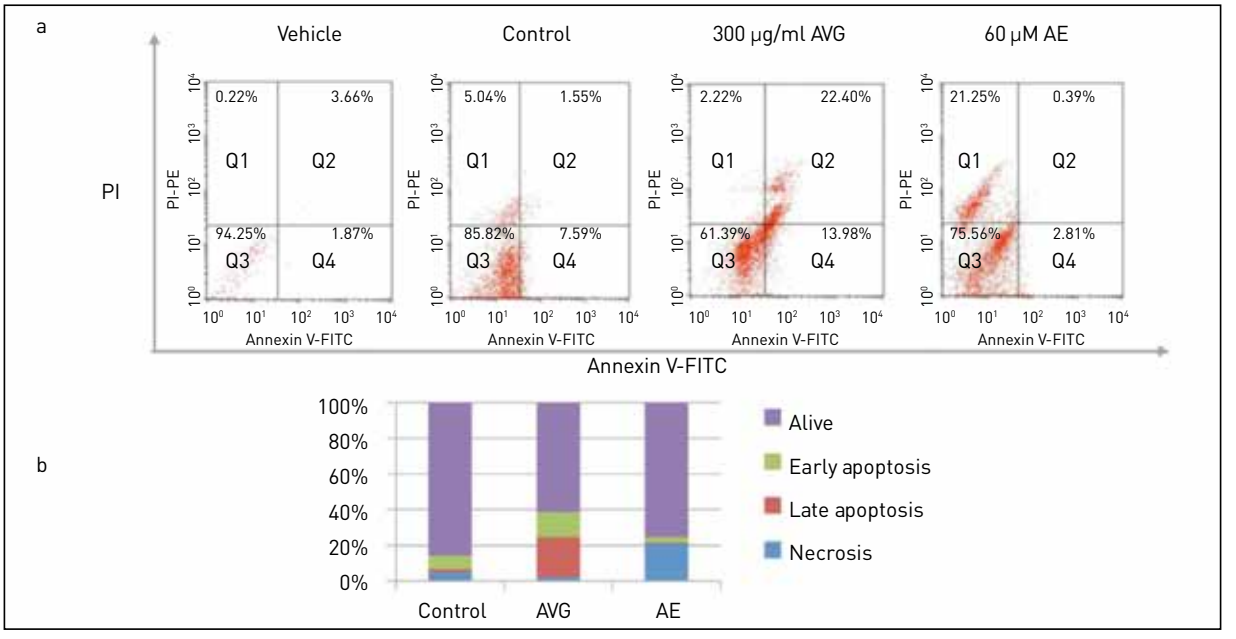

Figure 3. a, b. (a) Flow Cytometry of B16F10 cells treated with Annexin V-FITC/PI. Vehicle: Control cells without the presence of Annexin V-FITC /PI. Control: Cells without the presence of *A. vera* gel extract (AVG) and aloe emodin (AE). The cells were incubated with *A. vera* gel extract (300 µg/ml) and AE (60 µM) for 72 h, stained with Annexin V-FITC /PI and analyzed by FACScan flow cytometer marked for apoptosis/necrosis. Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI negative; Q4: Annexin V/PI negative.The analysis of data from flow Cytometry was performed using the FlowJo software. (b) Histogram representation of the quantitative percentage of alive, early, late and necrotic cell populations

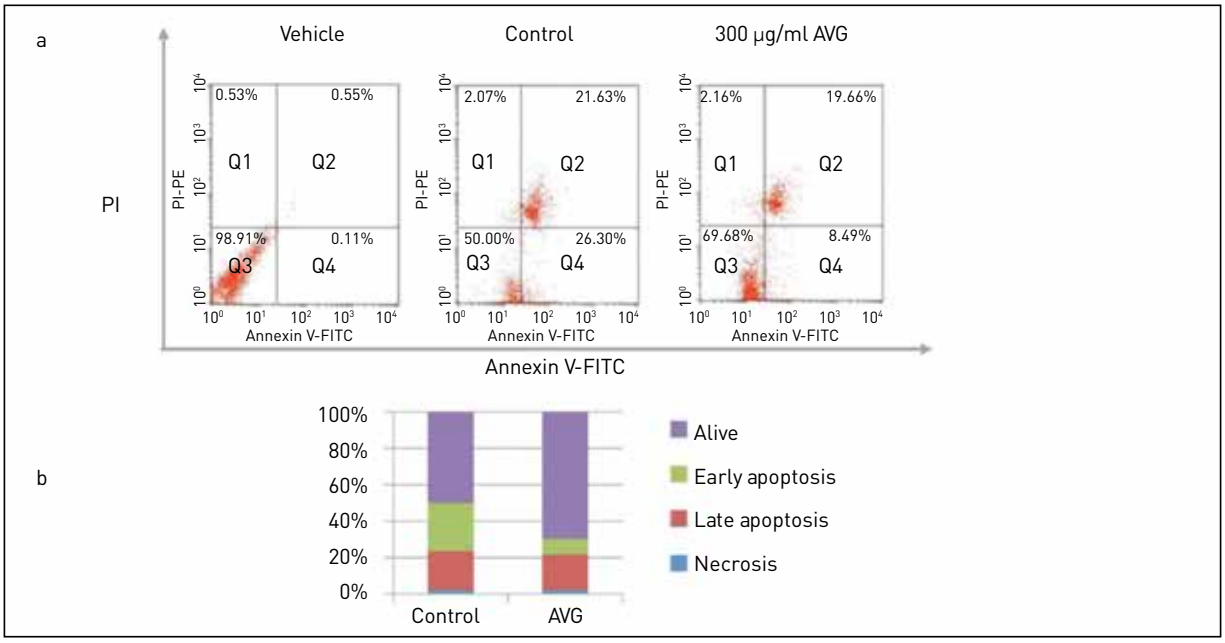

Figure 4. a, b. (a) Flow Cytometry of NIH3T3 cells treated with Annexin V-FITC/PI. Vehicle: Control cells without the presence of Annexin V-FITC /PI. Control: Cells without the presence of A. vera gel extract (AVG). The cells were incubated with A. vera gel extract (300 µg/ml) for 72 h, stained with Annexin V-FITC /PI and analyzed by FACScan flow cytometer marked for apoptosis/necrosis. Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI negative; Q4: Annexin V/PI negative. The analysis of data from flow Cytometry was performed using the FlowJo software. (b) Histogram representation of the quantitative percentage of alive, early, late and necrotic cell populations.

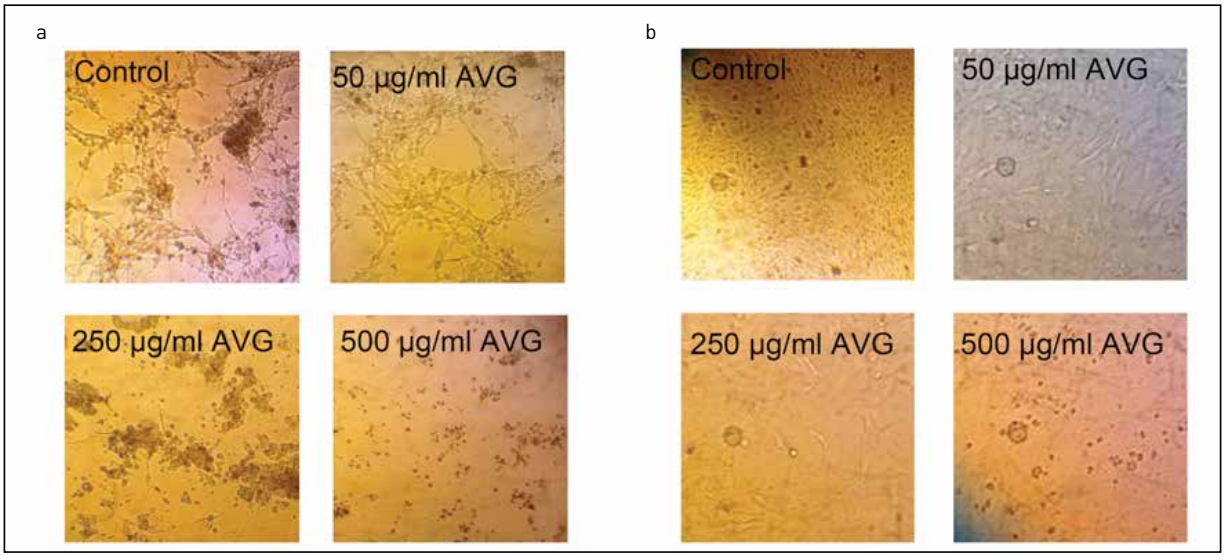

Figure 5. a, b. Visualization of (a) B16F10 and (b) NIH3T3 cells using inverted microscope after treatment with *A. vera* gel extract (AVG: 50, 250 and 500 µg/mL)

cells (Chandu et al. 2012). To our knowledge, this is the first study dealing with the mechanism of AVG and AE cytotoxicity. In this study, the mechanism of AVG cytotoxicity on B16F10 murine melanoma cells was found to be related to apaptosis while that of AE was necrosis. On the other hand, we can say that, in NIH3T3 mouse embryonic fibroblast cells, treatment with AVG, increased the percentage of viable cells by delaying apoptosis. This result could be considered in accordance with the use of aloe gel in cosmetics and in alternative medicine for centuries as a preservative or antiaging product. We can also suggest that the anticancer effect of aloe gel is better than that of the purified substance AE in terms of apoptotic/necrotic mechanims.

Aloe emodin, one of the main constituents of the plant, is in turn suggested as a novel anticancer drug (Ahirwar and Jain 2011; Yordanova and Koprinarova 2014). In terms of skin cancer, AE significantly stopped the proliferation process of irradiated keratinocytes (human skin cells). This confirmed Aloe's benefit in halting the progression of tumor formation after radiation

by the sun (Popadic et al. 2012). The apoptotic effect of AE was demonstrated in T24 human bladder cancer cells through the p53 dependent apoptotic pathway (Lin et al. 2006) and also in human gastric carcinoma cells (Chen et al. 2007). In our study, the IC_{50} value for B16F10 murine melanoma cells of AE was found to be 68.48 ± 9.85 µM similarily to the results of (Tabolacci et al. 2010) which was reported to be 60 µM.

The well documented immunostimulant effect of the plant leaf extracts (Im et al. 2010; Srivastava et al. 2014) as well as their antioxidant activity (Ozsoy et al. 2009) could also have had an influence on the selective cytotoxic effectiveness.

CONCLUSION

The benefits of *A. vera* extracts and aloe derivatives on skin as well as their demonstrated anticancer properties, makes the plant a good target for studies on skin related diseases and skin cancer. We have demonstrated in the present study the cytotoxic effect of *A. vera* extracts and AE and their apototic/necrotic mechanisms, on a skin cancer cell type. No difference was observed between the cytotoxic effects of aqueous and methanolic extracts as well extracts obtained from fresh or dried leaves. *A. vera* gel and AE are thus potential targets for anticancer drug research.

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Original Article

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The usage of the most frequently preferred herbal products in Turkey in nursing mothers, newborns, infants and children

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ABSTRACT

This study was conducted to identify the most frequently used herbal products available in the pharmacies for the healthcare of nursing mothers newborns, infants and children and to compare their efficacy and the safety of some herbal ingredients in comparison with those reported in the literature. Today, mothers visit the pharmacies with several complaints for themselves and their children. Although these complaints may appear simple at the first glance, they are of importance due to their frequency among people. In this context, herbal medications and chemical agents are being used for the treatment of such complaints. The most frequent problem of nursing mothers seems to be hypogalactia according to the interviews conducted with the pharmacists of 40 pharmacies from several municipalities of Istanbul and Ankara for this study. Pharmacies have several herbal medicines or medicinal teas that are being used for their galactagogue effect. On the other hand, the problems encountered in children generally include lack of appetite, cold, colic, insomnia, and weakness of the immune system. Therefore, herbal remedies and medicinal teas that used as dietary supplements, immune system strengtheners, anti-cold and anti-colic agents are preferably recommended by the pharmacists for the relief of these problems. In addition, externally used herbal preparations are also involved in the composition of various anti-rash creams or lotions, foam shampoos, hair and body shampoos, baby oil and lotions, and bath oils. Notably, these herbal preparations may not be as harmless as they are considered; therefore, their usage should be more conscious particularly in these special patient groups.

Keywords: Newborn, Infant, children, colic, nursing mothers, pharmacy, medicinal tea

INTRODUCTION

Herbal drugs have been used for centuries to cure disease and relieve symptoms. The findings on efficacy were based on the experiences and observations of healed people well into the 19th century. Since ideas about the mechanism of action were missing at that time, one documented purely empirical experiences and did not critically deal with the possibilities and limits of the healing power of a plant or drug. In modern times and increasingly in the 20th century, the areas of application enumerated in the old works have been critically examined and scrutinized (Grünwald and Jaenicke 2004). In Germany, this process took place from 1983 to 1994 in connection with the entry into force of the Pharmaceuticals Act (1978) and was documented in writing by the Commission E in the form of drug monographs (Siegfried 2007). At European level, the ESCOP has been active since 1989; it is constantly developing new and updated older drug monographs and publishes the recognized applications of herbal drugs (Siegfried 2007). As part of the approval of herbal medicines, the HMPC, an EMA committee, formulates the recognized uses for herbal drugs and drug formulations (Siegfried 2007). The WHO also creates plant monographs, giving non-European countries access to scientific knowledge about medicinal plants and their preparations (Siegfried 2007; Grünwald and Jaenicke 2004).

The aim of this study is to find out the most frequently used herbal products for nursing mothers or for newborns, infants and children health in the pharmacies and to compare their herbal contents with the above mentioned monographs regarding their efficacy and safety. Because there is no guarantee of strength, purity or safety of products their effects may vary. The main reason of this fact is that the Minis-

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try of Agriculture and Rural Affairs does not strictly regulate herbs and supplements. Manufacturers don't need to submit any proof of safety for ingredients and this causes incompetent studies on herbal products and insufficient knowledge about their side effects/interactions with drugs (Cupp MJ, 1999). Therefore, there is no guarantee of strength, purity or safety of products and effects may vary (Houghton and Mukherjee 2009). Always the product labels should be read carefully. If there is some medical condition, or is taking other drugs, herbs, or supplements, it should be spoken and consult with a qualified healthcare provider in detail (e.g side effects etc.) before starting a new therapy. This causes incompetent studies on herbal products and insufficient knowledge about their side effects/interactions with drugs (Cupp MJ, 1999). For this reason, more attention must be paid to usage of preparations in delicate patient groups such as newborns (0-1 month), infants (1-12 month) , children (1-12 years of age), adolescents (13-18 years of age) and nursing mothers. It is obviously more wisely to use herbal preparations under the doctor's control and supervision of the pharmacist.

In this study, the common suggested herbal products, product's contents and usages for this sensitive group by professionals are evaluated and reported.

MATERIAL AND METHODS

The most frequently used herbal products for lactation problems and childhood illnesses were determined and studied, based on the interviews with pharmacists of 40 pharmacies from several municipalities of Istanbul and Ankara, with help of monographs and current scientific literatures regarding their side effects, usage and drug interactions. Thereafter, the dosage forms, content, company names, forms of the mixture, and medicinal tea samples of the drugs were examined one by one (Tables 1-8).

RESULTS

The results of the study are given in Tables 1-8.

Table 1. Herbal products used as dietary supplements in children

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
Sambucol® syrup (Pharmacare)	*Sambucus nigra*	It is used as dietary supplements for children.	*Due to the insufficient data *Sambucus nigra* L. isn't recommended for use in children under 12 years of age. (EMEA/HMPC 2007) *The duration of treatment with *S. nigra* is 3-5 days in acute viral infections (Zakay-Rones et al. 2004). It should not be used longer than a week. (EMEA/HMPC 2007).	1-3 teaspoons per day.
Day&Night® syrup (Milenyum)	*Citrus sinensis*, *Lavandula angustifolia*, *Matricaria chamomilla*, *Melissa officinalis*, *Origanum majorana*, *Crataegus monogyna*	It is used as dietary supplements for children.	**L. angustifolia* has the potential to increase the effect of sedative and tranquilizing drugs (Brinker 1998). **L. angustifolia* should not be used in people with allergic nature (WHO Monographs 2007). **M. chamomilla* can effect synergistic when used in combination with anticoagulants such as warfarin, because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000). **O. majora* is not suitable for long term use because of the arbutin in its composition (PDR for Herbal Medicines 2004) **C. monogyna* or its drugs can increase the efficacy of cardiac glycosides. It also may effect antihypertansive when used in combination with beta blockers (Mills and Bone 2000). *Internal usage of Meliloti herba may potentiate the activity of anticoagulants (Arora RB, Marthur CN, 1963). In rare cases headaches have been reported after internal use (Bisset 1994). * The use of *M. officinalis* in children under 12 years of age has not been established due to lack of adequate data (HMPC 2013).	It is taken twice a day after meals as 1 teaspoon (5 ml). It is recommended to be used for 1 month regularly. If deemed necessary, the usage may continue as one month periods.
Herbazinc® Syrup (Milenyum)	*Echinacea purpurea*, *Panax ginseng*, *Rosa canina*, *Malpighia punicifolia*, *Brassica oleracea*	It is used as dietary supplements for children.	* *E. purpurea* should be used up to eight weeks (ESCOP Monographs 2003). Hypersensitive reactions in the form of rash, urticaria, itching, swelling of the face may occur. Cases of severe hypersensitivity reactions, such as Stevens-Johnson Syndrome,	

Table 1. Herbal products used as dietary supplements in children (continued)

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
			angioedema of the skin, Quincke oedema, bronchospasm with airway obstruction, asthma and anaphylactic shock have been reported. The frequency is not known (HMPC 2015). **P. ginseng* reduces the effect of warfarin when they are taken together and it cause INR values to decrease (Yuan et al. 2004). *There may be the same amount of mild cases of gastrointestinal discomfort because of *R. canina* usage (Warholm et al. 2003).	It is taken twice a day after meals as 1 teaspoon (5 mL) as dietary supplement. It is recommended to be used for 1 month regularly. If deemed necessary, the usage may continue as onemonth periods.
Floradix® Syrup (Allergo)	*Malva sylvestris*, *Rosa canina*, *Croton elutaria*, *Matricaria chamomilla*, *Ribes rubrum*, *Vitis vinifera*, *Foeniculum vulgare*	It is used as dietary supplement. It strengthens the immune system. It increases body resistance to infection.	**M. chamomilla* can effect synergistic when used in combination with anticoagulants such as warfarin, because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). *Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases. (Wichtl 2002) *There may be the same amount of mild cases of gastrointestinal discomfort because of *R. canina* usage (Warholm et al. 2003).	Children 6 to 12 years: 1 teaspoonful 2 times daily before the morning and evening meal.

Table 2. Herbal products used as anti-gas medication in newborn, infants, children and nursing mothers

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
OM-X® syrup (Agiç)	*Anethum graveolens*, *Zingiber officinale*	It is used in newborns and infants for the relief of abdominal pain and gas pains.	*Ginger is used under medical supervision in patients with gallstones (List of German Commission E Monographs, 2016). It has been reported that more than 6 grams of ginger can cause irritation to the stomach (Desai et al. 1990). **A. graveolens* is considered safe, but in sporadic cases, it causes allergic reactions, oral pruritus, tongue and throat swelling, urticaria, vomiting and diarrhea (Al Snafi 2014).	It can be given 4 times per day as ½ teaspoon (2.5 mL) for newborns between 15 days and 1 month, and 4 times per day as 1 teaspoon (5 mL) for infants between 1 month and 6 months, and 6 times per day in infants between 6 months and 12 months as 10 mL.
Nurse Harvey's® Syrup (Haks)	*Anethum graveolens*, *Carum carvi*	It is used in infants to relieve gas and colic pains.	**A. graveolens* is considered safe, but in sporadic cases, it causes allergic reactions, oral pruritus, tongue and throat swelling, urticaria, vomiting and diarrhea (Al Snafi 2014). *The oral of *C. carvi* use in children and adolescents under 18 years of age has not been established due to lack of adequate data. The use in patients with liver disease, cholangitis,achlorhydria, gallstones and any other biliary disorders is not recommended (HMPC 2014).	It is given as 5 mL (1 tsp) for babies of up to 6 months and 10 ml (2 tsp) after 6 months. 1 scale is given after or during feeding. More than 6 scales are not given per day.

Table 2. Herbal products used as anti-gas medication in newborns, infants, children and nursing mothers (continued)

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
Aguline® Syrup (Medfors farma)	*Zingiber officinale*, *Foeniculum vulgare*, *Carum carvi*, *Anethum graveolens*, *Pimpinella anisum*, *Mentha piperita*	It helps to ease digestion, and to alleviate the gas and abdominal pain in infants and children.	*_M. piperita_* and *_Z. officinale_* should not be used without consulting a doctor when gallstone is concerned (List of German Commission E Monographs 2016). * It has been reported that more than 6 grams of ginger can cause irritation to the stomach (Desai et al. 1990). *Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). *_A. graveolens_* is considered safe, but in sporadic cases, it causes allergic reactions, oral pruritus, tongue and throat swelling, urticarial, vomiting and diarrhea (Al Snafi 2014). *The oral use of *_C. carvi_* in children and adolescents under 18 years of age has not been established due to lack of adequate data. The use in patients with liver disease, cholangitis, achlorhydria, gallstones and any other biliary disorders is not recommended (HMPC 2014). *Ingestion of 1 to 5 millilitres of anise oil (*_Pimpinella anisum_*) has been associated with nausea, vomiting, seizures and pulmonary oedema (HMPC 2013).	It can also be given mixed with water or milk during or after feeding as ½ teaspoon 2-3 times a day for newborns up to 1 month old, 1 teaspoon 3-4 times a day for infants between 1 and 6 months old, and 1 teaspoon 4-5 times a day for infants between 6 and 12 months old.
Neo Baby® Gripe Mixture Syrup (Zima)	*Zingiber officinale*, *Anethum graveolens*	It is used to relieve gas pains and abdominal pain in infants and nursing mothers.	*Ginger is used under medical supervision in patients with gallstones (List of German Commission E Monographs 2016). It has been reported that more than 6 grams of ginger can cause irritation to the stomach (Desai et al. 1990). *_A. graveolens_* is considered safe, but in sporadic cases, it causes allergic reactions, oral pruritus, tongue and throat swelling, urticaria, vomiting and diarrhea (Al Snafi 2014).	It is given 3 times a day with the 2.5 mL scale for the infants up to 1 month old, and with a 5 mL scale for infants up to 1-year-old.
No Gass® Cream (Megamed)	*Laurus nobilis*, *Foeniculum vulgare*, *Salvia officinalis*, *Cinnamomum zeylaniccum*, *Malus domestica*	It is used externally as antigas cream for infants.	*_C. zeylanicum_* can cause allergic reactions in the skin and mucous membranes (List of German Commission E Monographs 2016). *_C. zeylanicum_* should be used with doctor consult if there is a recurrent case or if the usage lasts longer than a week (Wichtl 2004).	It is applied on the belly and soles in peasize by massaging with oval movements. It should be applied by massaging for 30 seconds.
Gazason® massage oil (Dr. Besnim)	*Cuminum cyminum*, *Origanum vulgare*, *Olea europaea*	Along with the massage on the baby's abdomen, it relieves the baby's gas pains.	Not applicable	It is dropped 3-4 times a day around the abdominal area as 4-8 drops and applied by massaging the belly.

Table 3. Medicinal teas used as anti-gas medication in newborns, infants and children

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
Milupa® Chamomile Tea (Numil)	*Matricaria chamomilla*	It is used as carminative in infants.	*_M. chamomilla_* can effect synergistic when used in combination with anticoagulants such as warfarin, because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000).	It is prepared by adding 2 teaspoon (10 mL to 100 mL of water).

Table 3. Medicinal teas used as anti-gas medication in newborns, infants, and children (continued)

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
Milupa® Fennel Tea (Numil)	*Foeniculum vulgare*	It is used as carminative in infants.	*Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016).	It is prepared by adding 2 teaspoon (10 mL to 100 mL of water).
Humana® Fennel Tea With Cumin (Mamsel İlaç)	*Foeniculum vulgare*, *Carum carvi*	It is used as carminative in infants.	*Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). *The oral use of *C. carvi* in children and adolescents under 18 years of age has not been established due to lack of adequate data. The use in patients with liver disease, cholangitis, achlorhydria, gallstones and any other biliary disorders is not recommended (HMPC 2014).	It is prepared by adding 2 teaspoon (10 mL to 100 mL of water).
Hipp® Fennel Tea (Hipp)	*Foeniculum vulgare*	It is used as gastrointestinal movement enhancer, and spasm reliever. It is used in digestive problems such as abdominal pain, gas, and indigestion.	*Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016).	It is used from the 1st week onwards.
Hipp® Mixed Herbal Tea (Hipp)	*Pimpinella anisum*, *Matricaria chamomilla*, *Foeniculum vulgare*	Chamomile is used in stomach discomfort and gas pains. The anise is known as a good carminative since it prevents formation of gas in the digestive system.	**M. chamomilla* can effect synergistic when used in combination with anticoagulants such as warfarin, because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000). *Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). *Ingestion of 1 to 5 millilitres of anise oil (Pimpinella anisum) has been associated with nausea, vomiting, seizures and pulmonary oedema (HMPC 2013).	It is used from the 2nd week onwards.
Günvit® Chamomile Baby Tea (Kurtsan)	*Matricaria chamomilla*	It has a gas removing and relaxing effect in infants and children.	**M. chamomilla* can effect synergistic when used in combination with anticoagulants such as warfarin, because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000).	It is prepared by adding 5 g baby tea into 100 mL of water.
Günvit® Baby Fennel Tea (Kurtsan)	*Foeniculum vulgare*	It has gas removing and pain-relieving effects in infants and children.	*Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016).	It is prepared by adding 5 g baby tea into 100 mL of water.
Günvit® Mixed Baby Tea (Kurtsan)	*Matricaria chamomilla*, *Melissa officinalis*, *Foeniculum vulgare*, *Pimpinella anisum*, *Thymus vulgaris*	It has gas removing and relaxing effect in infants and children.	* *T. vulgaris* (Timol) is contraindicated in enterocolitis, pregnancy and cardiac failure (Braun & Frohne 1987). **M. chamomilla* can effect synergistic when used in combination with anticoagulants such as warfarin,	It is prepared by adding 5 g baby tea into 100 mL of water.

Table 3. Medicinal teas used as anti-gas medication in newborns, infants and children (continued)

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
			because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000). *Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). *Ingestion of 1 to 5 millilitres of anise oil (*Pimpinella anisum*) has been associated with nausea, vomiting, seizures and pulmonary oedema (HMPC 2013).	
Günvit® Anise Tea (Kurtsan)	*Pimpinella anisum*	It is used as carminative.	*Ingestion of 1 to 5 millilitres of anise oil (*Pimpinella anisum*) has been associated with nausea, vomiting, seizures and pulmonary oedema (HMPC 2013).	Drunk 2-3 cups per day.
Günvit® Minivit Mixed Tea® (Kurtsan)	*Pimpinella anisum*, *Matricaria chamomill*, *Foeniculum vulgare*, *Rosa canina*, *Nigella sativa*	It is used as carminative for infants and young children in stomach and intestinal complaints.	**M. chamomilla* can effect synergistic when used in combination with anticoagulants such as warfarin, because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000). *Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). *Ingestion of 1 to 5 millilitres of anise oil (*Pimpinella anisum*) has been associated with nausea, vomiting, seizures and pulmonary oedema (HMPC 2013).	Drunk 2-3 cups per day.
Günvit® Mint & Lemon Tea® (Kurtsan)	*Mentha piperita*, *Citrus limonum*	It is used carminative in stomach complaints.	**M. piperita* should not be used without consulting a doctor when gallstones are concerned (List of German Commission E Monographs 2016).	Drunk 2-3 cups per day.
Günvit® Cinnamon & Clove Tea (Kurtsan)	*Matricaria chamomilla*, *Foeniculum vulgare*, *Rosa canina*, *Citrus sinensis*, *Malus domestica*, *Cinnamomum zeylanicum*, *Caryophyllus aromaticum*	It is used as carminative for stomach and intestinal gas.	**C. zeylanicum* can cause allergic reactions in the skin and mucous membranes (List of German Commission E Monographs 2016). **M. chamomilla* can affect synergistic when used in combination with anticoagulants such as warfarin, because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000). *Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). **C. zeylanicum* should be used with doctor consult if there is a recurrent case or if the usage lasts longer than a week (Wichtl 2004). *There may be the same amount of mild cases of gastrointestinal discomfort because of *R. canina* usage (Warholm et al. 2003).	Drunk 2-3 cups per day.

Table 4. Herbal preparations and medicinal teas used as galactagogue drugs in nursing mothers

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
Vita® Malt (Royal Unibrew)	*Hordeum distichon*, *Humulus lupulus*	It is used in nursing mothers to increase milk.	**H. lupulus* increases the effect of sedative drugs (Lee et al. 1993).	Not given
Humana® Still-tee (Mamsel)	*Trigonella foenumgraecum*, *Foeniculum vulgare*, *Galega officinalis*, *Verbena officinalis*, *Rubus idaeus*	It is used in nursing mothers to increase milk.	**G. officinalis* interaction with the hypoglycemic drugs is concerned. Care should be taken in use in diabetic patients (PDR For Herbal Medicine 2000).	10 g are added to 200 ml of water. 3-4 cups a day should be consumed.
Hipp® Natal Granular Herbal Tea for Nursing Mothers (Hipp)	*Foeniculum vulgare*, *Illicium verum*, *Citrus limonum*, *Melissa officinalis*, *Urtica diocia*, *Carum carvi*, *Ruta graveolens*	It is used in nursing mothers to increase milk.	*As a result of the active substances passage to milk, sedation can be seen in infants when *M. officinalis* is consumed by mother (Mills and Bone 2005). *Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). *The oral use of *C. carvi* in children and adolescents under 18 years of age has not been established due to lack of adequate data. The use in patients with liver disease, cholangitis, achlorhydria, gallstones and any other biliary disorders is not recommended (HMPC 2014).	4 teaspoons are added to 200 mL of water and 2-3 cups are consumed per day.

Table 5. Herbal products used for colds in children and adoloescents

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
Propolsaft® Syrup (Marnys)	*Mentha piperita*, *Thymus vulgaris*, *Propolis*, *Malpighia punicifolia*	It is helpful in the treatment of upper respiratory tract infections. It helps to alleviate the uncomfortable symptoms of respiratory tract infection (cough, nasal congestion etc.).	**M. piperita* should not be used without consulting a doctor when gallstones are concerned (List of German Commission E Monographs 2016). **T. vulgaris* (Timol) is contraindicated in enterocolitis, pregnancy and cardiac failure (Braun and Frohne 1987).	Up to 6 years old: 5 ml before meals; 6 - 12 years old: 10 ml before meals; 2 years old and adults: 15 mL before meals.
Umca® Solution (Abdi İbrahim)	*Pelargonium sidoides*	It is suitable for the treatment of acute and chronic infections, respiratory tract infections, earnose-throat infections, sinusitis, and arginine. It helps in treating symptoms such as cough, fever, sore throat, and fatigue.	*The use of *P. sidoides* in children under 6 years of age has not been established due to lack of adequate data. Hepatotoxicity and hepatitis cases were reported in association with the administration of the medicinal product. In case signs of hepatotoxicity occur, the administration of the medicinal product should be stopped immediately and a medical doctor should be consulted (HMPC 2012).	Acute infections: 20-30 drops 3 times a day for adults and children older than 12 years; 10-20 drops 3 timesa day for children in the age group of 6-12 years; 5-10 drops 3 times a day in children younger than 6 years. Chronic infections: It is recommended to be used as 10-20 drops 3 times a day in adults and children older than

Table 5. Herbal products used for colds in children and adoloescents (continued)

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
				12 years. The drops should be taken with some liquid 30 minutes before meals. In order to prevent the relapse of the disorder, the use of the drug is recommended to be continued for several days after the symptoms alleviate. It should not be used in infants under 1 years old. Pregnant women and nursing mothers should not use it.
Prospan® Syrup (Biomeks)	*Hedera helix*	It helps the treatment of acute respiratory inflammation accompanied by cough and chronic inflammatory bronchial diseases.	**Hedera helix* may cause mild gastrointestinal disorders (Fazio et al 2009).	It is used as 2.5 mL 3 times a day in infants (under 1 year of age) and young children (1-5 years); 5 mL 3 times a day in schoolage children (6-9 years) and adolescents (10 years and older); and 5-7,5 mL 3 times a day in adults.
Strath® Cold Drops (Interpharm)	*Thymus vulgaris*, *Primula officinalis*	It is cough sedative, expectorant, and supportive in the treatment of colds, flu, bronchial catarrh and whooping cough.	** T. vulgaris* (Timol) is contraindicated in enterocolitis, pregnancy and cardiac failure (Braun & Frohne 1987).	Adults use it by dropping 20-30 drops into a small amount of water every 2 hours. 10 drops are given every 2 hours children of 6 years old and older.

Table 6. Herbal teas used for colds in infants, children and adolescents

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
Günvit® Linden Tea (Kurtsan)	*Tilia cordata*	It is used as respiratory softener, and sweater and relaxer in fierce colds.	The use of *T. cordota* in children under 12 yearsof age has not been established due to lack of adequate data (HMPC 2012).	Drunk 2-3 cups per day.
Günvit® Rosehip Fruit Tea (Kurtsan)	*Rosa canina*	It is used as body protection against the cold.	As a specific adverse event in rare cases, allergy to *R. canina* may occur. Allergy with generalized exanthema and gastrointestinal complaints may even occur after drinking rose hip tea (Lleonart et al. 2007).	Drunk 2-3 cups per day.
Hipp® Mixed Fruit Tea (Hipp)	*Rosa canina*, *Melissa officinalis*, *Citrus sinensis*, *Citrus limonum*, *Malus domestica*, *Alcea rosea*	It is used as body protection against the cold.	*As a specific adverse event in rare cases,allergy to *R. canina* may occur. Allergy withgeneralized exanthema and gastrointestinalcomplaints may even occur after drinking rose hip tea (Lleonart et al. 2007). * The use of *M. officinalis* in children under 12 years of age has not been established due to lack of adequate data (HMPC 2013). *There may be the same amount of mild cases of gastrointestinal discomfort because of *R. canina* usage (Warholm et al. 2003).	It is used from 6th month onwards. Drunk 2-3 cups per day.

Table 7. Herbal products and medicinal teas used as an immune system booster in children and adolescents

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
Propolmar® Syrup (Marnys)	*Echinacea purpurea*, Propolis, *Mentha piperita*	It supports strengthening of the immune system. It reduces the risk of contracting colds, flu and lower and upper respiratory tract diseases.	* *M. piperita* should not be used without consulting a doctor when gallstones are concerned (List of German Commission E Monographs 2016). * *E. purpurea* should be used up to eight weeks (ESCOP Monographs 2003). Hypersensitive reactions in the form of rash, urticaria, itching, swelling of the face may occur. Cases of severe hypersensitivity reactions, such as Stevens-Johnson Syndrome, angioedema of the skin, Quincke oedema, bronchospasm with airway obstruction, asthma and anaphylactic shock have been reported. The frequency is not known (HMPC 2015).	Up to 6 years old: 5 mL before meals; 6 - 12 years old: 10 mL before meals; 12 years of age and adults: 15 mL before meals.
Immuzine® Syrup (Berko)	*Sambucus nigra*, *Echinacea purpurea*, *Propolis*	It supports strengthening of the immune system. It reduces the risk of contracting colds, flu and lower and upper respiratory tract diseases.	*Due to the insufficient data *Sambucus nigra* L. isn't recommended for use in children under 12 years of age. (EMEA/HMPC 2007) * *E. purpurea* should be used up to eight weeks (ESCOP Monographs 2003). Hypersensitive reactions in the form of rash, urticaria, itching, swelling of the face may occur. Cases of severe hypersensitivity reactions, such as Stevens-Johnson Syndrome, angioedema of the skin, Quincke oedema, bronchospasm with airway obstruction, asthma and anaphylactic shock have been reported. The frequency is not known (HMPC 2015).	½ scale per day for 1-3 year-olds, 1 scale per day for 4-6 yearolds, and 1.5 scale per day for 7-12 year-olds. *The duration of treatment with S. nigra is 3-5 days in acute viral infections (Zakay-Rones et al. 2004). It should not be used longer than a week. (EMEA/HMPC, 2007).
Echinol® Syrup (Mikro-gen)	*Echinacea purpurea*	It contributes to immune system function. It is used as a support against upper and lower respiratory tract diseases.	* *E. purpurea* should be used up to eight weeks (ESCOP Monographs 2003). Hypersensitive reactions in the form of rash, urticaria, itching, swelling of the face may occur. Cases of severe hypersensitivity reactions, such as Stevens-Johnson Syndrome, angioedema of the skin, Quincke oedema, bronchospasm with airway obstruction, asthma and anaphylactic shock have been reported. The frequency is not known (HMPC 2015). It should not be used more than 8 consecutive weeks.	It should be taken 3 times a day as 5 mL.
Strath® Syrup (Interpharm)	*Citrus sinensis*, *Mentha piperita*, *Melissa officinalis*, *Carum carvi*, *Thymus vulgaris*, *Matricaria chamomilla*, *Cinnamomum zeylanicum*, *Salvia officinalis*, *Ocimum basilicum*, *Sambucus nigra*, *Foeniculum vulgare*, *Armoracia rusticana*, *Hyssopus officinalis*, *Lavandula angustifolia*, *Glycyrrhiza glabra*, *Petroselinum crispum*	It supports strengthening of the immune system.	* *M. piperita* should not be used without consulting a doctor when gallstones are concerned (List of German Commission E Monographs 2016). * *C. zeylanicum* can cause allergic reactions in the skin and mucous membranes (List of German Commission E Monographs 2016). * *T. vulgaris* (Timol) is contraindicated in enterocolitis, pregnancy and cardiac failure (Braun and Frohne 1987). * *L. angustifolia* has the potential to increase the effect of sedative and tranquilizing drugs (Brinker 1998). *Due to the insufficient data *Sambucus nigra* L. isn't recommended for use in children under 12 years of age (EMEA/HMPC 2007). * *L. angustifolia* should not be used in people with allergic nature (WHO Monographs 2007).	It is used 2 times a day before meals as 5 mL.

Table 7. Herbal products and medicinal teas used as an immune system booster in children and adolescents (continued)

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
			*_M. chamomilla_ can effect synergistic when used in combination with anticoagulants such as warfarin, because it carries the hydroxy coumarins (PDR for Herbal Medicine 2000). *Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). *The oral use of _C. carvi_ in children and adolescents under 18 years of age has not been established due to lack of adequate data. The use in patients with liver disease, cholangitis, achlorhydria, gallstones and any other biliary disorders is not recommended (HMPC 2014). * The use of _M. officinalis_ in children under 12 years of age has not been established due to lack of adequate data (HMPC 2013). *_C. zeylanicum_ should be used with doctor consult if there is a recurrent case or if the usage lasts longer than a week (Wichtl 2004). *Fennel preparations should not be used for a long time without consulting a doctor or pharmacist (List of German Commission E Monographs 2016). *The duration of treatment with _S. nigra_ is 3-5 days in acute viral infections (Zakay-Rones et al. 2004). It should not be used longer than a week. (EMEA/HMPC 2007).	
Günvit® Rosehip Fruit Tea (Kurtsan)	_Rosa canina_	It is used as body resistance increaser and body protector against colds.	*There may be the same amount of mild cases of gastrointestinal discomfort because of _R. canina_ usage (Warholm et al, 2003).	Drunk 2-3 cups per day.

Table 8. Herbal products for external use in infants and children

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
99® Diaper Rash Preventive Cream (İstanbul Cosmetic)	_Echinacea purpurea_, _Hamamelis virginiana_, _Centella asiatica_	Echinacea purpurea strengthens the immune system. It boosts cell renewal. It helps heal wounds. It helps the healing of wounds such as witch hazel. It softens the baby skin. Gotu kola has antibacterial effects. It helps to remove redness and irritation. It is cell regenerative and softening.	* _E. purpurea_ can trigger allergic reactions in atopic patients (HMPC 2015). *Topical use of the _C. asiatica_ extract has led to reports of rash (Eun 1985). *Allergic contact dermatitis when using _H. virginiana_ has been reported. The frequency is not known (HPMC 2009). It is applied externally to sensitive areas each time the diaper is changed.	
Popishic® Diaper Rash Cream (Medicure)	_Anthemis nobilis_, _Primula veris_	It has relaxing and moisturizing qualities. It makes the skin look healthy.	Not applicable	It is applied externally to sensitive areas each time the diaper is changed.
Popolin® Diaper Rash Cream (İstanbul Cosmetic)	_Matricaria chamomilla_, _Calendula officinalis_, _Centella asiatica_, _Melissa officinalis_,	It is hypoallergenic. It prevents rash and redness.	*Matricariae flos can rarely cause allergic skin reactions (Wichtl 2004). *Skin sensitization to _C. officinalis_ is reported. The frequency is not known (HPMC 2008).	It is applied externally to sensitive areas each time the diaper is changed.

Table 8. Herbal products for external use in infants and children (continued)

Name of the Preparation	Content	Usage	Warnings (According to literature and/or Monographs)	Dosage Adjustment (According to its prospectus information)
	Hamamelis virginiana, *Tilia cordata*		*Topical use of the *C. asiatica* extract has led to reports of rash (Eun HC 1985). *Allergic contact dermatitis when using *H. virginiana* has been reported. The frequency is not known (HPMC 2009).	
99® Premature and Newborn Foam Shampoo (İstanbul Cosmetic)	*Persea americana*, *Calendula officinalis*, *Tilia cordata*, *Camellia sinensis*	Avocado provides intense hydration. Calendula has antibacterial properties. It protects the skin against irritation and redness. Green tea is a powerful antioxidant. It supports hair growth. Linden has restorative, moisturizing and soothing properties.	*Skin sensitization to *C. officinalis* is reported. The frequency is not known (HPMC 2008).	It is thoroughly rinsed after application to the baby's damp hair.
99® Baby Hair & Body Shampoo (İstanbul Cosmetic)	*Oleum olivea*, *Matricaria chamomilla*	Olive oil gives softness, vitality and shine to baby hair by softening dry and damaged hair skin. Chamomile soothes irritated skin and relieves it.	*Matricariae flos can rarely cause allergic skin reactions (Wichtl 2004).	It is thoroughly rinsed after application to the baby's damp hair.
99® Baby Oil (İstanbul Cosmetic)	*Macadamia integrifolia*, *Simmondsia chinensis*	It gives the baby skin brightness and vitality. It is used in the care of dry and damaged skin. It has intensive moisturizing and soothing qualities. It prevents dehydration of the skin.	Case reports of contact dermatitis confirmed by skin patch tests exist for jojoba oil (*Simmondsia chinensis*) (Wantke et al. 1996).	It is applied on the entire body of babies after the bath and at each diaper change.
99® Baby Lotion (İstanbul Cosmetic)	*Prunus amygdalus var. dulce*, *Calendula officinalis*, *Melissa officinalis*	It has antibacterial properties. It prevents dryness and itching on baby skin. It is cell regenerative and moisturizing.	*Skin sensitization to *C. officinalis* is reported. The frequency is not known (HPMC 2008).	It softens the skin. It is applied by massaging the entire body of the baby. It is used every day regularly.
99® Bath Oil (İstanbul Cosmetic)	*Matricaria chamomilla*, *Mercurialis annua*, *Pseudevernia prunastri*, *Mentha piperita*, *Eucalyptus globulus*	It has antibacterial properties. It is inhalant, skin refreshing and invigorating. It has soothing and itching reducing effect on the skin. It has softening effect.	*Matricariae flos can rarely cause allergic skin reactions (Wichtl 2004).	8-10 drops of bath oil is dripped into 20 liters of water. 3-5 minutes is sufficient for body bath. For the use before sleeping, 5 drops are dripped into 1 cup of warm water 1 hour before taking the baby to the room. Thus, it makes the baby's room refreshing and relaxing.

CONCLUSION

The complaints of the mothers coming to pharmacies regarding themselves and their children were examined based on a survey with 40 pharmacists from various municipalities of İstanbul and Ankara.

It was found that colic in children and hypogalactia in nursing mothers are relatively more common than others and therefore they were in this study more under focus. We have summarized on the tables the recommended numerous herbal preparations for these complaints in the pharmacies. According the monographs and the recent studies of the used me-

dicinal herbs in these preparations we can clearly say that the contents of these preparations have been carefully selected. It has been observed that these herbal preparations are not as harmless as they are considered.

As a result of the detailed review about the contents of the common used herbal products were showed there are serious drug interaction. *C. monogyna* may effect antihypertansive when used in combination with beta blockers (Mills and Bone 2000), Meliloti herba and *M. chamomilla* may strengthen the activity of anticoagulants (Arora RB, Marthur CN, 1963; PDR for Herbal Medicine 2000), *P. ginseng* reduces the effect of warfarin (Yuan et al. 2004), *H. lupulus* and *L. angustifolia* increase the effect of sedative drugs (Lee et al. 1993; Brinker 1998) and should not be used in people with allergic nature (WHO Monographs 2007), *G. officinalis* may interact with the hypoglycemic drugs (PDR For Herbal Medicine 2000) are found in product compositions without any warning on products.

In the leaflet of the some herbal products and medicinal teas and herbs, which are used for different purpose for children, have without sufficient data for usage. Due to the lack of of adequate data the usages of M. officinalis and Sambucus nigra L. under 12 years old have not been established (HMPC 2013; EMEA/HMPC 2007). The oral use of *C. carvi* under 18 years and the use of *P. sidoides* under 6 years old also has not been established because of same reason (HMPC 2014; HMPC 2012).

In contrast to popular belief should be used these products under an expert's control. It is the only way patients can reach sufficient knowledge of situations to encounter during treatment or duration of usage. The duration of treatment with *S. nigra* is 3-5 days in acute viral infections (Zakay-Rones et al. 2004). *E. purpurea* should be used up to eight weeks which may cause severe hypersensitivity reactions, such as Stevens-Johnson Syndrome, angioedema of the skin, Quincke oedema, bronchospasm with airway obstruction, asthma and anaphylactic shock (ESCOP Monographs 2003; HMPC 2015). Another missing information on the researched preparation is that *O. majora* is not suitable for long term use because of the arbutin regarding to its composition (PDR for Herbal Medicines 2004). *A. graveolens* is considered safe, but in sporadic cases, it causes allergic reactions, oral pruritus, tongue and throat swelling, urticaria, vomiting and diarrhea (Al Snafi 2014). Topical use of the *C. asiatica*, *C. zeylanicum* or *H. virginiana* extract has led to reports of rash (Eun 1985; List of German Commission E Monographs 2016; HPMC 2009). Case reports of contact dermatitis confirmed by skin patch tests exist for jojoba oil (Simmondsia chinensis) (Wantke et al. 1996).

There are several examples of herbal products and medicinal teas used for different purposes in children/breastfeeding mother includes *Zingiber officinalis*, *Pimpinella anisum*, *Hedara helix* and others with reported side effects. It has been reported that more than 6 grams of ginger can cause irritation to stomach (Desai et al. 1990). Ingestion of 1 to 5 millilitres of anise oil (*Pimpinella anisum*) has been associated with nausea, vomiting, seizures and pulmonary oedema (HMPC 2013). *Hedera helix* may cause mild gastrointestinal disorders (Fazio et al 2009). Allergy with generalized exanthema and gastrointestinal complaints may even occur after drinking rose hip (*Rosa canina*) tea (Lleonart et al. 2007). *T. vulgaris* (Timol) is contraindicated in enterocolitis, pregnancy and cardiac failure (Braun and Frohne 1987). Sedation can be seen in infants when *M. officinalis* is consumed by mother (Mills and Bone 2005). There may be the same amount of mild cases of gastrointestinal discomfort because of *R. canina* usage (Warholm et al. 2003). Allergic reactions to fennel preparations can affect the skin or respiratory tract by particular cases (Wichtl 2002). In rare cases headaches have been reported after internal use of Meliloti herba (Bisset 1994). But people intending to buy concerned products can not reach this kind of information on their packages.

Main side-effects of herbal ingredients are listed as a table. The main output of this survey and investigations result is that the field of herbal products needs to be supported with more detailed and comprehensive studies.

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Original Article

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Association between apolipoprotein E polymorphisms and gastric cancer in a hospital-based Turkish population

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ABSTRACT

Apolipoprotein E (ApoE) is an important secretory glycoprotein, and also a ligand for LDL receptors and ApoE receptors. It is involved in lipid transport and catabolism, nerve regeneration, immune system regulation, and cell proliferation. Moreover, both the causative and protective effects of *APOE* polymorphic variants and alleles have been correlated with several diseases, including cancer types such as gastric cancer. However, to the best of our knowledge, no data are available describing the association between gastric cancer and *APOE* polymorphisms in the Turkish population. In this context, this study was conducted to evaluate the association between the genotypes of *APOE* and the risk of gastric cancer and also to demonstrate the allele frequencies and genotype distributions of *APOE* gene in a hospital-based Turkish population with gastric cancer. A total of 59 patients with gastric cancer and 200 healthy controls were included in this study. Results showed the absence of any statistically significant association between *APOE* gene polymorphisms and the risk of gastric cancer (χ^2 =2.711, p=0.744), which could possibly be due to the small sample size of our study. Therefore, further investigations such as expression studies and meta-analysis comprising larger sample size and detailed demographic data are necessary to identify the alleles of APOE gene associated with the risk of gastric cancer.

Keywords: Gastric cancer, *APOE* polymorphisms, Turkish population

INTRODUCTION

Gastric cancer, also called stomach cancer, is the first cause of cancer-related mortality worldwide (Türkiye Halk Sağlığı Kurumu 2017). The incidence rate of gastric cancer is falling worldwide; however, still nearly 730,000 deaths are encountered per year (Parkin et al. 2001). Environmental and genetic factors play an important role in the development of gastric cancer. *Helicobacter Pylori* infection and infection related gastritis are the common environmental factors implicated in its causation, besides others such as diet, smoking, and geographical location (Sipponen 2002; Yaghoobi et al. 2010). However, genetic and familial contributors have not yet been extensively investigated in studies (Yaghoobi et al. 2010).

Recently, the attention has been focused on combined effect of low-penetrance alleles as a potential risk factor for causation of gastric cancer. In this respect, gastric cancer associated genes need to be further identified and expanded (Yaghoobi et al. 2010). Apolipoprotein E (*APOE*) is one of the genes (19q13.2) that is implicated in causation of gastric cancer (De Feo et al. 2012). The *APOE* gene is a high affinity ligand for low density lipoprotein (LDL) receptors and apoE receptors found in hepatic tissues and plays an important role in lipid transport and catabolism (Uniprot 2017; Sazci et al. 2008). The *APOE* gene consists of 3 introns and 4 exons, and due to two single nucleotide mutations in the coding region of *APOE* gene, three alleles (ε2, ε3, and ε4) and three homozygotes (apoE2/2, E3/3 and E4/4) and three heterozygotes (apoE2/3, E2/4, and E3/4) genotypes are formed (Sazci et al. 2008; De Feo et al. 2012). These isoforms are different in terms of amino acid constitution at positions 112 and 158; apoE2 (cys-

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teine112/cysteine158), apoE3 (cysteine112/arginine158) and apoE4 (arginine112/arginine158). These substitutions give rise to the difference in isoelectric points among isoforms, so that each differs by receptor binding capacity (Zannis et al. 1982; Sazci et al. 2008; De Feo et al. 2012). Based on these, *APOE* polymorphisms may affect plasma cholesterol and LDL concentrations or signaling pathways; thus, causing cancer. Studies on several populations have suggested that cholesterol and LDL levels differ from lowest to highest in people carrying ε2, ε3, and ε4 alleles, respectively. Also, some studies have supported that genetic polymorphism is responsible for 60% of variations in levels of plasma cholesterol and *APOE* polymorphisms accounts for nearly 14% of this ratio (Weisgraber 1990; Sakashita et al. 2008).

APOE gene ε3 allele is reported as the common and neutral allele found in 40%–90% of the populations and correlated with prostate cancer and polycystic ovary syndrome (Cetinkalp et al. 2009; Uen et al. 2015; Yencilek et al. 2016; Uniprot 2017). *APOE* gene ε2 allele was correlated with some other diseases such as type III hyperlipoproteinemia and Parkinson's disease. Nonetheless, it plays a protective role in gastric cancer (Cibeira et al. 2014; Uen et al. 2015). *APOE* gene ε4 allele was correlated with Alzheimer's disease, coronary heart disease and several cancer types including prostate, breast, head and neck cancers (Uen et al. 2015). On the other hand, studies have shown that people with low total serum cholesterol levels are more prone to develop cancer. The mechanism regarding susceptibility to gastric cancer in this population is unclear; nevertheless, some of available estimations are low cholesterol levels, effects of different structural stabilities of apoE isoforms on tumor growth, variable interactions between apoE isoforms and their targets. Moreover, possible variability in post-translational modifications of plasma apoE may affect diseases or cancer development (De Feo et al. 2012; Uen et al. 2015).

Based on these data, we investigated association between genotypes of the *APOE* gene and gastric cancer risk in a hospital-based Turkish population in a case-control study including 59 gastric cancer patients and 200 healthy controls.

MATERIALS AND METHODS

Study population

This study consists of 59 gastric cancer patients and 200 healthy controls. The 59 gastric cancer patients were obtained from the Kocaeli University Hospital. Male and female ratios were matched in the cases and controls. The study was approved by the Ethic Committee of Kocaeli University (GOKAEK 2017/14.42).

Genotyping

Genomic DNA was isolated by using the conventional salting-out method (Miller et al. 1988). Genotyping was done by a PCR-RFLP method. In the PCR reaction 5'-ATGGACGAGACCATGAAGGAGTTGAAG-3' was used as forward and 5'-TGTACCAGGCCGGGGCCCGCGA-3' was used as reverse primer. The PCR conditions were as follows: 96°C for 2 min followed by 35 cycles at 96°C for 1 min, 67°C for 2 min, 72°C for 2 min and final extension step at 72°C for 4 min. The 314 bp long PCR product was digested with 2U Hin6I enzyme and the digestion products were run in 10% PAGE for 30 min at 20 W. The corresponding fragments for *ApoE* genotypes were given in Table 1.

Statistical Analysis

Genotype frequencies between gastric cancer patients and controls were compared by $\chi 2$ test. The odds ratio (OR) and 95% confidence intervals (CI) were used to verify the effects of the genotypes and alleles. Statistical analysis were done by SPSS (version 21.0 IBM Corp.; Armonk, NY, USA) software package. p-value less than 0.05 was accepted as significant.

RESULTS AND DISCUSSION

Gastric cancer accounts for many cancer-related deaths. Some single nucleotide polymorphisms in the corresponding regions have been reported as a genetic risk factor on gastric cancer. Both genetic risk factor and protective effects of *APOE* polymorphic variants and alleles have been correlated with several disease and cancer types including gastric cancer. The current study including 59 gastric cancer patients and 200 controls revealed that there is no association between *APOE* genotypes and gastric cancer and this study is the first study in the Turkish population (Table 2).

Previous studies demonstrated the genotype distribution of *APOE* gene alleles on gastric cancer. When the genotype distribution of our study was compared with the study done in Italian population, E2/2 and E4/4 genotypes were not found in the cases for both population. Also, the frequencies of E2/3 and E3/4 in both populations are in consistent in terms of their distribution among cases and controls. Moreover, E3/3 genotype was reported as the most common genotype in cases; 71.71%, 76.3% and controls; 62.94%, 70.0% for the Italian and Turkish population studied herein, respectively (De Feo et al. 2012). Similarly, E3/3 genotype was found as common, E2/2 is observed rarely and E3/4 genotype frequencies are close to each other in the study done in the Chinese Han population for both groups. In contrast to Italian and Turkish population, E2/3 genotype frequency is higher in gastric cancer patients than controls and higher frequencies for E4/4 and E2/4 were obtained in the cases of Chinese Han population. On the other hand, high frequency of *APOE* gene ε2 allele and low serum total cholesterol are significantly correlated with gastric cancer in the Chinese Han population. Additionally, they proposed that higher risk of gastric cancer is due to the ε2 allele and it could be related with reduced serum cholesterol levels (Kang et al. 2016). Conversely, *ApoE* ε2 allele was reported as protective against gastric cancer in Italian population and the possible explanation of this phenomena was higher antioxidant properties of ε2 allele than other alleles (De Feo et al. 2012). The differences of genotype distribution between Chinese population and other populations may be due to the different ethnicity, geographical location, environmental factors and lifestyle. Also, variations among studies performed in different populations might be attributed to the study population size.

APOE gene polymorphic variants were studied in the Turkish population before, and they were correlated with various diseases such as type 2 diabetes (Erdogan et al. 2016), Alzheimer's disease (Acar Çinleti et al. 2015; Alaylıoğlu et al. 2016), coronary heart disease (Yılmaz-Aydogan et al. 2012), cerebrovascular disease (Tasdemir et al. 2008); however, there are only two studies regarding cancer that include breast (Yaylim et al. 2003) and prostate cancer (Yencilek et al. 2016). *APOE* gene polymorphic variants could affect the mechanism of cancer formation, thus it could be speculated that different cancer types might be associated with the same alleles in the same population. According to the study on breast cancer, no significant association was found between any alleles and breast cancer risk similar to some other studies and meta-analysis (Niemi et al. 2000; Yaylim et al. 2003; Liu et al. 2016). The distribution of E3/3 genotype is consistent with our results in terms of highest frequencies in both cases and controls, whereas the contrary results were obtained for ε4 allele in which its frequency is 9.25% in controls for the present study and 0% for the study done on breast cancer (Yaylim et al. 2003). In the other study focusing on prostate cancer in a Turkish population, E3/3 genotype was found as a risk factor, while ε4 allele was reported as protective (Yencilek et al. 2016). Even though some similarity was observed for different cancer types in the Turkish population, the obtained results have not high percentage consistency yet. The variability in results might be due to the small population size in addition to different demographic properties of the populations studied. Based on these data, it was not possible to speculate which alleles attribute as a risk factor or which one is protective against cancer. This leads to the idea that there might be other factors that contribute to the development of various cancer types.

Table 1. The fragments of *APOE* genotypes

Genotypes	Fragments
E2/2	109bp, 91bp, 79bp, 18bp, 16bp
E2/3	109bp, 91bp, 79bp, 48bp, 31bp, 18bp, 16bp
E2/4	109bp, 91bp, 79bp, 72bp, 48bp, 31bp, 19bp, 18bp, 16 bp
E3/3	109bp, 91bp, 48bp, 31bp, 18bp, 16bp
E3/4	109bp, 91bp, 72bp, 48bp, 31bp, 19bp, 18bp, 16bp
E4/4	109bp, 72bp, 48bp, 31bp, 19bp, 18bp, 16bp

In the literature, there are few expression studies focused on *APOE* gene allelic variants and gastric cancer association. As mentioned briefly before, ApoE protein is a secretory glycoprotein that is a ligand for some receptors and it stimulates different signalling pathways. Apart from regulating lipid metabolism, it is involved in nerve regeneration, immune system regulation, cell proliferation. Moreover, it is correlated with several cancer types. One study aimed to show function of ApoE in stimulation of lymph node metastasis in gastric cancer. Overexpression of ApoE mRNA and dominant expression of ApoE protein products were detected in gastric cancer. Besides, deeper tumor invasion and more lymph node metastasis were observed in the cases having high ApoE expression. Based on these, it has been indicated that ApoE might cause gastric cancer through stimulation of signalling pathways resulting in tumor growth after binding to the LDL receptors (Sakashita et al. 2008). Another study focused on the *APOE*-associated genes and transcription factors in gastric cancer development. The responsible genes have function in JAK-STAT cascade, acute inflammatory response, steroid hormone response and acute phase response. Thus effective pathways causing gastric cancer have been determined (Shi et al. 2015).

In addition to the abovementioned mechanisms of *APOE* gene on cancer susceptibility, expression studies also provide some clues for focusing on the role of expression levels of *APOE* gene. Moreover, meta-analysis with large population size and comprehensive demographic data could confirm the causative alleles of *APOE* gene in gastric cancer. Our study contributes for revealing the genotype and allelic distribution of *APOE* gene in the Turkish population and may provide a basis for further studies in this field.

Table 2. Genotype and allele frequencies of *APOE* gene in gastric cancer patients and controls

Genotypes	Gastric Cancer Patients, n (%)	Controls, n (%)	$\chi 2$	p	OR;95%CI
APOE	59 (100.0)	200 (100.0)	2.711	0.744	
E2/2	0 (0.0)	1 (0.5)	0.296	1.000	0.995 (0.985-1.005)
E2/3	6 (10.2)	23 (11.5)	0.081	0.960	0.871 (0.337-2.251)
E2/4	0 (0.0)	6 (3.0)	1.812	0.342	0.970 (0.947-0.994)
E3/3	45 (76.3)	140 (70.0)	0.878	0.440	1.378 (0.704-2.697)
E3/4	8 (13.6)	29 (14.5)	0.033	1.000	0.925 (0.398-2.149)
E4/4	0 (0.0)	1 (0.5)	0.296	1.000	0.995 (0.985-1.005)
Alleles					
ε2	6 (5.00)	31 (7.75)	0.888	0.466	0.642 (0.253-1.625)
ε3	104 (88.14)	332 (83.0)	2.435	0.205	0.960 (0.933-0.988)
ε4	8 (6.78)	37 (9.25)	0.637	0.548	0.715 (0.312-1.636)

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Original Article

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Antioxidant and Antimicrobial Activity of *Ferulago trojana* E. Akalın & Pimenov

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ABSTRACT

Ferulago W. Koch is a genus in the Apiaceae family comprising 34 species, of which 18 are endemic in Turkey. *Ferulago* species have been known since the time of Dioscorides and have been used in folk medicine for their sedative, tonic, digestive, carminative, and aphrodisiac effects, as well as for the treatment of intestinal worms and hemorrhoids. This study was conducted to evaluate the polyphenolic contents and antioxidant activities of methanol extracts of the aerial parts (HFT) and rhizomes (RFT) of *Ferulago* trojana E. Akalın & Pimenov by measuring their ability to inhibit lipid peroxidation induced by Fe^{3+}-ascorbate, their $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ scavenging activities, and their ferric reducing antioxidant power (FRAP value). The methanol extracts were also examined for their antimicrobial activity using the microbroth dilution technique. Results showed that the methanol extracts of the aerial parts of the plant, containing the highest amount of total phenolic content and flavonoids, exhibited antioxidative potential for the chain-breaking inhibition of lipid peroxidation and showed the strongest hydrogen and single electron donor activities, which could thus serve as a free radical scavenger, act as a reductant, and provide protection against oxidative stress. Although the methanol extract of rhizomes did not exhibit any inhibitory effect on lipid peroxidation, it is possible that it might also have protective effects against oxidative damage by scavenging free radicals and acting as a reductant. While both the methanol extracts of the aerial parts and rhizomes of *F. trojana* were effective against *Staphylococcus aureus, Methicillin-resistant S. aureus* (MRSA), and *S. epidermidis*, the extracts showed no activity against *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. In addition, the methanol extract of rhizomes of *F. trojana* exhibited antibacterial activity against *Proteus mirabilis* and antifungal activity against the yeast *Candida albicans*.

Keywords: *Ferulago, F. trojana*, antioxidant activity, antimicrobial activity

INTRODUCTION

Many medicinal plants and raw extracts, due to their traditional use in vitro antioxidants and antibacterial activities have been screened by many researchers. These activites have been observed on plants containing specially phenolic compounds (Wang et al. 1999; Kähkönen et al. 1999; Pietta 2000; Cushine & Lamb 2005; Rios & Recio 2005).

The genus *Ferulago* (Apiaceae/Umbelliferae) includes 49 species occuring throughout the Northern hemisphere. It is naturally grown mainly in Europe, Northwest and Central Asia, Caucasus, North and Northwest Africa and Turkey. 34 *Ferulago* species (18 endemic) are naturally grow in Turkey (Peşmen 1972; Davis et al. 1988; Pimenov and Leonov 1993; Özhatay and Akalın 2000; Solanas et al. 2000; Akalın and Pimenov 2004; Kandemir and Hedge 2007).

In different regions in Turkey, *Ferulago* species are known by different names, as the most common "çakşırotu", "kişniş", "asaotu", "kuzubaşı" ve "kuzukemirdi". Since Dioscorides, *Ferulago* species are used for the purpose tonic, digestive, carminative, aphrodi-

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siac as well as in the treatment of intestinal worms and hemorrhoids (Baytop 1999; Akalın 1999).

Ferulago trojana is endemic to Mount Ida which was first identified in 2004. On ISTE's samples, collected in Çanakkale and Balıkesir, previously determined as *F. sylvatica* (also this name was specified from same areas in the Flora of Turkey and East Aegean Islands). Later studies showed that they were different from the European sample. The results of the detailed examination of the Turkish '*F. sylvatica*' samples, they have been identified as a new species, *F. trojana*. The species of *F. sylvatica* is not considered to be in Turkey (Akalın and Pimenov 2004).

To date, there have been some studies on chemical composition of various *Ferulago* species. Essential oils, coumarins, flavonoids, sesquiterpenes, fatty acids and phytosterols were reported as the chemical constituents of the *Ferulago* plants. In these studies with respect to the essential oils of *Ferulago* genus, coumarins, monoterpenes and sesquiterpenes were characterized as the main components (Miski et al. 1990; Doğanca et al. 1991; Yoti&Assenov 1995; Rustaiyan et al. 1999; Jiménez et al. 2000; Başer et al. ; 2001; 2002; Erdurak et al. 2006; Kılıç et al. 2006; Erdemoğlu et al. 2008; Alkhatib et al. 2009).

In previous study, five compounds have been isolated from *F.trojana*. From these compounds bergapten, isoimperatorin, 3'-epidecursin and isomaltol are known compounds, isomaltol-3β-O-glucoside and 3,6-dimethoxy-7-isopropilcoumarin-4-tetradeca-13"-one have been isolated for the first time. Also antioxidant activities and anticholinesterase activities of dichloromethane and methanol extracts of *F. trojana* were determined (Çakar 2010). GS/MS analysis has resulted in the characterization of 19 compounds representing 99.3% of the oil with p-cymene (45.8%) as the main costituents. Monoterpenes and sesquiterpenes were reported from aerial parts of the essential oil of *F. sylvatica* (Chalchat et al. 1992).

The aim of this study was to evaluate and compare the antioxidant and antimicrobial activities of methanol extracts from the aerial parts (HFT) and rhizomes (RFT) of *F. trojana*. There are no reports on antimicrobial activity of *F.trojana*. The most active extract and parts of the plant will be detected and the compounds responsible for the antimicrobial activity in another study will be isolated.

MATERIALS AND METHODS

Plant material

Ferulago trojana E. Akalın & Pimenov a species growing in Turkey was collected from Kaz Dağları (Balıkesir) in June 2007, and identified by Dr. Emine Akalın. A voucher specimen (ISTE No: 74316) is deposited in the Herbarium of Faculty of Pharmacy, Istanbul University (ISTE).

Preparation of extracts

The dried and powdered aerial parts (30 g) (HFT) and rhizomes (30 g) (RFT) of the *F.trojana* were percolated with 600 mL methanol. The methanol extracts were evaporated to dryness under reduced pressure and controlled temperature (45 to 50°C) in a rotary evaporator. The extracts were lyophilized. The obtained methanol extracts (HFT, RFT) were used for antioxidant and antimicrobial activity determinations.

Chemicals

Soybean lecithin (L-a-phosphatidylcholine Type IV-S), 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{+}$), 6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Fluka Chemical Co. (Bushs, Switzerland). Thiobarbituric acid (TBA), trichloroacetic acid (TCA) and ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

Determination of total phenolic compounds

Total soluble phenolics in the methanol extract of *F.trojana* were determined with Folin-Ciocalteu reagent according to the method of (Slinkard and Singleton 1977) with some modifications. Aliquots (0.1 mL) of extracts were transferred into the test tubes and their volumes made up to 4.6 mL with distilled water. After addition of 0.1 mL Folin-Ciocalteu reagent (previously diluted 3-fold with distilled water) and 0.3 mL 2% Na_2CO_3 solution, tubes were vortexed and absorbance of mixture recorded after 2 h at 760 nm against a blank containing 0.1 mL of extraction solvent. Gallic acid (0.05 mg/mL–0.4 mg/mL) was used for calibration of a standard curve. The results were expressed as gallic acid equivalents (GAE)/g of extract. The data were presented as the average of triplicate analyses.

Determination of total flavonoid content

Total flavonoid content was determined by using a colorimetric method described by (Sakanaka et al. 2005). Briefly, 0.25 mL of the extract (0.625 mg/mL extract or (+)-catechin standard solution was mixed with 1.25 mL of distilled water or solvent in a test tube, followed by addition of 75 µL of a 5% (w/v) sodium nitrite solution. After 6 min, 150 µL of a 10% (w/v) $AlCl_3$ solution was added and the mixture was allowed to stand for a further 5 min before 0.5 mL of 1 M NaOH was added. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was measured immediately at 510 nm, using a spectrophotometer (Shimadzu UV-1208). (+)-Catechin standard solution (15–250 µL/mL) was used for the calibration of a standard curve. The results were expressed as means (± SD) mg of (+)-catechin equivalents per gram of extract.

Antioxidant activity

Antioxidant activity on liposome peroxidation

Lipid peroxidation assay was based on the method described by (Duh et al. 1999). Lecithin (300 mg) was suspended in 30 mL phosphate buffer (10 mmol/L, pH 7.4). This suspension was then sonicated with a rod using an ultrasonic homogenizer (Bandelin, Berlin, Germany) at 30 s intervals for 10 min until an opalescent suspension was obtained.

The sonicated solution (10 mg/mL), $FeCl_3$, ascorbic acid and the extracts (from 0.625 to 10 mg/mL) or quercetin (from 0.005 to 0.08 mg/mL) used as a reference antioxidant were mixed to produce a final concentration of 3.08 mg liposome/mL, 123.2 µmol $FeCl_3$ and 123.2 µmol ascorbic acid. After 1 h incubation at 37°C, the formation of lipid peroxidation products was assayed by the measurement of malondialdehyde (MDA) levels on the basis that MDA reacted with TBA at 532 nm according

to (Buege and Aust 1978). Briefly, 500 µL of this reaction mixture was mixed with 1000 µL TCA-TBA reagent (consisting of 15% w/v TCA and 0.375% TBA in 0.25 N HCl) and 14 µL BHT (2% in absolute ethanol). The mixture was vortexed and heated for 10 min in a boiling water bath. After cooling, an equal volume of n-butanol was added and the mixture was shaken vigorously. The n-butanol layer was separated by centrifugation at 3000 rpm for 5 min. The absorbance of the sample was read at 532 nm against a blank which contained all reagents except lecithin. The percentage inhibition of lipid peroxidation was calculated by comparing the results of the samples with those of controls not treated with the extract using the following equation: Inhibition effect (%) = [1-(Absorbance of sample at 532 nm/Absorbance of control at 532 nm)] x 100.

DPPH radical scavenging activity

The DPPH radical scavenging activity of the extract was measured according to the procedure described by (Brand-Williams et al. 1995). A 0.1 mL aliquot of extracts (from 0.16 to 15 mg/mL) or quercetin (from 0.01 to 0.16 mg/mL) in methanol was added to 3.9 mL of 6 x 10^{-5} M methanolic solution of DPPH. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The decrease in absorbance of the resulting solution was then measured spectrophotometrically at 517 nm against methanol. All measurements were made in triplicate and averaged. Two controls were used for this test, a negative control (containing all reagents except the test sample) and positive controls (using the reference antioxidants). The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = [1 - (Absorbance of sample at 517 nm/Absorbance of control at 517 nm)] x 100.

Total radical antioxidant potential (TRAP) assay

The total radical antioxidant potential of the extract was measured using the Trolox equivalent antioxidant coefficient (TEAC) assay as described by (Re et al. 1999) with minor modifications. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation was produced by reacting $ABTS^{+}$ stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 hours before use. At the begining of the analysis day, an $ABTS^{\bullet+}$ working solution was obtained by the dilution in 96 % ethanol of the stock solution to an absorbance of 0.70 (± 0.02) at 734 nm. After addition of 990 µL of $ABTS^{\bullet+}$ solution to 10 µL of the extracts (from 0.625 to 15 mg/mL) or quercetin (from 0.01 to 0.16 mg/mL) or trolox standards (final concentration 0 - 20 µM/l) in absolute ethanol the decrease in absorbance at 734 nm was monitored exactly 6 min after the initial mixing. Appropriate methanol blanks were run in each assay. All determinations were carried out in triplicate.

The ability to scavenge $ABTS^{+}$ radical was calculated by the following equation: $ABTS^{+}$ radical scavenging activity (%) = [1 - (Absorbance of sample at 734 nm/Absorbance of control at 734 nm)] x 100.

The total antioxidant capacity value in a sample was assessed as TEAC. The TEAC value was calculated by using a regression equation between the Trolox concentration and the percentage of inhibition of absorbance at 734 nm at 6 minutes of incubation and was expressed as mmol TEAC.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure of (Benzie and Strain 1996). The FRAP reagent contained 2.5 mL of 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3.6H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6. FRAP reagent (900 µL), prepared freshly and incubated at 37°C, was mixed with 90 µL of distilled water and 30 µL of the extracts (from 1.25 to 10 mg/mL) or quercetin (from 0.02 to 0.31 mg/mL) or water for the reagent blank. The increase in absorbance at 593 nm was measured at 4 min. The standard curve was constructed using iron sulfate heptahydrate solution (125–2000 µM), and the results were expressed as mM Fe^{2+} equivalents. All the measurements were taken in triplicate and the mean values were calculated.

Statistical Analysis

All measurements were made in triplicate. The results were statistically analyzed using GraphPad Prism version 7.00. Results were considered significant at $p<0.05$.

Antimicrobial activitiy

Antimicrobial activity against *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153, Methicillin-resistant (MRSA) ATCC 43300 and *Candida albicans* ATCC 10231 were determined by the microbroth dilutions technique the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI 2008; 2015). Mueller-Hinton broth for bacteria, RPMI-1640 medium buffered to PH 7.0 with MOPS for yeast strain were used as the test medium. Serial two-fold dilutions ranging from 5000 µg/mL to 4.9 µg/mL were prepared in medium. The inoculum was prepared using a 4-6h broth culture of each bacteria and 24h culture of yeast strains adjusted to a turbidity equivalent to a 0.5 Mc Farland standard, diluted in broth media to give a final concentration of $5x10^5$ cfu/mL for bacteria and $0.5x10^3$ to $2.5x10^3$ cfu/mL for yeast in the test tray. The trays were covered and placed in plastic bags to prevent evaporation. The trays containing Mueller-Hinton broth were incubated at 35 °C for18-20h and the trays containing RPMI-1640 medium were incubated at 35 °C for 46-50h. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of compound giving complete inhibition of visible growth. As control, antimicrobial effects of the solvents were investigated against test microorganisms. According to values of the controls, the results were evaluated.

RESULTS AND DISCUSSION

Antioxidant activity

It was reported that the oil, rhizomes, stems, leaves, and flowers of different *Ferulago* species contain phenolic compounds and show antioxidant activity (Azarbani et al. 2012; Mileski et al. 2015; Dikpınar 2017; Kiziltas et al. 2017).

The results given in Table 1 showed that the amount of extractable phenolic compounds and flavonoids in HFT extract is higher than that detected in RFT extract ($p<0.05$), so the aerial

parts of *Ferulago trojana* is a rich source of phenolics and flavonoids. A similar content of flavonoids was reported by (Kiziltas et al. 2017) for the flowers of *Ferulago angulata* (Schlecht.) Boiss. (Apiaceae).

The antioxidant activity was tested using four *in vitro* assays including, lipid peroxidation inhibition, scavenging effect on DPPH and ABTS radicals, and FRAP assays. For comparison, Table 2 presents the results of the antioxidant activities, expressed as EC_{50}, TEAC and FRAP values. As can be seen from the EC_{50} values, the methanol extract of aerial parts showed a higher scavenging effect on DPPH and ABTS radicals, and reducing power when compared to its capability to inhibit lipid peroxidation. TEAC value was similar to the FRAP value, which indicates that the extract is effective in donating of electrons. The aerial parts showed the better antioxidant activity than rhizomes in DPPH and FRAP assays. However, the results showed a weak antioxidant activity of both the extract compared to the referance antioxidant quercetin. Although the extract was less active than the quercetin ($p<0.05$), it was seen that it has hydrogen and a single electron donor activities, thus could serve as antioxidant.

These results are in accordance with previous studies, which reported the efficiacy of *Ferulago* species to scavenge free radicals (Azarbani et al. 2012; Mileski et al. 2015; Dikpınar 2017; Kiziltas et al. 2017). Antioxidant activity against lipid peroxidation (LPO) has been reported for the dichloromethane extract of *F. trojana*, which is attributed to the richness of the coumarin in the extract (Çakar, 2010). Çakar (2010) also reported that the pure compounds isolated from the *Ferulago trojana* (bergapten, isoimperatorin, 3'-epidecursin,

Table 1. Total phenolic compounds (PC) (as gallic acid equivalents) and total flavonoids (as catechin equivalents) in methanol extracts from *F. trojana*

Extract	PC (mg/g extract)	Flavonoids (mg/g extract)
HFT	64.49±4.47[a]	58.89±4.11[a]
RFT	6.51±0.82[b]	4.56±0.70[b]

HFT: Aerial parts of *F. trojana*
RFT: Rhizomes of *F. trojana*
Values were the means of three replicates ± standard deviation.
Values with different letters in the same column were significantly ($p<0.05$) different.

Table 2. EC_{50}, TEAC and FRAP values of methanol extracts from *F. trojana*

Extracts	Lipid peroxidation[a] EC_{50} (mg/mL)	DPPH[a] EC_{50} (mg/mL)	ABTS[a] EC_{50} (mg/mL)	Reducing power[a] EC_{50} (mg/mL)	Total Antioxidant potential[b]* (mM/L TEAC)	FRAP value[c]* (mM/L Fe^{2+})
HFT	3.01±0.045[a]	1.35±0.069[a]	1.79±0.22[a]	1.18±0.34[a]	2.15±0.005[a]	1.87±0.08[a]
RFT	N.d	16.69±0.34[b]	N.d.	10.73±0.54[b]	0.26±0.03[b]	0.27±0.03[b]
quercetin	0.034±0.006[b]	0.069±0.001[f]	0.113±0.002[d]	0.019±0.003[e]	2.15±2.42[a] (at 0.16 mg/mL)	2.15±0.011[a] (at 0.16 mg/mL)

HFT: Aerial parts of *F. trojana*; RFT: Rhizomes of *Etrojana*
[a] EC_{50} value: The effective concentration at which the antioxidant activity was 50%; DPPH and ABTS radicals were scavenged by 50% and the absorbance was 0.5 for reducing power. EC_{50} value was obtained by interpolation from linear regression analysis.
[b] Expressed as mmol Trolox equivalents per gram of dry weight
[c] Expressed as mmol ferrous ions eqivalents per gram of dry weight
* - Determined at 5 mg/mL
N.d. Not determined
Values were the means of three replicates ± standard deviation

Table 3. Antimicrobial activities (MIC in mg/L) of methanol extracts from *F. trojana*

Extracts/ Pozitive control (mg/L)	*Staphylococcus aureus* ATCC 6538	Methicillin-resistant Staphylococcus aureus ATCC 33591	*Staphylococcus epidermidis* ATCC 12228	*Escherichia coli* ATCC 8739	*Klebsiella pneumoniae* ATCC 4352	*Pseudomonas aeruginosa* ATCC 1539	*Proteus mirabilis* ATCC 14153	*Candida albicans* ATCC 10231
HFT	4.8	48	78	-	-	-	-	-
RFT	78	19	625	-	-	-	156	312
Cefuroxime- Na	1.2	nt	9.8	4.9	4.9	nt	2.4	nt
Vancomycin	nt	2	nt	nt	nt	nt	nt	nt
Ceftazidime	nt	nt	nt	nt	nt	2.4	nt	nt
Klotrimazole	nt	nt	nt	nt	nt	nt	nt	4.9

MIC: miniumum inhibitory concentration; HFT: Aerial parts of *Etrojana*; RFT: Rhizomes of *Etrojana*
(–): Not active
(nt): Not tested

isomaltol, isomaltol-3β-O-glucoside and 3,6-dimethoxy-7-isopropilcoumarin-4- tetradeca-13"-one) showed antioxidant activity against lipid peroxidation investigated in a β-carotene-linoleic acid model system, but do not have free radical scavenging ability.

Antimicrobial activitiy

The antimicrobial activity of *F. trojana* has been studied for the first time. The antimicrobial activity results of methanol extracts prepared from aerial parts (HFT) and rhizomes (RFT) of *F. trojana* are shown in Table 3. In this study, both methanol extracts from aerial parts and rhizomes of *F. trojana* showed antibacterial activity against Gram positive bacteria such as *S. aureus,* MRSA, *S. epidermidis* while no activity was observed against *E. coli, K. pneumoniae* and *P. aeruginosa* for any of the extracts. Methanol extract from rhizomes of *F. trojana* showed antibacterial activity against *P. mirabilis* and antifungal activity the yeast *C. albicans*. When the results of the antimicrobial activity were evaluated, it was found that the RFT extract showed moderate antimicrobial activity against 4 bacteria and 1 fungal strain while the HFT extract showed activity against three bacterial strains. When compared with positive control results, the best activity was the HFT extract against the *S. aureus* strain with an MIC value of 4.8 mg/L; *S. epidermidis* with 78 mg/L. It is planned to identify the active compounds and to exhibit antimicrobial activity of the HFT extract.

CONCLUSION

It was concluded that methanol extract from the aerial parts of the plant, containing the highest amount of total phenolics and flavonoids, has the antioxidative potential for chain-breaking inhibition of lipid peroxidation and shows the strongest hydrogen and a single electron donor activities, thus could serves as free radical scavenger, acts as reductant and provide protection against oxidative stress. Although the methanol extract from rhizomes did not show any inhibitory effect on lipid peroxidation, it may also be expected to protect against oxidative damage by scavenging free radicals and acting as reductant. The results demonstrated the health promoting potential of aerial parts from *F. trojana*. On the other hand, because of the high antimicrobial activity of the plant, the aerial parts of the plant and its rhizomes could be source of antimicrobial effective new molecules.

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Original Article

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Botanical origin and antioxidant activities of propolis from the Irano-Turanian region

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ABSTRACT

Propolis is a natural bioactive mix and a traditional medicine that has been used for treating several complications. The bioactive properties of propolis are dependent on its botanical origin. This study investigated the pollen composition, antioxidant activities, and the total phenol and total flavonoid content of a propolis sample from the Refahiye (Erzincan, Turkey) region. Melissopalynological analysis conducted according to the relevant literature revealed that the pollen profile of the sample primarily indicated the presence of the Fabaceae (38.4%), Asteraceae (20.2%), and Fagaceae (11.2%) families. The antioxidant ability of propolis extract was analyzed by the hydrogen peroxide scavenging activity (HPSA) (in terms of SC50), ferric reducing antioxidant power capacity (FRAP) (%), DPPH radical scavenging activity (in terms of SC50), metal-chelating activity (%), total phenol content (TPC), and total flavonoid content (TFC), which showed the following values: 11.72±0.04 µg/mL, 90.73% ± 0.24%, 18.34±0.08 µg/mL, 89.69% ± 0.12%, 10673.4±3.30 mg GAE/100 g of propolis sample (PS), and 170.65±1.12 mg QE/100 g of PS, respectively. These results were compared using butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol (TOC) as standard antioxidant compounds. The high biological activity of propolis from the Refahiye region could be attributed to its rich pollen composition. These results indicate that propolis is an important source in terms of its antioxidant activities.

Keywords: Bioactive properties, melissopalynological analysis, propolis, Refahiye (Erzincan)

INTRODUCTION

Propolis (bee glue) is a sticky dark-colored material that is collected from honeybee plants (buds and leaves), which is mixed with pollen as well as enzymes secreted by bees (Marcucci 1995). The term "propolis" has been derived from two Greek words, i.e., "pro" stands for "at the entrance to" and polis for "community" or "city" (Wagh 2013). Bees use propolis in their hives as a protection against predators and microorganisms, to repair damage, as a thermal isolator, and to build aseptic locals to prevent microbial infection of larvae (Bankova et al. 2000; Huang et al. 2014). Propolis has a wide spectrum of biological activities and has been used for various purposes by the people. Several studies have investigated the antibacterial (Sforcin et al. 2000; Hegazi and Abd El Hady 2001), antifungal (Ota et al. 2001; Herrera et al. 2010), anti-inflammatory (Borrelli et al. 2002), anticancer (Sawicka et al. 2012), antioxidant (Perveen and Qaiser 2007; Kalogeropoulos et al. 2009; Silva et al. 2013), and antitumor (Oršolić & Bašić 2003; Sobočanec et al. 2011) properties of propolis. The chemical ingredients of propolis have been reported to be highly variable and dependent on the native flora (Bankova et al. 2000; Kumazawa et al. 2004; Silva et al. 2008). Turkey has a great diversity of plants comprising more than 10,000 taxa with 173 families and about 2,650 endemic species (Davis 1965-1985; Özhatay 2013). However, propolis production has been generally ignored in Turkey, where several beekeepers focus on only the production of honey. The aim of the present study was to determine the quality of a propolis sample from the Irano-Turanian phytogeographic region of Turkey in terms of its antioxidant activity and botanical origin.

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MATERIALS AND METHODS

Reagents and standards

All the following reagents used were of proanalysis grade: 2,2-diphenyl-1-picrylhydrazyl, butylated hydroxyanisole, butylated hydroxytoluene, gallic acid, quercetin, α-tocopherol (Sigma), Folin–Ciocalteu reagent, and absolute ethyl alcohol (Merck). All other chemicals were of analytical grade.

Sample collection

Propolis sample was collected from the East Anatolia Region of Turkey, which covers the Irano-Turanian floral region (Davis 1965-1985).

Sample solution

The sample solution was prepared by mixing 1.33 g propolis with 100 mL absolute ethanol. This suspension was shaken at room temperature on a magnetic stirrer for 24 h. Then, the extract solution was filtered through a Whatman no. 4 filter paper and stored at -4°C.

Palynological identification

The study material was prepared for examination under the microscope according to the method of Warakomska and Maciejewic (1992). The sample was ground into powder, mixed with ethanol-ether-acetone (1:1:1), and then shaken. This mixture was filtered through a strainer with 0.3-mm holes. The suspension was then centrifuged at 3500–4000 rpm for 20 min, after which the supernatant was discarded. Then, using the residual sediment, two slides were prepared for each sample using basic fuchsin glycerin gelatin and were examined simultaneously for determining the pollen count.

The identification of the stages of pollen grains was performed using an optical microscope (Nikon Eclipse Ci, Japan) at 400× and 1000× magnifications.

Antioxidant analyses

Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The DPPH radical scavenging activity was determined according to a previously reported method of Blois (1958) with few modifications. Serially diluted samples (3.0 mL) at different concentrations (10–100 µg/mL) were added to DPPH solutions (1.0 mL, 0.2 mM) with ethanol. The mixtures were vigorously shaken and allowed to stand at room temperature for 30 min. Then, the absorbance was recorded at 517 nm using a spectrophotometer, and the results were expressed as SC_{50} (the concentration required for scavenging 50% of DPPH) (µg/mL) by a linear regression analysis and represented as mean of the data.

Determination of hydrogen peroxide scavenging activity (HPSA)

The HPSA was determined according to the method described by Ruch et al. (1989). Briefly, the samples were dissolved in 0.04 M phosphate buffer (pH=7.4) and 3.4 mL of the sample was mixed with 0.6 mL of 40 mM H_2O_2 solution (prepared using the same buffer). The absorbance of the mixture was measured at 230 nm versus the blind sample after 10 min using a UV/VIS spectrophotometer. Phosphate buffer without hydrogen peroxide was used as blank. A decrease in the absorbance value indicated a high level of hydrogen peroxide scavenging activity. The results were expressed as SC_{50} values (µg/mL).

Ferric reducing antioxidant power (FRAP) assay

The reducing ability of the sample was investigated following a method using a ferric ion, with minor modifications (Güder et al. 2014). About 2.0 mL of the sample or standards was mixed with PBS (phosphate-buffered saline) (2.0 mL, 0.2 mol L^{-1}, pH 6.6) and potassium ferricyanide (2.0 mL, 1.0%). This mixture was incubated at 50°C for 20 min, followed by the addition of trichloroacetic acid (2.0 mL, 10%). Then, 2.0 mL of this solution was mixed with distilled water (2.0 mL) and $FeCl_3$ (0.5 mL, 0.1%). The Fe^{3+}/Fe^{2+} transformation was determined due to the presence of samples at 700 nm.

$$FRAP\ (\%) = (A_s/A_c) \times 100$$

Where, A_c is the absorbance of the control, and A_s is the absorbance of the sample or standards.

Determination of metal-chelating activity

The metal-chelating activities of the propolis extract and the standard antioxidant materials were estimated according to the method described by Dinis et al. (1994). Briefly, 0.05 mL of 2 mM $FeCl_2$ and 0.4 mL of the extract solution were mixed. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine solution. This mixture was vigorously shaken and kept at room temperature for 10 min, after which the absorbance of the mixture was measured at 562 nm using a UV/VIS spectrophotometer. A decrease in the absorbance value demonstrated a high level of metal-chelating activities of the extract solution and the standard antioxidant materials. The metal-chelating activities of the extract solution and the standard antioxidant material were calculated according to the following formula:

$$\text{Ferrous ion chelating activity } (\%) = [1 - (A_s/A_c)] \times 100$$

Where, A_c is the absorbance value of the control, and A_s is the absorbance value of the extract solution or the standard antioxidant material

Determination of total flavonoid content (TFC)

The TFC of the extracts was determined according to the colorimetric method described by Chung (2002) with minor modifications. Sample solutions (0.5 mL) were added to a tube containing 1.5 mL of absolute ethanol. $AlCl_3.6H_2O$ solution (0.1 mL, 10.0%) and potassium acetate (0.1 mL, 1.0 mol L^{-1}) were subsequently added to prepare the mixture. Distilled water was added to make up the total volume to 5.0 mL, and then the absorbance was read after 30 min at 415 nm. The TFC values were expressed as microgram of quercetin equivalent that was obtained from the standard graph ($R^2 = 0.9979$).

Determination of total phenolic content (TPC)

The TPC of the samples was determined by the Folin–Ciocalteu phenol reagent (Folin C) colorimetric method described by Slinkard and Singleton (1977). The sample solutions (0.5 mL) were mixed with 7.0 mL of distilled water and subsequently with Folin C reagent (0.5 mL). After 3 min, Na_2CO_3 solution (3.0

mL, 2.0%) was added to the mixture. The color developed after 1 h, and then the absorbance was measured at 760 nm using a spectrophotometer. Gallic acid was used as the standard, and TPC was expressed as microgram of gallic acid equivalent using an equation that was obtained from the standard gallic acid graph ($R^2 = 0.9995$).

RESULTS AND DISCUSSION

All the 18 pollen types (Table 1) belonging to 13 families were identified in the propolis sample. The identified pollen samples generally belonged to the Fabaceae (38.4%), Asteraceae (20.2%), and Fagaceae (11.2%) families (Figure 1). The pollen spectra of the sample were found to overlap with those of the Refahiye vegetation. Gençay and Sorkun (2006) stated that 32 different plant families have been identified by the pollen analysis of 30 propolis samples from Kemaliye (Erzincan), and Apiaceae, Asteraceae, Campanulaceae, Fabaceae, Fagaceae, Lamiaceae, Liliaceae, Pinaceae, Rosaceae, Salicaceae, Rhamnaceae, and Scrophulariaceae families were primarily determined as the botanical origins of propolis. Çelemli and Sorkun (2012) analyzed the pollen spectra of 92 propolis samples collected from Tekirdağ and reported that the frequently observed pollen grains belonged to the Asteraceae, Boraginaceae, Brassicaceae, Fabaceae, and Salicaceae families. These results are consistent with those of our study.

The HPSA, FRAP, DPPH radical scavenging activity, metal-chelating activity, TPC and TFC values are as follows: 11.72±0.04 µg/mL, 90.73% ± 0.24%, 18.34±0.08 µg/mL, 89.69% ± 0.12%, 10673.4±3.30 mg GAE/100 g of PS, and 170.65±1.12 mg QE/100 g of PS, respectively. These results were compared using butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol (TOC) as standard antioxidant compounds. The obtained results of the standards are presented in Table 2. Moreira et al. (2008) reported the DPPH radical scavenging activities of two propolis samples (Bornes and Fundão) as 6±3 and 52±3 µg/mL, respectively. In addition, they reported the TPC of the same samples as 32900 and 15100 mg GAE/100 g, respectively. Based on these literature data, our results showed an average DPPH radical scavenging activity but a lower TPC.

Popova et al. (2005) investigated the TPC of Turkish propolis samples (from Adana, Artvin, Erzurum, İzmir, Kayseri, and Yozgat) and reported TPC values of 8.2%–30.4%. In the Irano-Turanian samples (Erzurum, Kayseri, and Yozgat), the TPC values were 10.5%, 27.5%, and 26.4% respectively. Our results were found to be consistent with these literature data.

Lima et al. (2009) reported the TPC values of the methanolic propolis extract as 25700–39300 mg GAE/100 g. In addition, the TFC was found to be between 6600 and 13300 mg QE/100 g. Wali et al. (2015) analyzed the propolis samples collected from the Kashmir–Himalayan region using different extraction solvents (ethanol, water–ethanol, and water) and reported TPC and TFC values of 18000–26000 mg GAE/100 g and 4500–10500 mg QE/100 g, respectively. Ahn et al. (2007) studied the propolis samples collected from different parts of China and reported that the TPC values ranged from 42.9±0.8 to 302±4.3 mg GAE/g of samples and the TFC values ranged from 8.3±3.7 to 188±6.6 mg QE/g of samples. Choi et al. (2006) found the TPC value of Korean propolis samples collected from Yeosu to be 212.7±7.4 mg GAE/g, and Kumazawa et al. (2004) reported that the TPC and TFC values of propolis ranged from 31.2±0.7 to 299±0.5 mg GAE/g and from 2.5±0.8 to 176±1.7 mg QE/g, respectively, collected from different geographic regions. Laskar et al. (2010) showed that the TPC and TFC values ranged from 159.10±0.26 to 269.10±0.17 mg GAE/g and from 57.25±0.24 to 25.50±0.36 mg QE/g, respectively, in Indian propolis samples. The TPC and TFC values are comparable with the literature data because of the average contents. Furthermore, the DPPH radical scavenging activity and the HPSA were found to be 18.34 and 11.72 µg/mL, respectively. Gülçin et al. (2010) have also reported the DPPH radical scavenging activity and the HPSA of the lyophilized aqueous extract of propolis collected from

Table 1. Pollen types identified from Refahiye propolis

Taxa/Family	%	Taxa/Family	%
Apiaceae	1.2	Fagaceae	
Asteraceae		*Quercus*	11.2
Echinate type	9.2	Lamiaceae	2.6
Scabrate type	1.2	*Thymus*	1
Xanthium	2	*Phlomis*	0.6
Taraxacum	1.8	Rosaceae	1.8
Carthamus	4.2	*Sanguisorba*	0.6
Centaurea	1.8	Salicaceae	
Betulaceae		Salix	5
Betula	0.4	*Populus*	4
Boraginaceae	8.8	Pinaceae	
Onosma	0.6	*Pinus*	0.2
Brassicaceae	0.6	Poaceae	0.6
Campanulaceae	1	Unidentified	1.2
Fabaceae	20.2		
Astragalus	10.2		
Onobrychis	8		

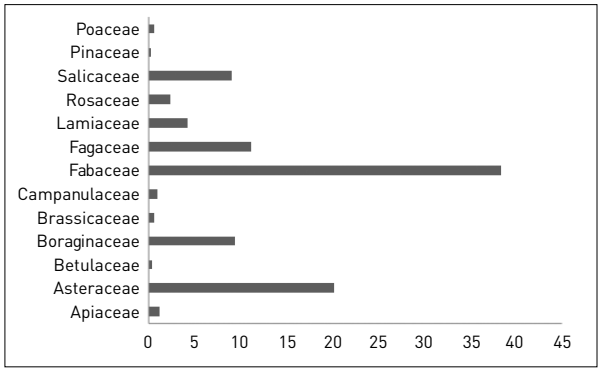

Figure 1. Distribution of families of which the pollens were identified from Refahiye propolis

Table 2. Antioxidant test results of the sample and standards

	HPSA[1]	FRAP[2]	DPPH[1]	TPC[3]	TFC[4]	MCA[2]
Propolis	11.72±0.04	90.73±0.24	18.34±0.08	10673.4±3.30	170.65±1.12	89.69±0.12
BHA	184.13±1.19	92.02±0.80	8.53±0.39	-	-	89.95±0.05
BHT	147.49±0.09	54.16±0.10	9.01±0.02	-	-	86.26±0.15
TOC	216.26±0.47	32.98±0.21	11.97±0.07	-	-	93.41±0.06

[1] SC_{50} (µg/mL)
[2] % activity
[3] mg GAE/100 g of PS
[4] mg QE/100 g of PS
BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene; TOC: α-tocopherol

Erzurum as 31.81 and 6.54 µg/mL in terms of IC_{50} after analyzing the polyphenol contents and the antioxidant activity. Our sample showed a higher DPPH radical scavenging activity than that of the Erzurum sample, but lower HPSA. Moreira et al. (2008) studied two Portugal propolis samples collected from different regions and determined the DPPH radical scavenging activities to be 0.006 and 0.052 mg/mL, respectively (in terms of EC_{50}). Laskar et al. (2010) determined the DPPH radical scavenging activities of propolis in terms of IC_{50} values to be 0.05–0.07 mg/mL. The DPPH radical scavenging activities of Brazil propolis was found to be 3.17–8.79 mg/mL (Pontis et al. 2014). The DPPH radical scavenging activity of our sample was the highest among all the literature samples, except the Portugal propolis sample. Gülçin et al. (2010) found the FRAP activity to be 0.568 (absorbance value at 700 nm). Compare this value with our result, it was very lower than that of our sample. The metal-chelating activity of propolis was determined as 89.69%, and those of the standard compounds (BHA, BHT, and TOC) were found to be 89.95%, 86.26%, and 93.41%, respectively. Gülçin et al. (2010) calculated the metal-chelating activity using EDTA as a reference standard and reported a value of 12.04 µg/mL of Fe^{+2}-chelating activity for the Erzurum propolis sample, which was lower than those of the standard compounds (BHA, BHT, and TOC). However, our sample showed a similar activity as those of the standard compounds, especially BHA. Geckil et al. (2005) determined the metal-chelating activities of different extracts of Malatya propolis and reported values of 56%–70%. Geckil et al. (2005) also reported lower metal-chelating activities of propolis samples than those of the standard compounds (BHA and BHT). Subsequently, the Erzincan propolis sample demonstrated effective chelating activity than that by the Erzurum propolis sample. Therefore, the results of this study show that our propolis samples exhibited highly effective antioxidant activities.

CONCLUSION

This study showed that the propolis sample collected from the Refahiye (Erzincan, Turkey) region has an average antioxidant activity in comparison with the literature data. Therefore, it can be used as a natural source in the medicine and food industry. Especially, the active components in the propolis sample can be isolated and characterized. In this context, these active components can be used as potential treatment agents for certain diseases.

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Review

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Targeted drug delivery and vaccinology approaches using virus-like particles for cancer

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ABSTRACT

Nanotechnology has the potential to make significant alterations in the treatment of diseases such as cancer through targeted drug delivery nanoparticles. Virus-like particles (VLPs) are composed of the capsid proteins that do not carry the viral genome and are also noninfectious. VLPs are self-assembling competent protein structures with identical or highly related structures to their corresponding native viruses. VLPs that have precise 3D nanostructures exhibit a notable diversity in shapes and structures. They can be produced in large quantities through biological amplification and growth. External protein inserts can be displayed through genetic methods or chemical modifications. Functionalized VLPs when used as delivery systems have the ability to target with specificity and can attract macrophages for the destruction of cancer cells. The capability to target tumors for the delivery of therapeutic agents is an important goal of the design approaches of VLPs. Against the current problems in cancer therapies, delivery systems using VLPs are an arising and promising field with the potential to exhibit solutions. Cancer therapies require specific targeting of the diagnostic element or the drug to tumor cells without binding to or affecting healthy cells and tissues. Specialization of the VLPs provides an opportunity for using them as site-specific drug delivery systems in cancer therapy while reducing the systemic toxicity and the overall damage to healthy cells. With fewer side effects, immunotherapy is also a promising alternative for cancer treatment by primarily activating the host's immune system. Cancer vaccines are aimed at inducing an immune response in the host, thereby generating a defensive mechanism against tumor cells. VLPs can be used as a vaccine without the requirement of any adjuvant due to their naturally optimized particle size and their repetitive structural order. Therefore, the aim of this review is to provide basic information about VLPs and describe previous research on VLPs used as drug and vaccine delivery systems and their applications in different types of cancer.

Keywords: Nanocarriers, virus-like particles, drug delivery, cancer vaccine, cancer therapy

INTRODUCTION

Virus-like particles (VLPs) are constituted of one or more viral proteins which are self-assembling particles expressed in vitro through recombinant technologies. The self-assembling feature of these proteins results in the generation of subviral or viral particles in the 20–100 nm size range, typically (Ghasparian et al. 2011). The high stability of VLPs, their symmetric spatial organization, ease of obtaining and purification, safety, and the possibility of directed modification make these nanoparticles ideal carriers of drugs, biologically active peptides and whole proteins (antigens, receptors, enzymes, etc.) (Blokhina et al. 2013).

Previously, VLPs have been produced for over thirty different infectious viruses in humans and animals. VLPs contain one or more structural proteins and they have features such as self-assembly. Morphological properties of VLPs are similar to native viruses. Due to being lack of infectious genetic material, VLPs are not amenable to replication and being infective, unlike native viruses. The general features of VLPs are summarized in Figure 1 as a scheme. In case of usage in vaccines, virus-like particles require no adjuvant as a safe vaccine candidate. To produce virus-like particles, different viruses offer different structures. For example, parvoviruses have viral capsids containing one or two major proteins are simple while viral capsids of picornaviruses are complex

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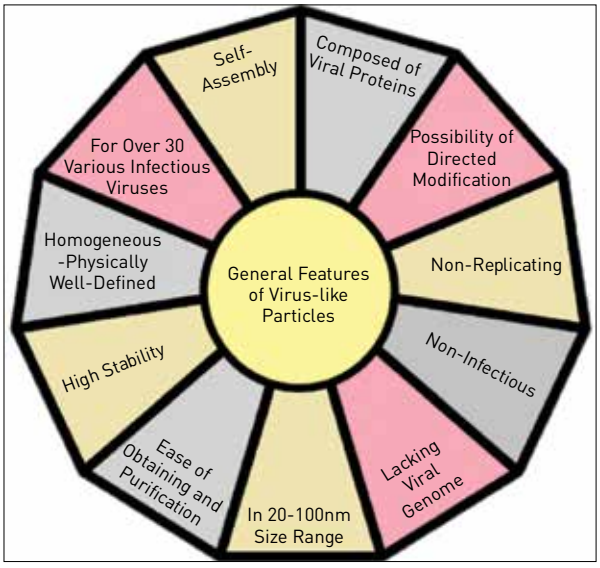

Figure 1. General features of virus-like particles as hollow nanocages

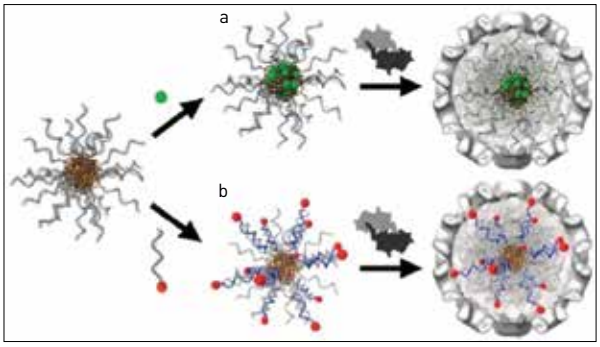

Figure 2.. DNA micelle-templated virus-like particle formations. Green shows hydrophobic molecules loaded into the core of the micelle in (a). Red shows hydrophilic molecules attached to complementary DNA strands by hybridization and equipping the micelles in (b) (Ma et al. 2012)

and these complex viral capsids showed diversity in protein layers which are encoded by many different mRNAs or viral capsid of picornaviruses is from a single polyprotein. Viral capsids of some viruses' such as influenza, HIV, and hepatitis C are acquired from the host cell, and also viral glycoprotein spikes. They have lipid bilayer and lipid envelopes (Shirbaghaee and Bolhassani 2016).

VLPs have definite 3D nanostructures and they show a diversity of shapes and structures. By using biological amplification and growth, production of VLPs can be in great amounts. They can display external protein inserts through modifications/techniques as genetically or chemically. The feature of being selective about the deposition of organic or inorganic materials, at specific locations on the VLP provides fine adjustment controlling of the assembly of nanomaterial, size and spacing that results in uniform and reproducible nanoarchitectures (Zhou et al. 2014).

Some VLPs have proved more immunogenic than recombinant protein immunogens vaccines, and are capable to induce both the humoral and cellular response of the immune system. VLPs structurally mimic their parent virus thus they could be used as a safe alternative for inactivated virus vaccines. Additionally, binding to pattern-recognition receptors and B-cell receptors of VLPs can induce direct immune system. Due to their naturally-optimized particle size and repetitive structural order, VLPs can be used as a vaccine without any adjuvant requirement. Immunogenicity of the VLPs also generates great potential to use them for immune therapy, targeted drug delivery, and gene therapy, aside from their applications in vaccination (Teunissen et al. 2013; Smith et al. 2015).

VLPs have biocompatible and monodisperse properties, also are capable of scale-up production and they are amenable to multiple functionalization strategies. These properties made VLPs advantageous to be used as delivery system. Beside chemical engineering, affinity tags or targeting peptides can be introduced by genetic engineering.As an example, Figure 2 represents DNA micelle templated VLP formation (Ma et al. 2012).

Modifications of VLPs

In some cases, VLPs could be modified chemically and genetically in order to gain extra functionalities. Drugs, epitopes, peptide fragments have been added to the surface of the particle by genetic engineering to use VLPs as nanocarriers. Disassembly and reassembly features of some VLPs provide a crucial way for encapsulation of drugs, peptide fragments, genome components. These unique characteristics give many advantages in comparison with synthetic nanoparticles (Yildiz et al. 2011).

Both the outer and inner surfaces of the VLPs can be functionalized by modification of the protein subunits genetically or chemically. Such modifications can cause considerable changes in the physicochemical properties and offer novel functions for VLPs (Smith et al. 2015). VLPs' inner surface can be modified to increase the loading efficiency and new affinity properties are acquired for a specific size of the foreign molecules. Shen et al. (2015) have demonstrated a method for encapsulation of magnetic nanoparticles into VLPs by hypothesizing a mutant HBc144 VLPs, which is produced by interpolation of six histidine residues (Histag) at the N-terminal ends and showed at the inner surface of the VLPs. It depends on the affinity of histidine tags to metal-ion complexes, such as nickel-nitrilotriacetic acid (nickel-NTA) chelate. By this method, the magnetic core containing VLPs were obtained at high efficiency with the properties of uniformly sized and monodispersed and the cellular uptake ability of Fe_3O_4-NTA-Ni^{2+} core-containing HBc-144-His VLPs was higher than pure Fe_3O_4 nanoparticles. Core size strong affects the efficiency of the VLPs encapsulation and correlation between the T-number (Triangulation number, facets per triangular face of an icosahedron) and the self-assembly efficiency could be the reason of this effect. It is concluded that 20 nm is the maximum size limit for VLP formation because VLP formation with large cores are failed. In case of exceeding this limit, it prevents obtaining stable particles. As a crucial result of this study, these core-containing VLPs have been shown to be very effective cellular T2 contrast agents for MRI applications (Shen et al. 2015).

Cancer Drug Delivery with VLPs

Virus-like nanoparticles are promising technologies as a new delivery platform. Their various features render them a convenient and potential candidate for targeted delivery of nucleic acids, peptide fragments, and therapeutic drugs within the protein structure. They have hollow structures which are composed of self-assembling protein subunits and considered as protein cages. Protein nanocages are often produced in living hosts. To precisely control over particle stability, drug encapsulation, surface charge and ligand display, functional elements can be applied as internal, external, and inter-subunit by performing protein engineering techniques (Molino and Wang 2014).

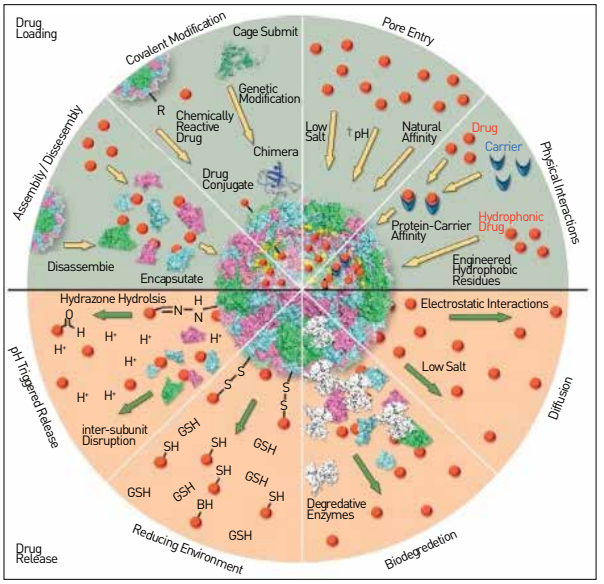

Figure 3. Methods that were used for drug loading (upper panel) and methods that were used for drug release (lower panel) in VLPs. R: any reactive amino acid side chain; GSH: glutathione. Cowpea chlorotic mottle virus is used as the representative scaffold. (Molino and Wang 2014)

Some VLPs are more capable in case of using as a nanocarrier. For example, Bacteriophage MS2 VLPs possess many properties as a delivery platform. These particles can be obtained in a very easy way by the recombinant-protein technology. On the other hand, the MS2 capsid sympathizes with the pac site of DNA or RNA and can encapsulate the target RNA or DNA by locating at the five terminus of the pac site. This inhibits degradation risks of the target RNA or DNA by nucleases. Antigenic components, epitopes can be delivered by designed MS2 VLPs for clinical approach MS2CP gene has a specific site to insert DNA oligonucleotide and the epitope peptide can be presented to the immune system after the expression. In addition, VLPs can display viral antigens on their surface of the capsid, and stimulate a higher immunologic response. Major advantages of MS2 VLPs are their great stability and appropriate size for presenting of viral antigenic epitopes. Moreover, MS2 VLPs can also be used as potential nanocarriers for targeted and passive drug delivery. Drugs can be packaged to MS2CP, MS2 during the self-assembly step (Fua and Li 2016).

The present strategies for a particulate system are defined by dynamics, the type of drug that is loaded, its structure and the environment that the nanoparticle is targeted. The strategies are described in Figure 3 (Molino and Wang 2014).

In cancer treatment, tumour-targeted drug delivery is an attractive strategy. The various VLPs delivery systems which are investigated before in treatment of cancer were shown in Table 1. For different types of cancer drugs, different VLPs systems were developed such as MS2 bacteriophage to deliver 5- fluorouracil and doxorubicin; and murine polyomavirus for methotrexate. (Ashley et al., 2011; Abbing et al., 2004) To deliver Paclitaxel, a JC polyomavirus VLP system was also synthesized which encapsulates the coupled drug with cyclo-

Table 1. Summary of various VLPs, which have been investigated as delivery platforms for cancer therapeutics

Drug	Virus-Like Particle	Target of Drug	References
Paclitaxel	Adenovirus JC polyomavirus	AIDS-related Kaposi sarcoma, breast cancer, and ovarian cancer	Shan et al. 2012 Niikura et al. 2013
Bleomycin	Adenovirus	Lymphoma, penile cancer, squamous cell carcinoma of the cervix, head-neck, and vulva, testicular cancer	Zochowska et al. 2009
5- Fluorouracil	MS2 bacteriophage Avian sarcoma leukosis virus	Some skin cancers, head and neck cancers, breast cancer, stomach cancer, anal cancer, colon cancer	Ashley et al. 2011 Kaczmarczyk et al. 2011
Doxorubicin	Cowpea mosaic virus Cucumber mosaic virus Hibiscus ringspot virus MS2 bacteriophage Red clover necrotic mosaic virus	Leukemia, lymphoma, neuroblastoma, sarcoma, Wilms tumour, and cancers of the breast, lung, thyroid, stomach, ovary, and bladder	Aljabali et al. 2013 Zeng et al. 2013 Ren et al. 2007 Ashley et al. 2011 Lockney et al. 2011
siRNA	Hepatit B virus JC polyomavirus MS2 bacteriophage	By disease-related gene suppression- specifically inhibit oncogene overexpression or gene mutation	Choi et al. 2013 Chou et al. 2010 Galaway&Stockley 2013
Methotrexate	Murine polyomavirus	Certain types of cancer of the lung, breast, head-neck, or skin	Abbing et al. 2004

dextrins as hydrophobic pockets through disulfide bonds inside the VLPs and has capable of glutathione (GSH)- triggered release of drug molecules. (Niikura et al., 2013). In a previous study, Shan et al. explained that modified adenovirus can be strongly targeted to tumour and has less toxicity effect to normal tissues. In a further study, commonly used clinical anticancer drug, Paclitaxel was conjugated to folate-modified adenovirus nanoparticles by using linkers to form two prodrugs. The results showed that the targeting and residence time of Paclitaxel can be improved by Paclitaxel-conjugated vector in tumour site. In vitro and in vivo studies show that Coxsackie adenovirus receptor or foliate receptor-mediated uptake of Paclitaxel induced highly anti-tumour activity. The results showed that chemically modified adenovirus vector has the potential to be used as a drug-loaded tumour-targeting delivery system (Shan et al. 2012). In chemotherapeutic approaches, inactive prodrugs together with enzymes have been widely used. These combinations convert the prodrugs to an active form. In another study, 5-Fluorocytosine has been introduced as a prodrug. Fcy protein converts 5-fluorocytosine into a highly cytotoxic compound mostly used in cancer chemotherapy, which is 5-fluorouracil. Then, Fur protein converts 5-FU to 5-F UMP, which blocks DNA synthesis. Yet this approach depends on expression of the enzymes required to convert the prodrug into the cytotoxic component. This problem was tried to solve by generating VLPs that composed of Gag-Fcy-Fur fusion for the delivery of enzymes that could be used for prodrug-to-active drug conversion (Kaczmarczyk et al. 2011).

Bleomycin is an anticancer antibiotic and has systemic toxicity and dose-dependent pneumonitis able to progress to lung fibrosis although its usage for wide range of cancer types such as lymphoma, penile cancer and testicular cancer. To enhance bleomycin delivery, adenovirus VLP vector was used by Zochowska et al. (Zochowska et al. 2009). Doxorubicin, which damages DNA and may kill cancer cells and cure many types of cancer such as lymphoma, leukemia and breast cancer, was encapsulated into different VLPs types (Aljabali et al. 2013; Ren et al. 2007; Ashley et al. 2011; Lockney et al. 2011). For example, encapsulated doxorubicin into cucumber mosaic virus was targeted to folate-expressing cancer cells in vivo. The results showed that cardiotoxicity was reduced and antitumour responses were increased, compared to free drug (Zeng et al. 2013).

Another treatment method for cancer is to silence gene expression by RNA interference (RNAi) however delivering sequences of RNAi in vivo remains a problem. To overcome this problem JC virus VLPs has been used as a vector for delivering RNAi in silencing the cytokine gene of IL-10 which resulted in reducing IL-10 expression by 85 to 89%, when compared with VLPs alone. (Chou et al. 2010) Galaway&Stockley also showed that VLPs reassembled in vitro with the RNA bacteriophage MS2 coat protein and an RNA conjugate encompassing a siRNA and a known capsid assembly signal can be targeted to HeLa cells by protecting from nuclease. (Galaway&Stockley, 2013).

Nanovaccinology with VLPs against Cancer

The use of nanotechnology in vaccinology, in particular, has been increasing exponentially in the past decade, leading to the birth of "nanovaccinology". In therapeutic approaches, nanoparticles are used as either a delivery system to improve antigen processing or as an immunostimulant adjuvant to activate or increase immunity. Therapeutic nanovaccinology is mostly applied for cancer treatment and is increasingly explored to treat other diseases or conditions, such as Alzheimer's, hypertension, and nicotine addiction (Zhao et al. 2014). The self-assembly of the viral particle needs a single viral capsid protein To make VLP vaccines. Mostly, production of VLPs are based on insect and yeast cell-based systems due to their advantageous for commercial vaccine manufacturing, ease of production and ability to produce complex viral protein targets (Rosenthal et al. 2014). Moreover, VLP vaccines do not need a chemical treatment step for inactivation which could affect the structure of the antigen epitopes of the surface glycoproteins conformationally (Matassov et al. 2007).

Several viruses are linked to cancer in humans. In the year 2002, 1.9 million cases that represent 17.8% of the global cancer casesare estimated as cancer associated with viral infections (Parkin 2006). Viruses can play roles at different stages of the cancer development, and the association of a virus with a given cancer can occur anywhere from 15 to 100%. It is thought that 15 and 20% of all human cancers may have a viral cause (Parkin 2006; zur Hausen 2001). Preventing cancer via vaccination became very popular starting in the late 1980s. Human vaccines against human papillomavirus, hepatitis E virus and hepatitis B virus use recombinant virus-like particles as the antigen (Zhao et al. 2013). Recombinant hepatitis B virus has licensed VLP vaccines. The first two of these vaccines are Recombivax and Engerix-B and they are both HBV recombinant DNA vaccines. They consist of purified non-infectious subunits of the HBV surface antigen (i.e., HBsAg) (Lacson et al. 2005). After the invention of Recombivax-HB (Merck&Co.) and Engerix-B (GlaxoSmithKline, GSK), Human Papillomavirus (HPV) vaccines are produced by the same approach. Gardasil (Merck & Co.; produced in yeast) and Cervarix (GSK; produced in insect cells) are examples of Human Papillomavirus vaccines which were produced in the late 2000s (Dillner et al. 2010; Lehtinen et al. 2012; Kaufmann et al. 2010; Kreimer et al. 2011). Both the HBV and HPV vaccines containrecombinant virus-like particles (Glaxosmithkline Vaccine HPVSG et al. 2009; Mao et al. 2006). Clinical trials of these vaccines showed that they prevent infection by inducing protective and neutralizing antibodies (Schiller et al. 2012). Recombinant HBsAg is produced in yeast by cloning of a part of the HBV DNA into the yeast *Saccharomyces cerevisiae (Hauser et al. 1987)*. The same antibodies to HBsAg are induced to be produced by Recombivax and Engerix-B and both vaccines have similar immunogenic properties (West and Calandra 1996). More immunogenic VLP vaccines containing Pre-S1, Pre-S2 and hepatitis B surface antigens have been developed. The third generation hepatitis B vaccine, which can produce a strong antibody response, is Bio-Hep B (Alpar et al. 2014). Production of Bio-Hep B is in mammalian Chinese hamster ovary (CHO) cells and contains Pre-S1 (large), Pre-S2 (middle) and the small (s) surface proteins of HBV (Hourvitz et

Table 2. VLP vaccines with license (modified from Kushnir et al. 2012)

Vaccine Name	Disease	Company	Expression System
Epaxal	Hepatitis A	Crucell	Cell-free
GenHevac B	Hepatitis B	Pasteur-Merieux Aventis	Mammalian (CHO cells)
Bio-Hep-B	Hepatitis B	BTG (SciGen, FDS Pharma)	Mammalian (CHO cells)
DTP-Hep B	Hepatitis B	P.T. Bio Farma	Yeast (*P. pastoris*)
Engerix B	Hepatitis B	GSK	Yeast (*S. cerevisiae*)
Enivac HB	Hepatitis B	Panacea Biotec	Yeast (*P. pastoris*)
Euvax B	Hepatitis B	LG Life Sciences	Yeast (*S. cerevisiae*)
Gene Vac B	Hepatitis B	Serum Inst. of India	Yeast (*H. polymorpha*)
Heberbiovac HB	Hepatitis B	CIGB-Heber Biotec	Yeast (*P. pastoris*)
Hepavax-Gene	Hepatitis B	Crucell	Yeast (*H. polymorpha*)
Recombivax HB	Hepatitis B	Merck	Yeast (*S. cerevisiae*)
Revac-B	Hepatitis B	Bharat Biotech International	Yeast (*P. pastoris*)
Shanvac-B	Hepatitis B	Shanta Biotechnics	Yeast (*P. pastoris*)
Gardasil	HPV	Merck	Yeast (*S. cerevisiae*)
Cervarix	HPV	GSK	Insect (High FiveTM cells)
Inflexal V	Influenza	Crucell	Cell-free
HeberNasvac	Hepatitis B	The Cuban regulatory authorities	*E.coli*
Hecolin	Hepatitis B	Xiamen Innovax Biotech	*E.coli*

al. 1996). Yeast (Venters et al. 2004), insects, bacteria *Escherichia coli* (Wei et al. 2014; Li et al. 2015), plant (Kapusta et al. 1999) and mammalian cells were used for the production of the approved VLP-based vaccines (Table 2). Several recombinant protein-based products were derived from *E.coli* such as the first recombinant human insulin (Kyriakopoulos et al. 2013). There are over 50 VLP based vaccines which are produced in different expression hosts between 1986 and 2015 (Huang et al. 2017). Hecolin and Heber Nasvac are produced in *E.coli* and are licensed VLP-based vaccines (Lua et al. 2014; Huang et al. 2017). Hecolin is the first commercialized *E.coli* derived hepatitis E vaccine (Proffitt, 2012). ABX203 (trade name HeberNasvac) is a VLP vaccine for hepatitis B treatment and is the first marketing authorized vaccine by the Cuban regulatory authorities in 2015 (Lobaina et al. 2015; http://www.abivax.com/images/pdf/ 151208_ABX203_Cuban_Authorization.).

For vaccinology in cancer, Human Papilloma Virus (HPV) VLPs in GARDASIL vaccine is a good example. It prevents HPV infections which can be cause of cervical cancer and warts. The major capsid protein L1 of HPV are expressed into VLPs either in yeast or Baculovirus systems. It mimicks the original epitopes of virions and shows protective immune responses when properly adjuvanted. The adsorbed VLPs of each HPV type is prepared and combined to produce GARDASIL, an aluminum-containing adjuvant is used for adsorbtion of all of the VLPs. The VLP morphology is checked to figure out whethertheinteraction with the aluminum adjuvant surface modifies the VLP morphology. According to results, adsorption onto adjuvants did not affect the morphology (Zhao et al. 2014).

Goldinger et al. (2010) have designed an anticancer vaccine MelQbG10 virus-like nanoparticle loaded with CpG-oligonucleotides and coupled to a peptide derived from Melan-A. This vaccine may trigger cytotoxic T lymphocyte response of the immune system against Melan A expressed melanoma cancer cells. As a result, vaccination of patients at phase II clinical trial resulted in an increase of T-cells at the injection site. The biopsies of the injection site demonstrated an enhanced expression of CD4 and CD8 which confirms the flow of T-cells. Furthermore, in case of evaluation of Melan A expression, it exhibited a down-regulation in the tumour tissue after vaccination. (Goldinger et al. 2010).

Chimeric Virus-Like Particles and Cancer

The lacking genomic material of VLPs provides great safety profile to use in numerous fields, such as vaccines, drug delivery, in vitro imaging systems (Grgacic and Anderson, 2006). On the other hand, chimeric VLPs, occurring from two distinct capsid proteins, are the specific approach for both of vaccination and drug delivery system at the same time. As an example, gag and M1 are two capsid proteins from influenza virus and Deo and his colleagues used these two capsid proteins to express colon carcinoma cell-targeting chimeric virus-like particles. These chimeric VLPs displayed a variable fragment region targeting colon carcinoma cells. chimeric VLPs were packaged by Large unilamellar vesicles containing calcein-AM or doxorubicin. The dye and the drug were delivered to the cells by VLPs successfully and targeted cancer cells with high specificity (Deo et al. 2015).

Choi et al. also used chimeric VLPs and demonstrated how a chimeric VLP can be used in gene silencing. Previously, lentiviral vectors were the effective vectors in gene silencing for a long period in mice. However, the usage of these vectors have some risks due to mutagenesis and carcinogenesis which may occur during the integration of their DNA into the host's genomic DNA. This limits their use for clinical applications. So in this study, chimeric siRNA/capsid nanocarrier complexes were produced. In the cell culture system, these nanocarriers suppressed RFP gene expression efficiently. As a result, they proved tumor-specific targeting ability of the chimeric nanocarrier in vivo. Moreover, the siRNAs in capsid shell are protected against nucleases in plasma by encapsulation, so the enhanced stability of siRNA during body circulation was also proved. Due to overexpression of the RGD-mediated binding to integrin receptors on tumour cells, delivery of siRNA is accomplished to the tumour tissues in vivo efficiently by the multivalent RGD peptides on shell surface (Choi et al. 2013).

Conclusion

In recent years, studies were shown that VLPs are excellent candidates for vaccination and targeted drug delivery systems owing to their inherent properties; multimeric antigens, particulate structure, not being infectious. Here, we summarize some of them in vaccinology and drug delivery areas. The assembly, disassembly/reassembly, self-assembly features of the VLPs are known as a key point to design with modifications and encapsulation in order to provide extra immunogenic and functional properties. All these properties have made possible different mechanisms of drug encapsulation and specific targeting (Molino and Wang 2014).

Major advantages of VLPs are being appropriate for the induction of safe and efficient humoral and cellular immune responses. Thus, they can utilize in vivo applications without any toxicity and inflammatory response. Chemically and genetically modifications of VLPs make them more useful for specific applications than native forms. Recently, especially when targeted to a therapeutic site, the success of drugs inside a VLP system can be better than free drug while reducing side effects.

Recombinant VLP systems can be arranged to gain more functionalities to present drugs, imaging reagents, antigenic epitopes of a corresponding virus or another disease-associated antigen and specific targeting peptides to the internal and external surfaces of the particle. It supplies an important additional advantage because in the future, vaccinology should be able to produce vaccines which have both preventive and therapeutic effects. It is considered that by the use of VLP-based delivery systems, enhanced immunogenicity could be achieved.

Traditionally, against solid tumours only chemotherapy or accompanied radiotherapy is applied. However, traditional treatments which have lack of specificity for each cancer types results in side effects. In further doses of chemotherapy, tumour cells can become drug resistance and normal cells affected by toxins. In cases that require more dose intake, toxicity limits the chemotherapy-based treatment. Even just self-assembly and encapsulation features, the VLPs can be loaded according to the conditions required by the treatment. This distinctive flexibility provides many advantages over synthetic nanoparticles and consequently traditional treatments.

We hope that immunotherapy and preventative cancer vaccines control cancer in humans with fewer side effects than chemotherapy-based methods. Although all novel approaches, there are some drawbacks to overcome related with production processes, or with the formation of chimeric VLPs. Currently, the absence of reliable preclinical animal models and issues such as cost obstructs rapid and effective vaccine development. Also, further researches are required to understand behavioral features of VLP systems in vivo and to carry this technology from the laboratory to the clinic. However, with further studies, VLP-based technologies will continue to progress due to their predominant and great advantages and will be more applicable in future.

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