MELLIFERA RESEARCH ARTICLE

Genotoxic and Antigenotoxic Evaluation of Propolis by Using *in vitro* Bacterial Assay Systems

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ABSTRACT

Propolis (bee glue) has been widely used as a therapeutic agent for centuries. Because of its pharmacological and biological properties, scientific interest in this issue has increased during the last years. The aims of the study were to determine chemical composition of ethanol extract of propolis (EEP) and evaluate its genotoxicity and antigenotoxicity by using Ames/*Salmonella* and *E. coli* WP2 short term test systems. Results that obtained from the viability assay for the tester strains, EEP at concentrations of greater than 1 mg/plate was highly toxic however lower concentration doesn't have any toxicity. According to genotoxicity test results, any concentration of EEP up to 1 mg/plate did not show genotoxic effect on *S. typhimurium* TA1535, 1537 and *E. coli* WP2*uvrA* tester strains. On the other hand, antigenotoxicity assay results showed that EEP has significantly antigenotoxic activity against NaN_3, 9-AA and MNNG induced mutagenicity on the same tester strains.

Key words: Propolis, GC-MS, Genotoxicity, Antigenotoxicity, Ames test.

Introduction

Propolis is a resinous substance that is gathered by worker bees from many different plant sources and enriched with wax, sallivary and enzymatic secretions [1]. Chemical composition of propolis intimately related to the ecology of the flora of each region visited by the honeybees, for this reason physical appearance of propolis has different properties depending on various factors [2]. The composition of propolis varies and in general, it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances [3].

Propolis have a wide range of biological activities such as antibacterial [4,5], antiviral [6,7], anti-inflammatory [8], anticariogenic [9,10], anticancer [11], antioxidative [12], tumoricidal [13] and antimutagenic [14,15].

Because of its healing properties, it has been used as an antiseptic, wound healer and

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therapeutic substance from ancient times to the present [1]. During the last years, propolis has become the subject of scientific interest because of its bioactive properties. Therefore, in this study it has been determined the major compounds of Hakkari-Turkey propolis by using gas chromatography-mass spectrometry (GC-MS) and tested its genotoxicity.

Material and Methods

Preparation of propolis sample

The propolis sample used in this study was collected from Hakkari region of Turkey. The collected propolis sample was frozen in the fridge, crushed into pieces and then weighed 100 g was mixed with 96% ethanol in a ratio of 1 g: 3 mL (w/v) and then sealed in a bottle at 30°C for two weeks. After two weeks, the supernatant was filtered twice with Whatman No. 4 and No.1 filter paper, respectively. The final solution (1:10, w/v) called Ethanol Extracts of Propolis (EEP) was evaporated until complete dryness. About 5 mg of dry substance was mixed with 75 µl of dry pyridine and 50 µl bis (trimethylsilyl) trifluoroacetamide (BSTFA) heated at 80°C for 20 min and then the final supernatant was analyzed by GC-MS [16].

GC-MS analysis

GC-MS analysis of EEP samples were performed using a GC 6890N from Agilent (Palo Alto, CA, USA) coupled with mass detector (MS5973, Agilent) fitted with a DB-5 MS capillary column (30 mx 0.25mm and 0.25 µm of film thickness). The column oven temperature was initially held at 50°C for 1 min, then programmed to rise to 150°C at a rate of 10°C/min and held for 2 min. Finally, temperature was increased to 280 with 20°C/min. heating ramp and kept at 280°C for 30 min. Helium was used as the carrier gas at a flow rate of 0.7 mL/min.

Chemicals and bacterial tester strains

Direct acting mutagens sodium azide (NaN_3), 9-aminoacridine (9-AA) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) from ABCR GmbH & Co. KG, Sigma-Aldrich and Merck. Other solvents and pure chemicals, including magnesium sulfate ($MgSO_4$), sodium ammonium phosphate ($Na_2NH_2PO_4$), D-glucose, D-biotin, sodium chloride (NaCl), L-histidine HCl, L-tryptophane, sodium phosphate-dibasic (Na_2HPO_4), crystal violet, citric acid monohydrate, potassium phosphate-dibasic (K_2HPO_4), sodium phosphate-monobasic (NaH_2PO_4) were also obtained from Difco, Fluka, Merck and Sigma.

S. typhimurium TA1535 (ATCC® Number: 29629) and TA1537 (ATCC® Number: 29630) strains were provided by The American Type Culture Collection – Bacteria Department of Georgetown University, Washington, USA, and *E. coli* WP2*uvrA* (ATCC® Number: 49979) strain was provided by LGC standards Middlesex, UK. All strains were stored at -80 °C. Working cultures were prepared by inoculating nutrient broth with the frozen cultures, followed by an overnight incubation at 37°C with gentle agitation [17].

Viability assays and determination of test concentrations

The toxicity of the propolis extract toward *S. typhimurium* TA1535, TA1537 and *E. coli* WP2*uvrA* tester strains was determined as described in detail elsewhere [18,19]. These tests confirmed that there was normal growth of the background lawn, spontaneous colony numbers within the regular range, and no significant reduction in cell survival. In these tests, 100 µl of the overnight bacterial culture diluted with nutrient broth to give 300–500 bacterial colonies per plate and treated with

increasing concentrations of EEP was spread onto nutrient agar plates. Then, the colonies from viable cells were scored on the plates after incubation at 37°C for 24 h. Thus, for the concentrations and conditions reported here, no toxicity or other adverse effects were observed.

in vitro genotoxicity and antigenotoxicity determination by bacterial reversion assays

The bacterial genotoxicity and antigenotoxicity assays with the tester strains were performed according to the described by in detail elsewhere [20,14]. The known mutagens sodium azide (NaN_3 in water–5 μg/plate) for *S. typhimurium* TA1535, 9-aminoacridine (9-AA in methanol–50 μg/plate) for *S. typhimurium* TA1537, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG in 10% DMSO–1 μg/plate) for *E. coli* WP2*uvrA* were used as positive controls and 10% DMSO was used as negative control in these studies [21,22].

In the genotoxicity test performed with *S. typhimurium* tester strains, 100 μl of the overnight bacterial culture, 50 μl of the EEP at different concentrations (0.2, 0.4, 0.6, 0.8 and 1 mg/plate in 10% DMSO), and 500 μl of sodium phosphate buffer (0.1 mM and pH 7.4) were mixed gently and the mixture was transferred into a water bath at 37°C for 20 min. Then, it was added to 2 ml of the top agar containing 0.5 mM histidine/biotin. The mixture was poured onto minimal glucose agar plates. Histidine independent revertant colonies and viable cells were scored on plates after incubation at 37°C for 48 h.

In the antigenotoxicity test performed with the same tester strains, 100 μl of the overnight bacterial culture, 50 μl of the EEP at different concentrations (0.2, 0.4, 0.6, 0.8 and 1 mg/plate in 10% DMSO), 50 μl of the appropriate mutagen solution (NaN_3 in water–5 μg/plate for *S. typhimurium* TA1535, 9-AA in methanol–50 μg/plate for *S. typhimurium* TA1537, and MNNG in 10% DMSO–1 μg/plate for *E. coli* WP2*uvrA*) and 500 μl of buffer solution were mixed gently and the mixture was transferred into a water bath at 37°C for 20 min. Then, it was added to 2 ml of the top agar containing 0.5 mM histidine/biotin. The mixture was poured onto minimal glucose agar plates. Histidine independent revertant colonies and viable cells were scored on plates after incubation at 37°C for 48 h.

The procedure of the genotoxicity and antigenotoxicity tests described for the Ames-*Salmonella* assay is applicable to the *E. coli* WP2 reverse mutation assay. The only procedural difference is the addition of limited tryptophan (0.01 mM) instead of histidine to the top agar [21].

The plate incorporation method was used to assess the results of genotoxicity and antigenotoxicity assays [23].

In this procedure, the mutagenic index was calculated for each concentration, which is the average number of revertants per plate divided by the average number of revertants per plate with the negative (solvent) control. A sample is considered genotoxic when a dose-response relationship was observed and a two-fold increase in the number of mutants with at least one concentration was observed [17].

For the antimutagenicity assays, the inhibition rate of mutagenicity was calculated by using the following equation (M: number of revertants/plate induced by mutagen alone, S_0: number of spontaneous revertants, S_1: number of revertants/plate induced by the extract plus the mutagen):

$$\text{Inhibition \%} = 1 - [(M - S_1) / (M - S_0)] \times 100$$

25–40% inhibition was defined as moderate antimutagenicity; 40% or more inhibition as strong antimutagenicity; and 25% inhibition as no antimutagenicity [24].

Statistical analysis

The results are presented as the average and standard deviation of three experiments with triplicate plates/dose experiment. The data were further analyzed for statistical significance using analysis of variance (ANOVA), and the difference among means was compared by high-range statistical domain using Tukey's test. A level of probability was taken as $P<0.05$ indicating statistical significance [17,24].

Results and Discussion

Chemical analysis of EEP from Hakkari-Turkey showed that the propolis sample had different concentrations of compounds that belong to aliphatic acids and their esters (7.93%), flavonoids (20.06%), hydrocarbons (6.06%), carboxylic acids and esters (0.29%), ketones (3.88), terpenes (0.07%) groups.

According to the GC-MS results, flavonoid group compouds (20.06%) were found in high ratios in propolis sample. These flavonoids are tectochrysin (6.25%), pinocembrin (6.11%), pinostrobin chalcone (5.72%) and chrysin (1.98%). Chemical content of Hakkari-Turkey propolis is given in Table 1.

Propolis sample from Hakkari-Turkey evaluated in this study showed genotoxic and antigenotoxic against to test microorganisms. The bacterial genotoxicity assay results performed with the EEP from the Hakkari region of Turkey and the tester strains showed that any concentration of the propolis extract did not induce mutagenic activity on *S. typhimurium* TA1535, *S. typhimurium* TA1537 and *E. coli* WP2*uvrA* strains. Moreover, the

Table 1. Chemical composition of the EEP from Hakkari-Turkey

Compound Groups	Compounds	% of Total Ion Current
Aliphatic acids and their esters	Palmitic acid	0.26
	Palmitic acid,ethyl ester	0.77
	Ethyl Oleate	6.90
	Total	**7.93**
Flavonoids	Pinostrobin chalcone	
	Pinocembrin	5.72
	Tectochrysin	6.11
	Chrysin	6.25
	Total	1.98
		20.06
Hydrocarbons	Eicosane	0.13
	Heneicosane	0.36
	9-Tricosene, (Z)-	0.33
	Docosane	1.80
	Nonadecane	1.00
	17-Pentatriacontene	2.44
	Total	**6.06**
Carboxylic acids and their esters	Pentadecanoic acid, ethyl ester	0.29
	Total	**0.29**
Ketones	2-Heptadecanone	1.09
	2-Nonadecanone	2.79
	Total	**3.88**
Terpenes	DELTA.3-Carene	0.04
	1R-.alpha.-Pinene	0.03
	Total	**0.07**
Others	(R)-(-)-(Z)-14-Methyl-8-hexadecen-1-ol	0.24
	(s)(+)-Z-13-Methyl-11-pentadecen-1-ol acetate	0.60
	Total	**0.84**

EEP showed significant activity against NaN_3, 9-AA and MNNG induced mutagenicity on the same tester strains in the antigenotoxicity assays. All of the detailed genotoxicity and antigenotoxicity results were given in Table 2.

Table 2. The genotoxicity and antigenotoxicity assay results of the ethanolic propolis extract for bacterial tester strains.

Propolis Ethanol Extract	Dose (mg/plate)	Count of Revertant Colonies		
		S. typhimurium TA1535	S. typhimurium TA1537	E. coli WP2uvrA
		Mean ±S.D.	Mean ±S.D.	Mean ±S.D.
Genotoxicity Assay	0.2	24.2 ±2.54	26.5 ±3.20	31.7 ±1.70
	0.4	25.0 ±1.63	24.2 ±2.11	27.8 ±1.67
	0.6	25.0 ±2.08	26.2 ±2.27	28.8 ±2.41
	0.8	22.8 ±2.67	26.0 ±3.00	29.7 ±2.21
	1.0	22.8 ±2.54	23.2 ±2.27	27.5 ±2.63
Antigenotoxicity Assay	0.2	323.3 ±12.27	266.8 ±14.10	691.1 ±10.77
	0.4	289.6 ±09.68	265.6 ±11.37	660.0 ±09.93
	0.6	205.8 ±09.90*	193.1 ±08.40*	549.6 ±12.72
	0.8	120.2 ±09.38*	112.8 ±09.57*	317.5 ±09.35*
	1.0	74.6 ±08.54*	65.0 ±14.83*	180.5 ±09.91*
NaN3**	5	314.8 ±9.32		
9-AA**	50		267.6 ±10.63	
MNNG* (µg/plate)	1			686.8 ±14.66
DMSO*** (µl/plate)	100	25.8 ±2.92	26.2 ±3.37	32.3 ±3.44

* $p<0.05$

** NaN_3, 9-AA and MNNG were used as positive controls for S. *typhimurium* TA1535, S. *typhimurium* TA1537 and *E. coli* WP2*uvrA* strains, respectively.

*** DMSO (dimethylsulfoxide; 100 µl/plate) was used as negative control.

Propolis that is known possess many different bioactive properties has been used as a therapeutic agent since ancient times [25,26,27,28]. Chemical composition of propolis is very complex and the main chemical groups present in its: phenolic acids or their esters, flavonoids, terpenes, aromatic aldehydes, aromatic alcohols, fatty acids, β-steroids and stilbenes. As already mentioned, there are many constituents in propolis, just like any other natural products. Therefore, chemical constituents of propolis extract vary greatly depending on solvent types, climate, location and years. In light of this information, as a matter of course, it could be said that, biological activity of propolis should be linked to its chemical composition [29,30].

Our investigation showed that the main components of Hakkari-Turkey propolis are mainly flavonoids. Previous studies have demonstrated that the main compounds of Turkish propolis are flavonoids [16,31,32,33, 34]. There are only limited research about genotoxicity and antigenotoxicity of propolis. Tavares et al. (2006) has examined genotoxic and antigenotoxic effects of propolis in hamster ovary cell. The authors demonstrated that low concentrations of propolis shows antimutagenicity and high concentrations shows mutagenicity effects [35]. Also, different

researchers concluded that antimutagenic activity of EEP might be due to the presence of flavonoids in view of their well known antioxidant activity [2,35,36].

In recent years, *in-vitro* and *in-vivo* assays give us many information about biological activity of propolis [25,28,37,38]. But it is hard to explain which constituents of propolis is the active substance as antigenotoxic. When we discuss about main compound groups of propolis and their antigenotoxic activities, it could be seen there is lack of *in-vitro* and *in-vivo* assays about this issue. In addition to, it is very clear that all of main compound groups of propolis have a large variety. For this reason, using all of these constituents for *in-vitro* and *in-vivo* assays will not be easy. Therefore, further *in-vitro* and *in-vivo* assays are needed to evaluate which compounds have genotoxic or antigenotoxic effects.

In an experiment to determine the effects of a propolis extract on immunomodulatory and antimetastatic action, it has been reported that polyphenolic compounds isolated from propolis and propolis itself significantly decreased the number of tumor nodules in the lung [39]. Additionally, it has been reported that caffeic acid phenethyl ester isolated from propolis has preferential cytotoxicity on tumor cells [40]. In addition to these studies, it has been demonstrated 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C) isolated from propolis has highly anti-tumor activity. In this study, artepillin C has exhibited preferential cytotoxicity to tumor cells cultured *in-vitro* [41]. Besides, it has been shown that a new clerodane diterpenoid isolated from propolis on chemically induced skin tumors in mice [42]. Finally, it has been demonstrated, PM-3, a benzo-gamma-pyran derivative isolated from propolis, inhibits growth of MCF-7 human breast cancer cells [43]. It is possible to replicate the examples above. Consequently, there are many different type constituents in propolis and each of these constituents has different chemical structure. Naturally, several components isolated from propolis have been shown to have different bioactive properties.

Conclusion

The results of our study show that, Hakkari-Turkey propolis has highly antigenotoxic effects. At the same time, in addition to this, it has not been observed any mutagenic activity. As discussed above, there is no enough information about which constituents of propolis has antigenotoxic effect exactly. But it is well known that the flavonoid concentration has important effect on biological activity of propolis [44]. For this reason, it is recommended that repeating *in-vitro* and *in-vivo* assays with isolated different main compounds such as flavonoids, carboxylic acids and their esters or aliphatic acids and their esters etc. from propolis.

in vitro Bakteriyel Test Sistemleri Kullanılarak Propolisin Genotoksisite ve Antigenotoksisitesinin Değerlendirilmesi

Öz: Propolis (arı tutkalı) yüzyıllardır terapötik bir ajan olarak yaygın bir şekilde kullanılmaktadır. Farmakolojik ve biyolojik özelliklerinden dolayı bu konu üzerindeki bilimsel ilgi son yıllarda giderek artmaktadır. Bu çalışmanın amacı, propolisin ethanol ekstraktının (EEP) kimyasal komposizyonunu belirlemek ve ayrıca Ames/*Salmonella* ve *E. coli* WP2 test sistemlerini kullanarak propolisin genotoksik ve antigenotoksik etkilerini değerlendirmektir. Canlılık testlerinden elde edilen sonuçlar, EEP'nin 1mg/plate ve daha yüksek konsantrasyonlarının oldukça toksik

olduğunu, daha düşük konsantrasyonların ise toksik etkisinin olmadığını göstermiştir. Genotoksisite test sonuçları ise, EEP'nin 1 mg/plate ve daha düşük konsantrasyonlarda, *S. typhimurium* TA1535, 1537 ve *E. coli* WP2*uvrA* test suşları için genotoksik etkisinin olmadığını göstermiştir. Ayrıca, aynı test suşları için mutajen etkiye sahip NaN_3, 9-AA and MNNG gibi mutajenlere karşı EEP'nin, belirgin bir şekilde antigenotoksik etkiye sahip olduğu gözlemlenmiştir.

Anahtar Kelimeler: Propolis, GC-MS, Genotoksisite, Antigenotoksisite, Ames testi

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