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Anticytotoxic and Antimutagenic Effects of Propolis on Human Lymphocytes In Vitro

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ABSTRACT

Propolis is a natural product which is collected from different plants by honeybees to provide antiseptic environment for colonies. It has been used as a therapeutical agent in alternative medicine since ancient times. The aim of this study was to investigate antimutagenic and anticytotoxic effects of propolis extract from Turkey's Hakkari region against aflatoxin B1 (AFB1) on human lymphocytes *in vitro*. Chemical content of propolis extract which was determined by Gas Chromatography–Mass Spectrometry (GC-MS) and it was observed to contain high amounts of flavonoids. The mutagenicity test results showed that AFB_1 caused DNA damages and increased sister chromatid exchange (SCE) frequency. Propolis reduced SCE frequency and showed strong antimutagenic effect against AFB1 on human lymphocytes. In addition, Cytotoxic and anticytotoxic effect of propolis was examined by LDH (lactate dehydrogenase) leakage test. Consequently, our findings showed that propolis had strong anticytotoxic and antigenotoxic properties against aflatoxin B_1.

Keywords: Aflatoxin B1, Propolis; GC/MS analysis, Sister Chromatid Exchange (SCE) Test, LDH (lactate dehydrogenase) leakage assay

Introduction

Propolis, a resinous material collected by honeybees from plant exudates, has recently aroused the interest of scientists for the study of its constituents and biological activities [1]. It has been used since ancient times by people in alternative medicine for its antibacterial, antiviral, antifungal, cytotoxic, antioxidant and many other properties [2, 3, 4, 5, 6]. Propolis contains a variety of substances including phenolic compounds such as flavonoids, aromatic acids and their derivatives, esters, alcohols and trace elements [7,8]. To date, over 500 chemical components have been defined in the chemical structure of propolis. Its chemical content differs according to the season and the region in which it is collected [9].

Aspergillus species, producing aflatoxins,

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can cause contamination of many foods including oilseeds, groundnuts, maize, pistachios, hazelnuts, wheat, barley, soya, rice and dried fruits [10]. Aflatoxin contamination of the food and food products are mainly caused by improper storage and transportation conditions [11]. Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds and for this reason they are considered as threat for human health [12]. Aflatoxins can cause serious health problems such as chronic and acute aflatoxicosis, cirrohosis and hepatic cancer [13]. AFB1 is the most potent carcinogenic agent among aflatoxins. Previous studies indicated that AFB1 is mutagenic in many test systems like chromosomal aberrations, micronuclei, sister chromatid exchange , unscheduled DNA synthesis and chromosomal strand [14, 15, 16].

It has been known for a long time that some substances exhibit mutagenic effect on genomic material in living organisms. Several quantitative tests are used to show such genotoxic effects. They are used in studies conducted both in vitro and in vivo. One of the most frequently used system among these is sister chromatid exchange (SCE) test. Known to be the part exchange between sister chromatids during metaphase without altering the chromosome morphology, SCE test is a fast, easily-applicable, low-cost, sensitive and reliable test system [17, 18, 19, 20]. The test developed by Taylor et al. (1957) [21] is still confidentially used with some modifications. SCE test, which is utilized in the determination of the toxic dosages of mutagens and carcinogens that damage live genomic material, and the investigation of the mutagenic or antimutagenic traits of substances to be used for the prevention of cytogenetic damage, is a highly important test due to being used for the sensitive and quantitative analysis of genetic damage [22, 23].

In this study, we aimed to determine the genotoxic and cytotoxic effects of propolis on human lymphocyte *in vitro* using SCE assay and lactate dehydrogenase (LDH) leakage assay. Additionally, significant compounds that form propolis and their relative amounts were determined by using GC-MS.

Materials and Methods

Preparation of propolis sample

The propolis sample that used in this study was collected from Hakkari region of Turkey. The sample was hardened in a freezer and ground in a handy grinder. Then one hundred grams of sample was dissolved in 300 ml of 96% ethanol. This mixture was incubated for two weeks at 30 °C in a tightly closed dark colored bottle. After two weeks, the supernatant was filtered twice with Whatman No. 4 and No.1 filter paper, respectively. The final solution was diluted in 1:10 ratio (w/v) with etanol (96%). A portion of this final solution was evaporated to obtain completely dry sample. 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.

GC-MS analysis

GC-MS analysis of ethanol extract of propolis 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 m x 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.

Sister Chromatid Exchange (SCE) Test

Heparin, at a ratio of 1/10, was added to the 1 ml peripheral blood samples of donors. The blood samples were added to the 5 ml chromosome medium B supplemented with 6 µg/ml 5′ -bromo-2′ -deoxyuridine in sterile conditions. The cultures were incubated at 37 °C for 72 hours. 0.06 µg/ml colchicine was added at 2 h before the harvesting of the culture. The SCE tests were performed as described by Perry and Evans (1975) [24]; Evans (1984) [25]; Perry and Thompson (1984) [26]; but with some modifications [27]. Then, slides were stained with %5 Giemsa (pH = 6.8) prepared in Sorensen buffer solution, for 20–25 min; washed in distilled water; dried at room temperature. The slides were stained with Giemsa according to the method of Perry and Wolff (1974) [28]; Speit and Haupter (1985) [29], with some modifications Yüzbaşıoğlu et al., (2006) [27].

In order to determine the genotoxic and mutagenic effects of heat shock stress, peripheral blood samples taken from the volunteer donors were transplanted as 13 drops (0.5 mL) into chromosome media that were heparinized at a 1/10 ratio in sterile cabins (Labormed). To determine SCEs, the cells were incubated for 72 h at 37 ± 0.5 °C by adding fresh 10 µg/mL 5-bromo-2-deoxyuridine solution (Sigma, CAS number: 59-14-3) into culture tubes at the beginning of the incubation. At the end of 72 h, the length of culture time employed, cells were precipitated by centrifugation for 10 min at 1200 rpm and then the supernatant was removed. The precipitate was homogenized, a warmed (37 °C) hypotonic solution (0.4% KCl) was added, and the cells were treated at 37 °C for 20 min. At the end of the period, the suspension was precipitated by centrifuging for 10 min at 1200 rpm and the supernatant was removed. After the addition of cold fixative (1:3 glacial acetic acid and methanol), the cells were held at room temperature for 15 min and centrifugation was repeated three times so that the cell pellet in the tube was homogenized. Cell pellet was dropped onto a cold slide from a height of 25 cm. After the slides dried under room temperature, they were stained with a 5% Giemsa stain prepared in a Sorensen buffer and covered with Entellan. To investigate SCEs, the fluorescence plus Giemsa method, developed by Speit and Haupter (1985) [29] was modified and used.

Cytotoxicity assay

Lymphocyte cells were seeded into 96-well plates at a density of $1x10^4$ cells/mL. After 24 h of seeding, cells were treated with different concentrations of propolis or media alone as a control. The cytotoxicity of propolis on cultured human lymphocytes was also assayed at 48 h by using following method.

LDH (lactate dehydrogenase) leakage assay

LDH leakage assay was carried out with a LDH-cytotoxicity assay kit (Cayman Chemical Company) according to the manufacturer's protocol. In brief, 10^4 to 10^5 cells/well were seeded in 96-well plates and exposed to different concentrations of propolis (0–20 µg/mL) for 24 h. At the end of exposure, the 96-well plate was centrifuged at 400 × g for 5 min to settle the propolis present in the solution. Next, 100 µL of supernatant was transferred to a well of a 96-well plate that already contained 100 µL of reaction mixture from a Bio-Vision kit and was incubated for 30 min at room temperature. After incubation, the absorbance of

the solution was measured at 490 nm using a microplate reader (Synergy-HT, BioTek, Winooski, VT, USA). LDH levels in the medium versus the cells were quantified and compared with the control values according to the instructions of the kit.

Statistical analysis

Statistical analysis was performed using SPSS® software (version 18.0, SPSS, Chicago, IL, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA). The experimental values were expressed as the mean±standard error (SE). The statistical significances and comparisons between different groups were evaluated with Duncan's test. Dose-response relationships were determined from the correlation coefficients. P< 0.05 was considered as the level of significance.

Results and Discussion

Mutagenicity Tests

In the present study, different concentrations of propolis were performed with the sister chromatid exchange (SCE) and LDH tests which widely used as a short term test system. In SCEs test, positive control (AFB_1) used significantly increased the SCEs frequencies on peripheral lymphocytes when compared with the control as seen in Table 1. Such an increase was found to be statistically significant ($p < 0.05$). It was observed that treatment group with different concentrations (P-1: 5µg/ml; P-2: 10µg/ml; P-3: 20µg/ml) of propolis together with AFB_1 (5 µM) decreased the SCEs frequencies compared with intoxicated with AFB_1.

In smilar to our results, it was reported frequencies of SCEs in peripheral lymphocytes was significantly increased by the direct-acting mutagen AFB1 compared with controls [30]. Similarly, Türkez and Yousef (2009) [31] found that treatment with propolis provide antigenotoxic effects by AFB1 at different degree. Lima et al. (2005) [32] demonstrated the antimutagenic activity of propolis against DNA damage induced by 1,2-dimethylhydrazine in rat colon cells by using comet test. According to recent studies, the toxicity of AFB1 is mainly due to lipid peroxide and

Table 1. The frequencies of SCEs in human lymphocytes exposure to AFB_1 and propolis.

Groups	Metaphase	Range of SCEs	SCEs/Cell	SCEs/Cell ± S.E
Control	60	3-7	328	3,28±0,11[a]
AFB_1(5 µM)	60	3-10	519	5,19±0,54[d]
Propolis (10 µg/ml)	60	2-15	348	3,48±0,15[a]
AFB_1+P-1 (5 µg/ml)	60	3-11	469	4,70±0,07[c]
AFB_1+P-2 (10 µg/ml)	60	3-10	395	3,96±0,06[b]
AFB_1+P-3 (20 µg/ml)	60	2-12	340	3,40±0,15[a]

[a] $p < 0.05$ compared with control.

[b] $p < 0.05$ compared with control.

[c] $p < 0.05$ compared with control.

[d] $p < 0.05$ compared with AFB_1 (5 µM) group.

oxidative damage which causes different types of cellular damage, including DNA breaks [33, 34].

Cytotoxic Effect

Lactate dehydrogenase (LDH) leakage tests showed that propolis doesn't have cytotoxic effect but have strong anticytotoxic effect against AFB_1. Especially, the highest concentration (20 µg/ml) was determined as the effective concentration. LDH leakage test, which is a cell membrane damage test and the indicator of cytotoxic damage, showed that propolis did not damage the cell. In addition, propolis has prevented it against the cytotoxic effect of AFB_1, and reduced LDH enzymes activities.

Many studies and research groups have confirmed that propolis possesses anticancer activity [35]. Hasan et al. (2015) [36] showed that propolis (from Makassar region) exhibited anticytotoxic effect in Michigan Cancer Foundation-7 breast cancer cell line. Milosevic-Dordevic et al. (2015) [37] demonstrated that the tested ethanolic extracts of propolis exhibited antimutagenic effect on human peripheral blood lymphocytes and anticancer activity on breast cancer cell line (MDA-MB-23).

Determination of Chemical Composition

The chemical composition of propolis sample, which was collected from Turkey (Hakkari) was determined by GC–MS.

GC-MS analysis of propolis indicated that it contains different concentrations of compounds that belong to fatty acids and their esters, flavonoids, hydrocarbons, carboxylic acids and their esters, ketones and monoterpenes groups (Table 2). The following compounds were identified in high ratios in propolis sample; ethyl oleate (6.90%), tectochrysin (6.25%), pinocembrin(6.11%), pinostrobin chalcone (5.72%). The level of ethyl oleate (% 6.90) belong to fatty acids and their esters had the highest concentration. In addition, flavonoids had high level in propolis. These findings are in agreement with different researchers. It has been reported that propolis from Turkey-Artvin region, containıng flavonoids and ethyl oleate as domi-

Table 2: Chemical composition of the propolis from Turkey-Hakkari

Compound Groups	Compounds	% of Total Ion Current
Fatty acids and their esters	Palmitic acid	0.26
	Palmitic acid,ethyl ester	0.77
	Ethyl Oleate	6.90
	Total	**7.93**
Flavonoids	Pinostrobin chalcone	5.72
	Pinocembrin	6.11
	Tectochrysin	6.25
	Chrysin	1.98
	Total	**20.06**
Hydrocarbons	Eicosane	0.13
	Heneicosane	0.36
	9-Tricosene, (Z)-	0.33
	Docosane	1.80
	Nonadecane	1.00
	17-Pentatriacontene	2.44
	(Z)-14-Methyl-8-hexadecen-1-ol	0.24
	(Z)-13-Methyl-11-pentadecen-1-ol acetate	0.60
	Total	**6.90**
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**
Monoterpenes	Δ^3-Carene	0.04
	α-Pinene	0.03
	Total	**0.07**

nant, had antibacterial activity against Enterecoccus faecalis [38]. Likewise, many studies have shown that flavonoids were the main propolis components [39,40,41,42].

Flavonoids, one of the main groups of phenolic compounds, are the key compounds for determination of propolis quality. It is well known that the flavonoid concentration will affect the biological activity of propolis [3,43]. Most of the flavonoids, such as acacetin, chrysin, galangin, naringenin, and pinocembrin are important metabolites that activate the antioxidant system and use of in vitro experimental systems has shown that they also possess antioxidant, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic, properties [44]. In addition, Liu et al. (2008) [45] showed that pinocembrin increased neuronal viability, decreased lactate dehydrogenase release, inhibited the production of NO and ROS, increased glutathione levels, and down-regulated the expression of neuronal NO synthase (nNOS) and iNOS in primary cortical neurons subjected to oxygen–glucose deprivation/reoxygenation (OGD/R). Besides, pinocembrin is also able to regulate mitochondrial function and apoptosis [46]. Previous investigations have demonstrated that pinostrobin chalcone has various pharmacological activities, antioxidant activity, and neuroprotective effects [47]. Tectochrysin has previously been reported to have inhibitory effect on cell growth of colon cancer cells (SW480, HCT116) [48]. That is why propolis, due to the high amount of flavonoids content, may have prevented the mutagenic effect of AFB1 causing DNA damage, therefore preventing the sister chromatid exchange in DNA. Also, not having a cytotoxic effect, propolis exhibited a strong anticytotoxic effect by protecting the cell against the toxic effect of AFB1. This anticytotoxic effect may be based on the compounds of flavonoids and other chemical group in its content.

It was also revealed in the studies that secondary metabolites in the propolis content such as ketone and terpene have a protective effect. Some ketones and terpenes have biological activities such as strong antimicrobial and low cytotoxic activities [49]. In addition, the enormous structural diversity presented by this class of natural products ensures a broad range of biological properties ranging from anti-cancer and anti-malarial activities to tumor promotion and ion-channel binding [50].

In more detailed studies to be conducted in future, important active substances in the propolis content can be purified, and it can be identified what substance(s) cause(s) the anticytotoxic and antimutagenic effects. Hence, Hakkari propolis having strong anticytotoxic and antimutagenic effects can be used as an alternative drug in the cancer researches. The anticytotoxic and antimutagenic effects of Hakkari propolis were revealed in this study. More detailed studies are planned in future to investigate the antioxidant and anticancer effects of propolis.

Propolisin Antisitotoksik ve Antimutajenik Etkilerinin İnsan Lenfositlerinde *İn Vitro* Olarak Belirlenmesi

ÖZ

Propolis, koloniler için antiseptik bir çevre oluşturmak amacıyla bal arıları tarafından farklı bitkilerden toplanan, reçinemsi doğal bir üründür. Antik çağlardan beri alternatif tıpta terapötik bir ajan olarak kullanılmaktadır. Bu çalışmanın amacı Hakkari, Türkiye bölgesinden toplanan propolis ekstraktının AFBı'e (aflatoxin Bı) karşı insan lenfosit hücrel-

erinde *in vitro* olarak antisitotoksik ve antigenotoksik etkilerinin araştırılmasıdır. Propolisin kimyasal içeriği GC-MS (Gaz kromatografi – Kütle spektrometre) ile belirlenmiş ve yüksek oranda flavonoid içerdiği belirlenmiştir. Mutajenite test sonuçları AFB_1'in kardeş kromatid değişimi (SCE) frekansını arttırdığını ve DNA hasarlarına neden olduğunu göstermiştir. Propolis ise *in vitro* ortamda AFB1'e karşı insan lenfositlerinde güçlü antimutajenik etki göstermiş ve SCE frekansını azaltmıştır. Buna ek olarak propolis'in sitotoksik ve antisitotoksik etkisi LDH (Laktat dehidrogenaz) salınım testi ile belirlenmiştir. Testler sonucunda propolisin aflatoksin B_1'e karşı antisitotoksik ve antigenotoksik etkiye sahip olduğu görülmüştür.

Anahtar Kelimeler: Aflatoksin B1, Propolis, GC/MS analizi, Kardeş Kromatid Değişimi (SCE) Testi, LDH (laktat dehidrogenaz) salınım testi

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