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Comparative study of chemical composition and the antimutagenic activity of propolis extracts obtained by means of various solvents

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Abstract: The present study is aimed to evaluate the chemical characterization and antimutagenic potential of propolis extracted in three different solvents (ethanol, polyethylene glycol and water). The chemical properties of different extracts of propolis were identified using HPLC-DAD and LC–MS/MS and polyethylene glycol extract of propolis were found to be richer than the ethanolic and water extracts of propolis considering chemical composition. In addition, the antimutagenic activities of propolis extracts were determined using Ames assay. The concentrations of 3, 1.5 and 0.75 mg plate^{-1} of ethanolic and polyethylene glycol extracts, as well as 0.3, 0.15 и 0.075 mg plate^{-1} of water extract of propolis were used as active materials. Propolis extracted in three different solvents indicated strong antimutagenic activity against both 4-nitro--*o*-phenylendiamine and sodium azide mutagens in the *Salmonella typhimurium* TA98 and 100 strains at all concentrations. Ethanolic extract of propolis had the highest inhibition rates for both bacterial strains and these rates were 98.94 and 97.37 % for TA98 and TA100, respectively. The inhibition rates of polyethylene glycol extract of propolis ranged from 68.27 to 98.94%. Moreover, it was determined that water extract of propolis had the lowest inhibition rates, which were 56.86 and 55.35% for TA98 and TA100, respectively. The toxicological safety of natural products such as propolis has gained great importance due to extensive usage.

Keywords: HPLC-DAD; ames assay; genotoxicity test; *Salmonella typhimurium*.

INTRODUCTION

Propolis is a mixture of substances synthesized by combining the resin that bees collect from buds, exudates and other parts of plants with their own saliva

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enzymes, wax and bees use it to protect the hive.[1] Although the content of propolis has a different chemical composition according to the country, region and plant variety, similar action mechanisms such as antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, antiproliferative and antioxidant activity have been defined.[1]

The content of propolis varies according to the plant species used by the bees, the plant components collected, the harvesting season, the climate, the diet of the bees and the genetic differences in the queen bee. Chemical standardization is very difficult due to the wide variation in the chemical mix depending on the source of propolis.[2] It is also important to use suitable solvents in the extraction of propolis due to impurities such as wax, pollen and dead bees. Common solvents used for the extraction of propolis are ethanol, methanol and water in different concentrations. The solvents used during the extraction can affect the activity of propolis because different solvents dissolve and remove different compounds. Since most substances in the composition of propolis are lipophilic and lipophilic compounds are soluble in ethanol, ethanol has been the commonly used solvent for extracting propolis.[3] In addition, it has been known that flavonoids and phenolic acids are obtained by the extraction of propolis with different solvents such as methanol, acetone, chloroform, propylene glycol and especially water.[4] Medical and food technology processes are almost always carried out with ethanol or aqueous extracts. In addition, glycol extracts can be used for many cosmetic applications that improve dissolution in water-based emulsions.[5,6]

Many powerful chromatographic methods are used to determine and measure biologically active substances in complex mixtures such as propolis. HPLC, high performance thin layer chromatography (HPTLC), nuclear magnetic resonance (NMR), thin layer chromatography (TLC), gas chromatography (GC) and capillary electrophoresis (CE) are some of these methods.[7] Today, against the standardization of propolis, high-performance liquid chromatography with photodiode-array detection (HPLC-DAD) is one of the most widely used methods for the separation and quantification of phenolic compounds of propolis, thanks to its very high separation efficiency.[8]

The Ames test is one of the short-term test systems used to detect mutagens/ /antimutagens because it provides standard and reliable results.[9] The Ames *Salmonella* test is also accepted as a viable test by international organizations such as the Organization for Economic Co-operation and Development, International Conference on Harmonisation, the Japanese Ministry of Health and Welfare and the United States Environmental Protection Agency: Health Effects Test Guidelines International Workshop on the Standardization of Genotoxicity Test Procedures, and is one of the recommended tests to observe the mutagenicity/antimutagenicity of chemicals. Therefore, the aim of this study was to compare

chemical compositions of propolis extracted in three different solvents and to investigate the antimutagenic potential of them by Salmonella back mutation test.

EXPERIMENTAL

Chemicals

For the preparation of propolis extracts, ethanol (96.0 %) and polyethylene glycol 400 were purchased from Sigma Aldrich and Zag Chemistry®, respectively. For HPLC-DAD, caffeic acid (≥98.0 %), quercetin (≥95.0 %), apigenin (≥95.0 %), kaempferol (≥90.0 %), chrysin (≥97.0 %) and caffeic acid phenyl ester (≥97.0 %) were used as standards (Sigma Aldrich). For antimutagenicity assay, D-biotin, L-histidine, top agar, 4-nitro-*o*-phenylenediamine (NPD) and sodium azide (NaN_3) were bought at Sigma Aldrich. Nutrient agar was obtained from Merck.

Collection of propolis samples and preparation of extracts

Raw propolis samples was collected from Düzce (Yığılca) situated in Western Black Sea Region of Türkiye; latitude: 40° 57′ 28″ N, longitude: 31° 27′ 15″ E, in 2020 were kept in the freezer (–20 °C) until analysis. Raw propolis taken from the same hive was divided into 3 parts and used for different extraction.

Raw propolis samples were extracted in Duzce University, Traditional and Complementary Medicine Application and Research Center, Duzce, Türkiye. Briefly, for the ethanolic extract of propolis (EEP), a sample (50 g) was crushed into small pieces in a blender, extracted with 500 ml 96 % ethanol for 48 h, filtered and evaporated to dryness in a vacuum evaporator (Labfreeez®, China) at 40 °C. The resultant resinous product was added to 70 % ethanol.

For polyethylene glycol extract of propolis (PEGEP), preparation processes were as in the EEP and then dissolved in 60 % PEG400 (Zag Chemistry®, Türkiye).

For the water extract of propolis (WEP), 50 g of propolis was shaken (Allsheng® OS-200) in 500 ml distilled water for 10 days at room temperature at 1400 rpm and filtered. An orbital shaker was used for all process.

High-performance liquid chromatography with photodiode-array detection

The chemical characterizations of different extracts of propolis were identified by in-house method using HPLC-DAD at Duzce University, Scientific and Technological Research Application and Research Center Laboratory. HPLC-DAD analyzes (254 and 325 nm) were performed in HPLC (Shimadzu LC20A®) instrument with PDA detector. The HPLC conditions used in the study of Çakır *et al*.[10] were modified. Mobile phase (A) and acetonitrile (B) (HPLC grade, Isolab®) gradients containing 2 % acetic acid were applied on the C18 column (250mm×4.6mm×5um, Inertsil). In 20 µL injection volume, at 30 °C column temperature at 1.0 ml min^{-1} flow rate, initial volumes are 75 % of mobile phase A and 25 % of mobile phase B and programmed as A mobile phase 25 % and B mobile phase 75 % in 25 min linearly. The method was completed at 30 min and each reading was carried out under these conditions.

Liquid chromatography–mass/mass spectrometry (LC–MS/MS)

The samples were analyzed at Trakya University, Technology Research and Development Application and Research Center (TÜTAGEM) with triple quadrupole performance using liquid chromatography–mass/mass spectrometry (LC–MS/MS). In the analysis process, first of all, the certified standard compounds were prepared at 5 points between 5–100 ng/ml concentrations and a calibration curve was created. In this method, the emergence of the compounds in the chromatogram at a certain time interval, the ratios of parent ions and confirm-

ation ions are compared with the ratios of fragmented ions obtained from the matrix spike. The area of the peaks obtained as a result of the analysis is plotted against the concentration of the added standard in the sample. Thus, substance determination is made with high sensitivity and accuracy. Two different sample preparation processes were applied for the analysis of phenolic compounds.

Hydrolysis method

100 µl of the sample was added to 900 µl of the extraction solution (79 % ultrapure water + 20 % methanol + 1 % formic acid) and was vortexed for 30 s and then kept for 10 min in an ultrasonic bath at 45 °C. Then the sample was centrifuged at 9000 rpm for 5 min and the clear filtrate was poured into glass vials for analysis.

Acidic hydrolysis method

200 µl of 2 M HCl solution was added to 100 µl sample, vortexed for 30 s, and kept in an ultrasonic bath at 90 °C for 40 min. Then, 700 µl of extraction solution (79 % ultrapure water + 20 % methanol + 1 % formic acid) was added. The samples were centrifuged at 9000 rpm for 5 min and the clear filtrate was poured into insert glass vials for injection.

Analyzes of phenolic compounds were performed with Agilent 1260 infinity liquid chromatography, Agilent 6460 Triple Quadrupole MS/MS system (Jet Stream Electrospray ion source). Mobile phase A: 5 mM ammonium acetate in ultra pure water; mobile phase B: 50 % acetonitrile, 49 % methanol, 1 % acetic acid by volume; column: Agilent Zorbax SB-C8 3.0 mm×150 mm×3.5 µm.

Determination of toxicity

In order to determine the cytotoxic concentrations, 48, 24, 12, 6, 3, 1.5 and 0.75 mg plate^{-1} concentrations of EEP and PEGEP and 4.8, 2.4, 1.2, 0.6, 0.3, 0.15 and 0.075 mg plate^{-1} concentrations of WEP were prepared according to Dean *et al.*[11] with minor modifications. Propolis extracts (100 µl) and bacterial culture (100 µl) were added to tubes containing top agar, the mixture was shaken well. It was spilled into Nutrient Agar plates and spread rapidly. Plates were incubated at 37 °C for 24 h. Based on the results obtained, non-cytotoxic concentrations were determined as 3, 1.5 and 0.75 mg plate^{-1} concentrations for EEP and PEGEP; 0.3, 0.15 and 0.075 mg plate^{-1} concentrations for WEP.

Test strains

S. tpyhimurium TA98 strain, which is widely used to detect frame shift mutation and TA100 strain, which is widely used to detect point mutation, were provided by EBPI Bio-Detection Products (Mississauga, ON, Canada) and the genotypes of the test strains were checked for histidine requirement, biotin requirement, rfa mutation, uvrB mutation.[12]

4-Nitro-*o*-phenylendiamine-NPD and sodium azide were used as positive controls for *S. typhimurium* TA98 and TA100 strains, respectively, in the absence of S9 mix.[13]

Evaluation of antimutagenicity assay

For the antimutagenicity assay, the bacterial culture (100 µl), the test substance (100 µl), and the positive mutagens (100 µl) were added to top agar (3 ml) containing of histidine/biotin (0.30 ml) and they were spread homogeneously on Minimal Glucose Agar plates. After the incubation (48–72 h at 37 °C) the revertant colonies were scored on the plates for each sample.

The value of the propolis extracts to inhibit the effect of positive mutagens was evaluated between 0 and 100 %. Three plates were used for each variable in each experiment, and each experiment was repeated two times. The results obtained were evaluated using the % inhibition formula:

$$I = 100\left[1-(a-b)/(c-b)\right] \quad (1)$$

a: number of returned colonies in petri dishes with propolis extract, mutagen and bacteria, *b*: number of colonies returning spontaneously, *c*: number of colonies in petri dishes with mutagen.

To quantify antimutagenic effects by propolis extracts, the classification was made according to inhibition rates and according to this, antimutagenic effect is defined as: <20 %, negative; 20–40 %, moderate; >40 %, strong.[14]

Statistical analysis

The data were analyzed using SPSS 20 for Windows (SPSS Inc., Chicago, IL, USA) and the results obtained were expressed as the mean±standard deviation (*SD*). The Kruskal–Wallis test was carried out followed by the Mann–Whitney U-test to compare the statistical significance of the differences between the treated and control groups. The dose response relationship was determined using the Pearson correlation analysis. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

For the HPLC-DAD method, the amounts of the phenolic compounds were given in Table I. According to the results, chrysin (901.9 and 1371.0) and caffeic acid phenyl ester (CAPE, 1980.8 and 3196.1) were seen dominantly in EEP and PEGEP, respectively. PEGEP was found to be richer than the EEP and WEP in terms of phenolic compounds. The HPLC-DAD chromatograms of standard phenolic compounds are given in Fig. S-1 of the Supplementary material to this paper. There are many compounds that have not been identified as a result of the huge and different amounts of compounds within the propolis samples. While caffeic acid, quercetin and chrysin were determined in WEP, apigenin, kaempferol and CAPE were additionally detected in the EEP and PEGEP.

TABLE I. Chemical compositions (content in ppm) of different extracts of propolis by HPLC-DAD; ND: not determined

Compound	EEP	PEGEP	WEP
Caffeic acid	208.1	314.6	112.6
Quercetin	24.2	38.2	1.5
Apigenin	12.8	28.7	ND
Kaempferol	35.3	66.0	ND
Chrysin	901.9	1371.0	7.7
Caffeic acid phenyl ester	1980.8	3196.1	ND

For the LC–MS/MS method, the amounts of the phenolic compounds were given in the Supplementary material (Tables S-I and S-II of the Supplementary material).

The phenolic compounds of EEP, PEGEP and WEP were detected using LC–MS/MS hydrolysis and acid-hydrolysis methods using thirty-three phenolic standards. Of the 33 investigated phenolic compounds (Tables S-I and S-II), 22 were measured in the samples. In the hydrolysis method, when the concentrations

of phenolic compounds obtained in the EEP, PEGEP and WEP were evaluated, the phenolic compounds detected in the highest concentration were caffeic acid (1.74 mg/ml) and transferullic acid (0.64 mg/ml) for EEP; caffeic acid (3.95 mg/ml) and chlorogenic acid (0.23 mg/ml) for PEGEP; caffeic acid (1.06 mg/ml) and transferullic acid (0.07 mg/ml) for WEP.

In the acid hydrolysis method, when the concentrations of phenolic compounds obtained in the EEP, PEGEP and WEP were evaluated, the phenolic compounds detected in the highest concentration were caffeic acid (2.45 mg/ml) and transferullic acid (0.46 mg/ml) for EEP; caffeic acid (4.53 mg/ml) and transferullic acid (0.20 mg/ml) for PEGEP; caffeic acid (1.26 mg/ml) and protocatechuic acid (0.07 mg/ml) for WEP. In the results obtained from both methods, it can be said that caffeic acid and transferullic acid are the major components for all three solvents and there is no big difference between these two methods.

When the results were compared considering solvents, it was seen that the PEGEP had the richest content and this result was consistent with the result obtained by the HPLC-DAD method.

For the antimutagenicity assay, three different concentrations of EEP, PEGEP and WEP were used as material. The obtained results were showed in Table II. It was determined that the all of extracts of propolis had a strong antimutagenic effect at all concentrations on *S. typhimurium* TA 98 and 100 strains.

TABLE II. Antimutagenic effects as revertant colonies number of different concentrations of different propolis extracts on *S. typhimurium* TA98 and TA100 strains; NPD: 4-nitro-*o*-phenylendiamine

Group	Concentration mg/plate	Strain			
		S. typhimurium TA98		*S. typhimurium* TA100	
		Mean±*SD*	Inh. rate, %	Mean±*SD*	Inh. rate, %
Negative control	–	38.16±12.89	–	141.40±76.08	–
NPD	0.02	527.60±198.26	–	–	–
NaN_3	0.02	–	–	1625.60±993.14	–
EEP	3	63.33±4.93	94.86	541.00±470.53	73.07
	1.5	54.00±2.64	96.76	299.66±118.50	89.33
	0.75	43.33±3.51	98.94	180.66±100.50	97.35
PEGEP	3	119.00±64.37	83.48	612.33±75.63	68.27
	1.5	95.33±17.50	88.31	459.66±61.50	78.55
	0.75	43.33±11.37	98.94	340.00±37.24	86.61
WEP	0.3	249.33±50.52	56.86	804.00±96.50	55.35
	0.15	182.33±39.24	70.54	628.66±95.10	67.16
	0.075	121.66±36.74	82.94	456.33±27.39	78.78

EEP, PEGEP and WEP indicated strong antimutagenic activity against both 4-nitro-*o*-phenylendiamine and NaN_3 mutagens in the *S. typhimurium* TA98 and 100 strains at all concentrations. EEP had the highest inhibition rates for both bacterial strains and these rates were 98.94 and 97.37 % for TA98 and TA100,

respectively. Moreover, it was determined that WEP had the lowest inhibition rates, which were 56.86 and 55.35 % for TA98 and TA100, respectively (Fig. 1). However, it is thought that this low level in WEP may be due to the lower concentrations used than the others.

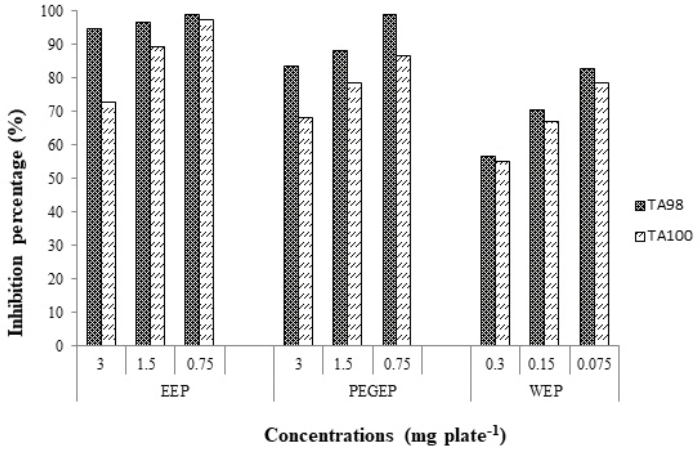

Fig. 1. The value of inhibition (%) of different concentrations of different propolis extracts on *S. typhimurium* TA98 and TA100.

The relationship between concentrations of EEP, PEGEP and WEP and the percentage of inhibition was evaluated by Pearson correlation test. It was observed that the percentage of inhibition significantly increased as the propolis concentrations decreased in *S. typhimurium* TA98 and TA100 strains (in *S. typhimurium* TA98 and TA100: $R^2 = 1$, $P \leq 0.05$ for EEP, PEGEP and WEP) (Fig. 2).

In the present study, the chemical compositions of propolis extracts were analyzed by HPLC-DAD and LC–MS/MS. PEGEP were found to be richer than the EEP and WEP in terms of chemical composition. The chemical characterization of many different extracts of propolis collected from different regions using many different methods (HPLC, GC–MS, LC–MS/MS, *etc*.) has been previously studied by many different researchers.[15–17] Furthermore, there are also many studies conducted with propolis collected from the Yığılca district of Düzce (Türkiye). Sevim *et al.*[18] determined by HPLC–UV method that EEP is rich in *p*-coumaric acid, ferulic acid, chrysin and pinocembrin components. Donmez *et al*.[19] investigated the volatile components of propolis in different solvents by LC–MS/MS and GC–MS–UV methods. It has been reported that the richest extract in terms of volatile component content is EEP. Ozdal *et al*.[20] reported that EEP has a rich content in their studies using the LC–MS/MS method. Rasgele and Kekecoglu[21] investigated the content of EEP with HPLC-DAD and they reported that biochanin, gallic acid, CAPE, pinostrobin and pinobankstin compounds were dominant. According to these data obtained from previous studies, propolis

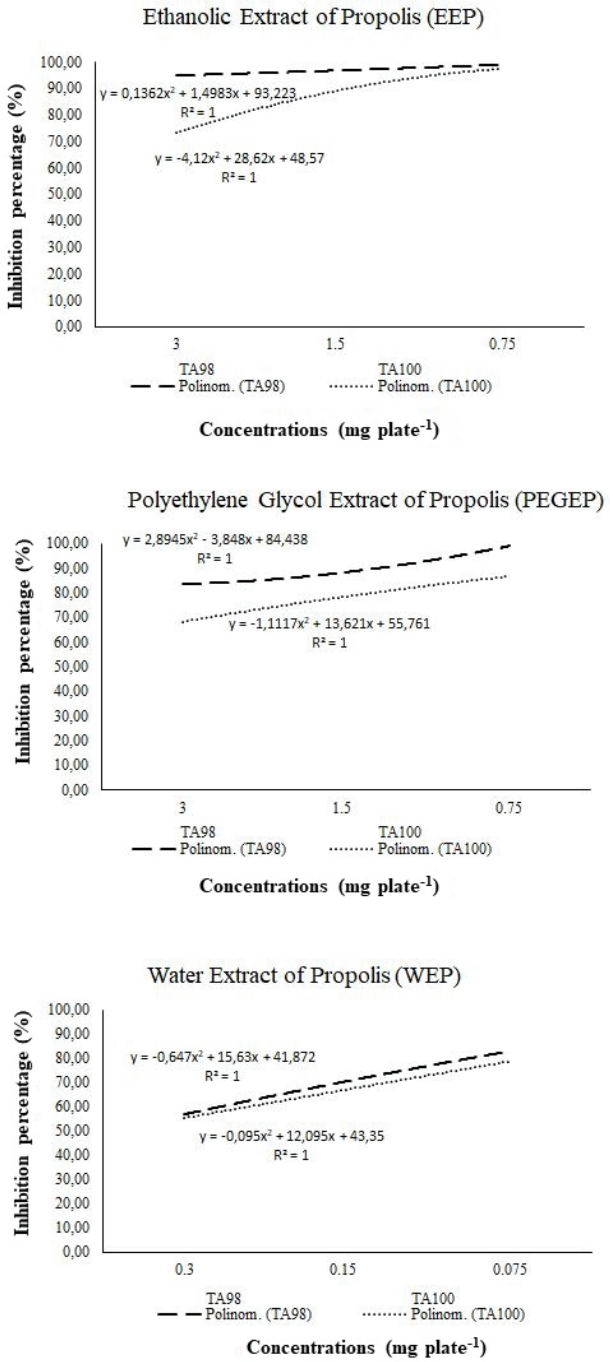

Fig. 2. Relationship between concentrations and inhibition rate (%) of test bacterial strains after exposure to different solvents of propolis.

has remarkable component richness in terms of content. The reasons for this diversity may be the region where propolis is collected, plant flora, bee race and solvent. When the results obtained from our study are discussed with others from literature, it can be noticed that although a diode array detector is used with the HPLCA-DAD method, the number of identified components is relatively low due to the peak overlap and the absence of compounds that do not absorb UV light.[22]

The Ames test is a useful method standardized in the 1970s for the mutagenicity of chemical compounds. It has also been used in the mutagenicity and antimutagenic effects of medicinal plants and natural products. However, due to the difficulty of working with natural products, there may be some technical problems and therefore sensitive and new guidelines are needed by regulatory agencies,[23] because the antimutagenic effect of natural extracts is a very important issue. Natural antimutagens can act against various mutagenic substances. On the other hand, antitumor effects of some compounds with antimutagenic effects have also been reported. Therefore, the search for antimutagenic compounds also represents a rapidly expanding field of cancer research. Therefore, it is very important to investigate both natural and synthetic antimutagens.[24] Especially in recent years, the preference of natural products such as propolis as a preservative in foods increases the importance of this. In general, it is desired to replace chemical compounds with active natural compounds.

Propolis is a complex bee product and contains very rich bioactive substances.[25] In this study, it is supposed that the antimutagenic effect of propolis is due to its rich phenol content,[26] because phenolics can act against mutagens through both intracellular and extracellular mechanisms.[27]

In the present study, EEP, PEGEP and WEP showed strong antimutagenic activity, being similar to the result obtained by many researchers using Ames assay. Varanda *et al*.[28] indicated that the ethanolic extract of propolis inhibited mutagenic potentials of mutagens in the *S. typhimurium* TA102, TA100 and TA98 strains. Jeng *et al*.[29] reported that the ethanolic extract of propolis showed an inhibitory effect against 4-nitro-*o*-phenylenediamine, 1-nitropyrene , 2-amino--3-methylimidazo[4,5-*f*]quinoline and benzo[*a*]pyrene mutagens on the *S. typhimurium* TA98 test system. Fu *et al*.[30] stated the antimutagenic effect of alcoholic propolis in the Ames assay *S. typhimurium* TA98 and TA100 strains, in which propolis inhibited the mutagenic activity of 2-aminofluorene and daunomycin. Moreno *et al.*[31] reported that extracts of propolis inhibited the effects of isoquinoline and 4-nitro-*o*-phenylenediamine mutagens in *S. typhimurium* TA98 and TA100 strains. Bayram *et al*.[32] observed that the ethanolic extract of propolis showed antigenotoxic effects against NaN_3, 9-aminoacridine and *N*-methyl-*N'*--nitro-*N*-nitrosoguanidine mutagens on *S. typhimurium* TA1535, TA1537 and *E. coli* WP2uvrA test strains.

Many antimutagenicity studies of propolis extracts, which were studied with different test systems, are also consistent with the results of our study. For example; Fu *et al.*[30] stated the antimutagenic effect of refined peanut oil in the micronucleus and chromosome aberration assays in mice, in which propolis inhibited the mutagenic activity of mitomycin and cyclophosphamide. Similarly, Lima *et al.*[33] reported that the aqueous extract of propolis had antimutagenic activity against DNA damage induced by 1,2-dimethylhydrazine in rats by aberrant crypt foci and comet methods. Also, Roberto *et al.*[34] reported that the ethanolic extract of propolis was not mutagenic and genotoxic in rat hepatoma cell lines, on the contrary, it caused a decrease in DNA damage in mutagen-treated groups. The researchers indicated that mechanisms of action of propolis might be related to either suppression of the mutagenic effects or antioxidant potentials of the components of propolis. It is thought that the antioxidant potential of propolis is due to its chemical components. The content of propolis varies according to the flora, harvest time, bee breed, etc., so it is one of the natural products that are very difficult to standardize.

CONCLUSION

Our study confirms the previous knowledge about the chemical composition of propolis. Chemically important and effectively bioactive components have been identified. Natural sources such as propolis remain an important component in drug development. Although propolis is used in traditional and complementary medicine, pharmacology and cosmetic industries, it is important to determine its antimutagenic potential in different solvents and more comprehensive studies are needed.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12280, or from the corresponding author on request.

ИЗВОД

УПОРЕЂИВАЊЕ ХЕМИЈСКОГ САСТАВА И АНТИМУТАГЕНЕ АКТИВНОСТИ ЕКСТРАКАТА ПРОПОЛИСА ДОБИЈЕНИХ ПРИМЕНОМ РАЗЛИЧИТИХ РАСТВАРАЧА

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Циљ студије је било испитивање хемијског састава и антимутагеног потенцијала прополиса екстрахованог применом три различита растварача (етанол, полиетилен гликол и вода). Идентификација хемијских састојака екстраката прополиса је изведена применом HPLC-DAD и LC–MS/MS метода. Већи број састојака је нађен у полиетилен-гликолном, него у етанолном и воденом екстракту. Антимутагена активност екстраката прополиса је одређена Ејмсовим (Ames) тестом. Као активни материјал су коришћени

екстракти прополиса у три концентрације: 3; 1,5 и 0,75 mg по плочи, у случају етанолног и полиетиленгликолног екстракта, односно 0,3; 0,15 и 0,075 mg по плочи, у случају воденог екстракта. Екстракти прополиса у сва три растварача и у свим тестираним концентрацијама су испољили јаку антимутагену активност када су бактеријски сојеви *Salmonella typhimurium* TA98 и TA100 изложени дејству мутагена 4-нитро-*o*-фенилендиамина и натријум азида. Највећи инхибиторни ефекат је имао етанолни екстракт прополиса, на оба соја бактерија, 98,94 и 97,37 % за TA98, односно TA100. Инхибиторни ефекат полиетиленгликолног екстракта је био од 68,27 до 98,94 %, док је ефекат воденог екстракта био најслабији, 56,86 и 55,35 % за сој TA98, односно TA100. Природни производи, као што је прополис, имају велики значај јер су токсиколошки безбедни за употребу.

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