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The cytotoxic effects of bio-transformed propolis on healthy and colon cancer cell lines

Biyotransforme propolisin sağlıklı ve kolon kanseri hücre hatları üzerindeki sitotoksik

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ABSTRACT

Aim: Propolis has a lot of properties related to human health: antioxidant, antimicrobial, antiseptic, antibacterial, anti-inflammatory, antimutagenic, and immunomodulating. Its possible effects on different cancers are among the priorities of these properties. In this study, the comparative cytotoxic effects of bio-transformed propolis samples on colon cancer cells and healthy colon epithelium cells were investigated.

Materials and Methods: *Lactobacillus plantarum* strains used in this study are based on their well-documented ability to metabolize phenolic compounds during fermentation, effectively modifying their structure and activity. These strains exhibit cinnamoyl esterase activity, which is critical for reducing allergenic compounds such as DMEA and CAPE in propolis. Additionally, *L. plantarum* is widely utilized in biotransformation processes due to its safety, adaptability, and efficiency in enhancing the bioactive properties of natural products, making it an ideal candidate for optimizing the cytotoxic potential of propolis against colon cancer cells.

Results: The highest reduction was determined for ferulic acid, and the lowest reduction was obtained by using ethanol as a solvent using ultrasound treatments in the presence of water at optimal conditions (300 W/40 kHz). It was found that propolis samples showed dose-dependent cytotoxic effects on the colon cancer cell line (HCT-116) and healthy colon epithelium cell line (CCD-841 CoN) at 24, 48, and 72 hours.

Conclusion: Our results show that the method of propolis extraction, and the type of biotransformation reaction are very important in terms of effect on the cytotoxicity of colon cancer cells. Consequently, in this study, the demonstration of the bio-transformed propolis to have the ability to destroy cancerous cells without causing severe damage to healthy cells reveals that it can have the potential that can be used in cancer treatment.

Keywords: Colon cancer, propolis, biotransformation, cytotoxicity, polyphenol.

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ÖZ

Amaç: *Propolisin insan sağlığıyla ilgili birçok özelliği vardır: antioksidan, antimikrobiyal, antiseptik, antibakteriyel, anti inflamatuar, antimutajenik ve immün modülatör. Farklı kanserler üzerindeki olası etkileri bu özelliklerin öncelikleri arasındadır. Bu nedenle, bu çalışmada biyotransforme edilmiş propolis örneklerinin kolon kanseri hücreleri ve sağlıklı kolon epitel hücreleri üzerindeki karşılaştırmalı sitotoksik etkileri araştırılmıştır.*

Gereç ve Yöntem: *Farklı çözücüler (etanol; polietilen glikol - PEG; su) ve farklı ultrason işlemleri (300 W/40 kHz (5, 10 ve 15 dakika) kullanılarak yapılan ekstraksiyon prosedürüne başlamadan önce, propolis örneklerinin biyotransformasyonu üç farklı L. plantarum suşu (ISLG-2, ATCC-8014 ve Visbyvac) ile çeşitli konsantrasyonlarda (%1,5; %2,5; %3,5) gerçekleştirildi. Örneklerin fenolik profili sıvı kromatografisi-kütle spektrometrisi/kütle spektrometrisi (LCMS/MS) ile analiz edildi. Propolisin HCT-116 ve CCD-841 CoN hücreleri üzerindeki sitotoksik etkileri WST-8 yöntemi ile belirlendi.*

Bulgular: *En yüksek indirgeme ferulik asit için belirlenirken, en düşük indirgeme optimum koşullarda (300 W/40 kHz) su varlığında ultrasonik işlemler kullanılarak çözücü olarak etanol kullanılarak elde edildi. Propolis örneklerinin 24, 48 ve 72. saatlerde kolon kanseri hücre hattı (HCT-116) ve sağlıklı kolon epitel hücre hattı (CCD-841 CoN) üzerinde doza bağlı sitotoksik etkiler gösterdiği bulundu.*

Sonuç: *Propolis ekstraksiyon yönteminin ve biyotransformasyon reaksiyon tipinin kolon kanseri hücrelerinin sitotoksisitesi üzerindeki etki açısından çok önemlidir. Bu çalışmada biyotransforme edilmiş propolisin Sağlıklı hücrelere ciddi zarar vermeden kanserli hücreleri yok edebilen bu yöntemin kanser tedavisinde de kullanılabilecek potansiyele sahip olduğu ortaya çıkarılmıştır.*

Anahtar Sözcükler: *Kolon kanseri, propolis, biyotransformasyon, sitotoksisite, polifenol.*

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide (6.1% of total cases) and ranks fourth among cancer-related deaths (9.2% of total cases) (1,2). It has been thought that its incidence can increase by 60% till 2030 (3). Increased age population, negative modern dietary habits, smoking, low physical exercise, and obesity have been pointed out to be among the causes of this increase in patients with colorectal cancers in developed countries. Nowadays, surgical, radiotherapy (rectal), neoadjuvant, and palliative chemotherapy methods have been used in the treatment of primary and metastatic colorectal cancer. However, these treatment options are known to have limited effects on treatment rates and survival (4). Not getting enough responses from late diagnosis, and chemotherapeutic drugs constitutes an important obstacle in the treatment of the disease (3).

Many drugs are isolated from natural products (3). Propolis is a complex natural product, enriched in terms of beeswax and bee secretions, and is collected from plant buds, and exudates by honey bees (5). The content of propolis varies depending on its geographical origin, and plant sources (6,7). Propolis, containing polyphenols, terpenes, and flavonoids, is a bioactive compound that has anti-inflammatory, antimicrobial, antioxidant, and antiviral activities (8).

The potential beneficial effects of propolis on human health are closely related to the polyphenol compounds in its ingredients. These compounds can be extracted with different solutions, such as water, ethanol, and polyethylene glycol (6). Propolis, which has been used for centuries due to its beneficial properties, nowadays commercially offered in various forms, such as capsules, creams, powders and mouthwash solutions (5,6). Although propolis possesses antioxidant, antimicrobial, antiseptic, antibacterial, anti-inflammatory, antimutagenic, and anti-tumorigenic effects (9–18), its utilization is limited since it causes various allergic reactions.

Approximately 10% of individuals are sensitive to propolis, primarily due to allergenic compounds such as caffeic acid esters, 1,1-dimethylallyl caffeic acid ester (DMEA), benzyl caffeates, geranyl caffeate, and related cinnamic acid derivatives (19). Among these, DMEA and its isomers constitute the majority of propolis content, accounting for 87% of its composition, with 63% of these isomers linked to DMEA. Another allergenic compound, caffeic acid phenethyl ester (CAPE), demonstrates allergenic properties similar to those of DMEA (20).

It has been suggested that a less allergenic form of propolis could be developed through biotransformation processes utilizing lactic acid bacteria (21). Previous studies have highlighted that certain strains of *Lactobacillus helveticus* and *Lactobacillus plantarum* with cinnamoyl

esterase activity are capable of reducing the allergenic compounds in propolis (Patent No: TR2015 16914B, dated 2018/07/23). Specifically, using *L. plantarum* for the extraction and biotransformation of propolis effectively decreases the levels of allergenic molecules such as DMEA and CAPE (6, 22).

Changes in phenolic compounds during the biotransformation are directly related to selected microorganisms and strains. Lactobacillus plantarum is the most preferred type of lactic acid bacteria, as it takes place in the metabolism of phenolic compounds during the fermentation of various plant materials (23). In our previous studies, it was shown that *L. lantarum* strains reduce the amount of allergen molecules in propolis.

In our study, *Lactobacillus plantarum* strains were selected to reduce the allergenic effects of propolis. These strains exhibit unique enzymatic activities, particularly on phenolic compounds, which are critical for their effectiveness. Through cinnamoyl esterase activity and related mechanisms, *L. plantarum* effectively reduces allergenic compounds such as DMEA and CAPE in propolis. These distinctive features make it an ideal candidate for enhancing both the safety and therapeutic potential of propolis.

Existing research on propolis presents notable limitations, particularly in addressing its allergenic effects. While many studies focus on the bioactive properties of propolis, there is a lack of innovative biotechnological approaches aimed at mitigating allergenic components (6, 21). Additionally, the variability of phenolic compounds in propolis, influenced by geographical and botanical origins, hinders standardization and therapeutic application (19). This variability often results in inconsistent efficacy and safety profiles in its clinical use.

Our approach is innovative in addressing these gaps by employing *L. plantarum* strains with specific enzymatic activities to bio-transform propolis. This process not only reduces allergenic compounds but also optimizes the antitumor properties of propolis, particularly in colon cancer cells. By focusing on reducing allergenicity and enhancing therapeutic efficacy, this study provides a novel strategy for improving the safety and clinical potential of propolis in cancer treatment and beyond.

One of the aims of this study is to improve the methods which will be used to increase the cytotoxic effect of propolis on colon cancer lines, besides investigating the cytotoxic effects of bio-transformed propolis on colon cancer and healthy cell lines.

In the present study, the cytotoxic effects of the propolis extracts obtained on both colon cancer cells and healthy colon epithelium cells by processing biotransformation with various *L. plantarum* strains were investigated.

In the present study, the cytotoxic effects of the propolis extracts, obtained by the biotransformation by various L. plantarum strains, were investigated on both colon cancer cells and healthy colon epithelium cells.

MATERIALS and METHODS

The Preparation of The Propolis Samples and The Process of The Biotechnological Transformation

Propolis samples were obtained from the Sarkikaraagac district of Isparta city (Coordinates: Latitude: 388040 45.9800 N and Longitude: 318210 59.0000 E) in the Mediterranean region of Turkey in July. Raw propolis samples were milled by conventional machines. Particle size was determined as 35 mesh (0.5 mm) by applying the sieve analysis method to the milled samples.

The biotransformation was carried out by using *L. plantarum* strains (ISLG-2, ATCC-8014, and Visbyvac) at different concentrations (1.5%, 2.5%, and 3.5%). The propolis samples (w/v:1/1) were treated with different solutions (ethanol: 10%; poly-ethylene glycol PEG: 40%; water) and ultrasonication treatment was applied at 300 W/40 kHz (5, 10, and 15 minutes) using water.

The incubation was performed at 30˚C for 24-72 hours under anaerobic conditions. After biotransformation, the obtained bioproducts were treated with 70 mL of ethyl acetate and incubated at room temperature for 10 minutes. The phenolic fraction extraction was followed by centrifugation of the mixture at 1500 × 3 rpm for 5 minutes. The solid residues were separated using ethyl acetate. The obtained solid extracts were dried and dissolved in 100 mL methanol. After centrifugation at 4000 × g for 1 minute, the supernatants were diluted in appropriate conditions for analyses.

Determination of Phenolic Content of The Propolis Extracts (LCMS/MS Analysis)

In this study, the analyses were realized using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). 1 mg/mL stock

solution of each molecule was prepared in acetonitrile and methanol. Chemical stock solution of 1 mg/mL (caffeic acid, caffeic acid phenethyl ester, 1,1-dimethyl allyl ester caffeic acid, benzyl ester caffeic acid, ferulic acid, salicylic acid, genticic acid, catechin, chlorogenic acid, vanicic acid, and ethyl ferlate) was prepared in acetonitrile, and 1 mg/mL chemical stock solution (gallic acid, synergic acid, catechol, kaempferol, epicatechin, quercetin, myricetin, beta carotene, routine, maleic acid, 4-hydroxybenzoic acid, trans cinnamic acid, protocatechuic acid, p-coumaric acid, ellagic acid, cyanidine, narenin, pelargonin, and deiphinin) was prepared in methanol. These stock standard solutions were diluted to the working concentrations of 1-1000 pg/mL (diluted with a water/acetonitrile (50/50%) mixture containing 0.1% formic acid up to the working concentrations of 1-10000 ng/mL except salicylic acid), and these solutions were used to obtain calibration curves for each chemical.

Quantitative analysis was performed by using a Waters® ACQUITY™ TQD tandem quadrupole UPLC-MS/MS system consisting of an ACQUITY Ultra Performance™ liquid chromatography system, an ACQUITY XEVO TQD, and multiple reaction monitoring (MRM) (Waters mode, Milford, MA) in electrospray ionization (ESI). This UPLC-MS/MS system was controlled by MassLynx™ 4.1 software.

Chromatographic analysis was performed using the Waters Acquity™ UPLC I-Class system. The essential separation was achieved using the Waters Analytical Acquity UPLC BEH C18 columns (2.1 mm x 50 mm, 1.7 um, Waters, Milford, MA, ABD). Mobile phase A (0.2% formic acid [v/v] in water) and mobile phase B (0.1% formic acid in ACN) were operated with a gradient elution at 0.4 mL/min as follows: %75 A (0-0.5 min.), %75 A → %2 A (0.5-2.1 min.), %2 A → %75 A (2.1-2.7 min.), %75 A (2.7-4.0 min.). The column temperature was adjusted to 60°C, and the autosampler temperature was kept at 10°C. The capillary voltage, the source temperature, the thaw temperature, the con gas flow, the thaw gas flow, and the ion energy were set to 3730 V, 150°C, 40 L/s, 600 L/s, and 0.5 V, respectively, for MRM data collection.

Cell Culture

HCT-116 human colon cancer and CCD-841 CoN human normal colon epithelial cell lines were provided from the American Type Culture Collection (ATCC). The cells were grown in McCoy's 5A (HCT-116) and Eagle's Minimum Essential Medium EMEM (CCD-841 CoN) media containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin and incubated in the incubator in a moist environment containing 5% CO_2 at 37 °C.

Cytotoxicity Experiments

WST-8 (water-soluble tetrazolium salt) assay was used for determination of cell viability and cytotoxicity studies. For this aim, HCT-116 and CCD-841 CoN cells ($2x10^4$) were seeded into 96-well plates at a density of $8x10^3$ cells per well, respectively. After 24 hours, different concentrations of the transformed and non-transformed propolis extracts (25–1000 µg/mL) were separately added to the cells. The wells into which no substances were added were considered as controls. 10 µl of WST-8 solution (CCK-8, Sigma-Aldrich, USA) was added to each well at the end of 24-, 48-, and 72-hour cycles. The optical density (OD) of each well was determined by absorbance at 450 nm wavelength (reference wavelength: 620 nm) using a microplate reader (Thermo-Scientific, Multiskan FC, Finland) for periods of 1-4 hours.

The percentage of cytotoxicity value was determined by using the absorbance data obtained from these experiments and the following formula: **% cytotoxicity = 100 – {[O.D. (experimental value) / O.D. (control value)] × 100}**

Statistical Analysis

The experimental results were indicated as the mean ± standard error of the mean (SD). For analyzing the data, a two-way ANOVA, and posthoc test were used as Bonferroni test. The p-values less than or equal to 0.05 are considered statistically significant.

The graphical and statistical analysis were done using the Graphpad v8 (GraphPad Software, Inc., Avenida de la Playa La Jolla, USA) program. IC_{50} values were calculated using CalcuSyn v2 (Biosoft) software. The experiments were studied in 3 replicates.

RESULTS

Effects of biotransformation on the phenolic compounds and the allergen molecules

The evaluation of allergic molecules in the bio-transformed propolis samples was performed after preliminary studies, including the determination and classification of important phenolic compounds.

The phenolic compounds were determined in the samples subjected to biotransformation by different *L. plantarum* strains (ISLG-2, ATCC-8014, and Visbyvac), used at different

concentrations (1.5%, 2.5%, and 3.5%), prior to the extraction procedure by using different solvents (ethanol, polyethylene glycol – PEG, water) and different ultrasound treatments (300 W/40 kHz) for 5, 10, and 15 minutes. It has been known that the ultrasound treatment enhances the extraction yield.

The conditions and design of testing model related to ultrasound treatments were optimized by using the other food matrix (unpublished).

Considering the importance of the reduction of allergenic molecules (DMAE-1,1-dimethyl allyl ester caffeic acid, CAPE-caffeic acid phenethyl ester, FA-ferulic acid, and TSA-trans cinnamic acid), the best prominent effects of biotransformation are summarized (14 design combinations) in Table-1.

As can be seen in Table-1, in all cases except the extraction done only with water treatment, the biotransformation process for the used *L. plantarum* strains, inoculum concentration, and the extraction treatment (ethanol, PEG, electrical treatment conditions) caused the reduction of allergenic molecules.

Comparing to the percentage of reduction effectiveness, the order from the highest to the lowest values could be ordered as followed: ferulic acid > DMAE > CAPE > trans-cinnamic acid.

The highest reduction was determined for ferulic acid using ultrasound treatments in the presence of water at optimal conditions (300 W/40 kHz). Increasing the retention time (5, 10, 15 minutes) leads to a lower reduction ratio of this compound. The best performance of *L. plantarum* was achieved with strain L2 with inoculum value of 1.5%. It is not required to increase the inoculum value up to 3.5% in order to reach the required reduction of this compound.

Considering the CAPE compound, the lowest reduction was obtained by using ethanol as a solvent during the extraction and ultrasound treatment with conditions of 300 W/40 kHz/10 and 15 minutes retention time. During biotransformation, the best results were obtained using *L. plantarum* L2 with inoculum concentration of 2.5%, followed by *L. plantarum* L1 with inoculum concentration of 2.5%.

The values related to DMAE reduction were found to be in a similar manner with ferulic acid. The highest reduction of this compound was obtained by applying ultrasound at conditions of 300 W/40 kHz/5 and 15 minutes retention time in addition to using the different solvent extraction procedures prior to the biotransformation.

Furthermore, in terms of the culture used, the best results and hence the best performance rate were provided with L. plantarum L2 with a 1.5% vaccination rate and with L. plantarum L1 with a 2.5% vaccination rate.

It was observed that trans-cinnamic acid reduction values are also related to the applied ultrasound treatment (300 W/40 kHz) with 5- and 10-minute retention times before the biotransformation.

Table-1. Quantities of allergen molecules were compared for all transformed samples with their non-transformed samples (the solvents of the samples are the same). The arrow sign indicates that the amount has increased, while the descending arrow indicates that the amount is decreasing. CAPE: caffeic acid phenethyl ester DMAE: DMAE caffeic acid

Propolis	DMAE	CAPE	Ferulic acid	Trans cinnamic acid
Water (Non-transformed sample)	250	170	530	30.63
Water, L2 2.5 Inoculum rate (%)	546 ↑	448 ↑	2270 ↑	464.6 ↑
Water, L3 3.5 Inoculum rate (%)	990 ↑	1501 ↑	1551 ↑	87.41 ↑
Ethanol (Non-transformed sample)	898	879	1363	94
Ethanol, L2 2.5 Inoculum rate (%)	308 ↓	272 ↓	2452 ↑	37 ↓
PEG (Non-transformed sample)	456	398	2112	48.3
PEG, L2 3.5 Inoculum rate (%)	84 ↓	78 ↓	422 ↓	41 ↓
Water, 40 kHz/5 min (Non-transformed)	1921	1510	4225	327
Water, 40 kHz/5 min, L2 1.5 Inoculum rate (%)	838 ↓	1120 ↓	2327 ↓	16.3 ↓
Water, 40 kHz/10 min (Non-transformed)	1181	963	3275	127
Water, 40 kHz/10 min, L1 2.5 Inoculum rate (%)	625 ↓	516 ↓	1871 ↓	24 ↓
Water, 40 kHz/10 min L3 3.5 Inoculum rate (%)	1004 ↓	773 ↓	1498 ↓	51 ↓
Water, 40 kHz/15 min (Non-transformed)	975	796	3355	133
Water, 40 kHz/15 min L2 2.5 Inoculum rate (%)	261 ↓	238 ↓	1565 ↓	143 ↑

Table-2. Samples with the most and least major polyphenolic ingredients (Specimens in which 12 major polyphenolic contents of more than 50 ng/ml were detected in propolis samples at the most and least were stated).

Polyphenols	The Most Detected Sample	The Least Detected Sample
1. Kaempferol	Ethanol, L2 2.5 Inoculum rate (%)-2113 ng/ml	PEG, L2 3.5 Inoculum rate (%) - 175 ng/ml
2. Vanillic acid	Ethanol, L2 2.5 Inoculum rate (%)-723 ng/ml	Water, L3 3.5 Inoculum rate (%) - 73 ng/ml
3. Myricetin	Ethanol, L2 2.5 Inoculum rate (%)-921 ng/ml	Water, 40 kHz/10 min, L1 2.5 Inoculum rate (%) - 18 ng/ml
4. Ellagic acid	Ethanol, L2 2.5 Inoculum rate (%)-156 ng/ml	PEG (non-transformed sample) - 25 ng/ml
5. Epicatechin	Ethanol, L2 2.5 Inoculum rate (%)-88 ng/ml	Water, 40 kHz/5 min, L2 1.5 Inoculum rate (%) - 1 ng/ml
6. Naringenin	Water, 40 kHz/5 min (non-transformed) - 369 ng/ml	PEG, L2 3.5 Inoculum rate (%) - 16 ng/ml
7. Pelargonin	Water, 40 kHz/5 min (non-transformed)- 119 ng/ml	Water, 40 kHz/15 min L2 2.5 Inoculum rate (%) - 13 ng/ml
8. Quercetin	Water, 40 kHz/5 min (non-transformed) - 393 ng/ml	Water, L3 3.5 Inoculum rate (%) - 81 ng/ml
9. P coumaric acid	Water, 40 kHz/5 min (non-transformed) - 506 ng/ml	Water (non-transformed sample) - 89 ng/ml
10. 4 hydroxybenzoic acid	Water, L2 2.5 Inoculum rate (%) - 69 ng/ml	Water, 40 kHz/10 min, L1 2.5 Inoculum rate (%) - 7 ng/ml
11. Caffeic acid	Water, L2 2.5 Inoculum rate (%) - 1029 ng/ml	PEG, L2 3.5 Inoculum rate (%) - 224 ng/ml
12. Chlorogenic acid	Water, 40 kHz/5 min, L2 1.5 Inoculum rate (%) - 259 ng/ml	Water, 40 kHz/15 min L2 2.5 Inoculum rate (%) - 23 ng/ml

Table-3. IC_{50} values of Propolis samples on HCT-116 cells at the 24th, the 48th and the 72nd hours.

Propolis (solvents)	Inoculum rate (%)	Ultrasound application	IC_{50} values (µg / mL)		
			24h	48h	72h
Ethanol + Phosphate buffer	No	No	1567	---	---
Ethanol + Phosphate buffer	L2 (2.5%)	No	---	---	---
PEG + Phosphate buffer	No	No	1345	1187	---
PEG + Phosphate buffer	L2 (3.5%)	No	1345	682.9	779.8
Water	No	No	1255	957	890.84
Water	L2 (2.5%)	No	1340	799.6	678.3
Water	L3 (3.5%)	No	1487	1563	1489
Water	No	40 kHz/5 min	1075	1046	596.77
Water	L2 (1.5%)	40 kHz/5 min	1012.7	841.5	772.5
Water	No	40 kHz/10 min	---	---	---
Water	L1 (2.5%)	40 kHz/10 min	1081	1486	1547
Water	L3 (3.5%)	40 kHz/10 min	>2000	1642	>2000
Water	No	40 kHz/15 min	525.7	395.8	552.3
Water	L2 (2.5%)	40 kHz/15 min	817.9	553.8	722.5

*__L1:__ ISLG-2, __L2:__ ATCC 8014, __L3:__ Visbyvac strains of *L. plantarum.*

In both non-transformed and transformed samples of propolis, we determined that 12 phenolic compounds were above 50 ng/ml in quantity, except for allergen molecules. These 12 polyphenols are: vanilic acid, paracoumaric acid, quercetin, kaempferol, caffeic acid, myricetin, epicatechin, ellagic acid, chlorogenic acid, 4-hydroxy benzoic acid, pelargonine and naringenin (4 polyphenols were found below 50 ng/ml; these were rutin, salicylic acid, gentisic

acid, and protocatechuic acid). The concentration of these compounds varies between each other, depending on the type of extraction used and the biotransformation conditions.

Table-2 gives the list of those having the most, the least, and the highest increase and the highest decrease in percent according to the percentages of the main polyphenolic components the samples contain.

Comparative Cytotoxic Effects of Propolis Samples on Colon Cancer and Healthy Colon Epithelium Cells

It was found that propolis samples showed dose-dependent cytotoxic effects on the colon cancer cell line (HCT-116) and healthy colon epithelium cell line (CCD-841 CoN) at 24, 48, and 72 hours. The results are shown in the following Figures (1-5) in a comparative manner. IC_{50} values of the propolis samples for colon cancer cells (HCT-116) are given in Table-3.

The propolis sample, dissolved in 20% ethyl alcohol, has shown 6.91, 2.93, 1.62, and 2.45-fold higher cytotoxic effects on colon cancer cells compared to healthy colon cells at the concentrations of 100 µg/mL, 500 µg/mL, 1000 µg/mL, and 1500 µg/mL at the 24th hour, respectively (Fig. 1). No such effect was observed at the 48th and 72nd hours.

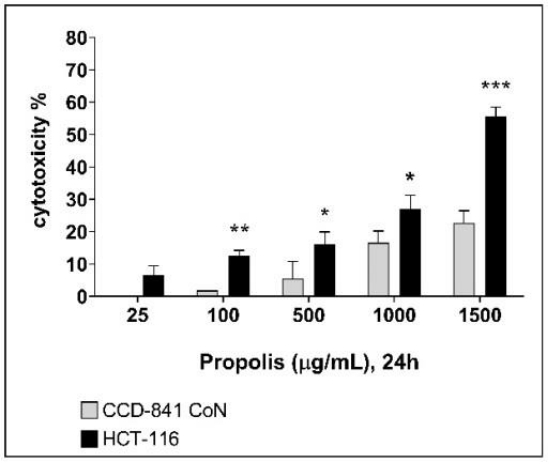

Figure-1. The cytotoxic effects of the propolis samples, dissolved in 20% ethyl alcohol, on colon cancer and the healthy colon epithelium cells at the 24th hour (**p<0.05, **p<0.01, ***p<0.001*).

Compared to healthy colon cells, the sample of propolis, dissolved in 50% PEG, showed 7.46- and 3.23-fold higher cytotoxic effects at the concentrations of 500 µg/mL and 1000 µg/mL at the 24th hour, respectively; higher cytotoxic effects became 2.76- and 2.29-fold at the concentrations of 1000 µg/mL and 1500 µg/mL at the 48th hour, respectively, and 1.94-fold at the concentrations of 1500 µg/mL at the 72nd hour on colon cancer cells (Fig. 2a, 2b and 2c).

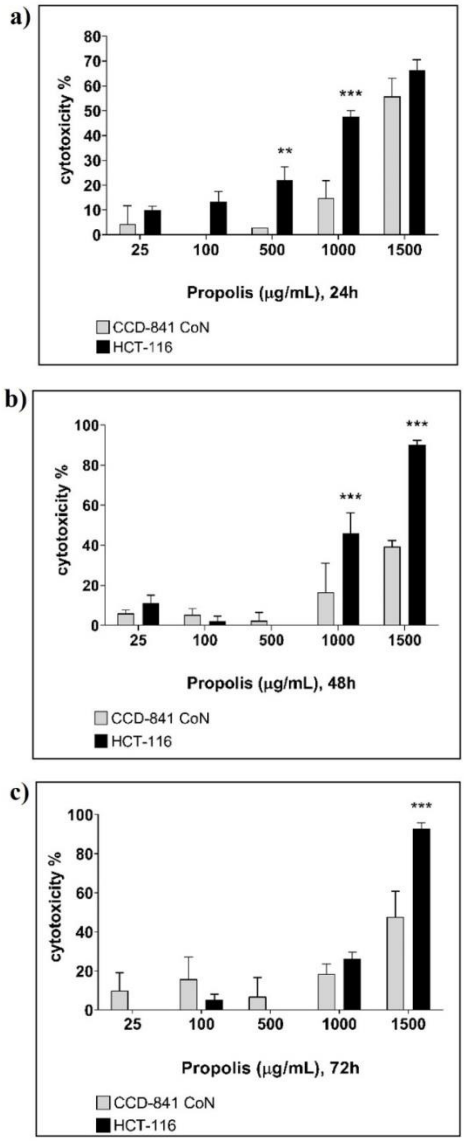

Figure-2a, 2b, 2c: The cytotoxic effects of the propolis sample, dissolved in 50% PEG, on colon cancer, and healthy colon epithelium cells at the 24th (2a), the 48th (2b), and the 72nd (2c) hours (**p<0.01, ***p<0.001*).

The sample, dissolved in water, on which ultrasound was applied at 40 kHz for 10 min and inoculated with 2.5% L. plantarum ISLG-2 strain, did not show a cytotoxic effect on CCD-841 CoN cells at the 24th hour and the 48th hour, but the significant cytotoxic effect was observed on HCT-116 cancer cells (Figure-3a, 3b).

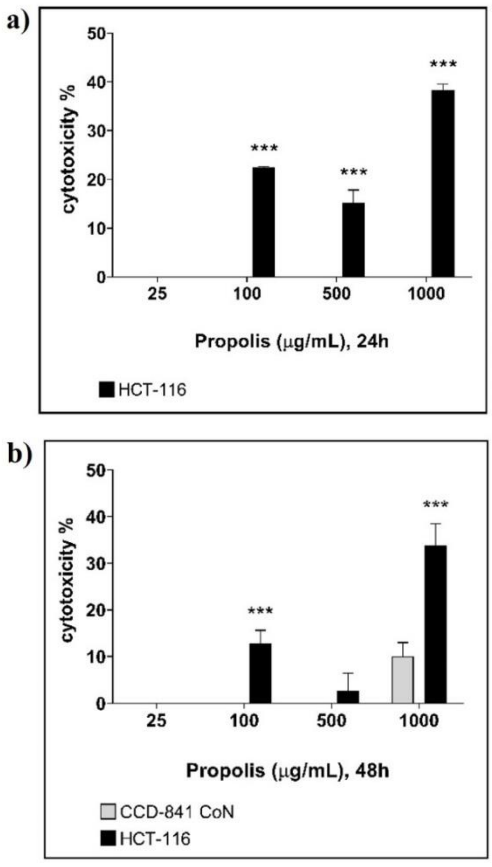

Figure-3a, 3b. The cytotoxic effect of the sample, dissolved in water, on which 40 kHz ultrasound was applied for 10 min, and which was inoculated with *L. plantarum* ISLG-2 strain of 2.5%, on colon cancer, and healthy colon epithelium cells at the 24th hour (3a), and the 48th hour (3b) (****p<0.001*).

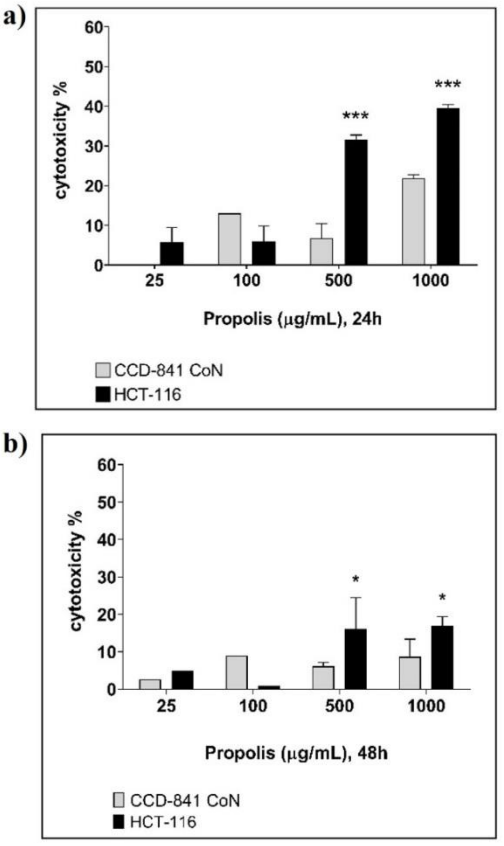

Figure-4a, and 4b: Cytotoxic effects of the propolis sample, dissolved in water, treated with 40 kHz ultrasound for 5 min, and which was inoculated with 1.5% *L. plantarum* ATCC® 8014 strain, both on colon cancer and healthy colon epithelium cells at the 24th (4a) and the 48th hour (4b) (**p<0.05, ***p<0.001*).

Compared to the healthy colon cells, the sample dissolved in water, on which 40 kHz ultrasound was applied for 5 min, and which was inoculated with 1.5% L. plantarum ATCC® 8014 strain, has shown 4.7 and 1.81 times higher cytotoxic effects on colon cancer cells at the concentrations of 500 and 1000 µg/mL at the 24th hour, respectively (Figure-4a).

The sample, treated as above, has shown 2.63 and 1.96 times higher cytotoxic effects, respectively, on colon cancer cells at the concentrations of 500 and 1000 µg/mL at the 48th hour compared to the healthy colon cells (Figure-4b). Such an effect was not observed at the 72nd hour.

The sample, dissolved in water, on which 40 kHz ultrasound was applied for 15 min, and which was inoculated with 2.5% L. plantarum ATCC® 8014 strain, has shown 4.32 and 2.83 times higher cytotoxic effects at the concentrations of 100 µg/mL, and 500 µg/mL on colon cancer cells compared to the healthy colon cells at the 24th hour, respectively.

While it has shown cytotoxic effects on HCT-116 colon cancer cells at the ratios of 35%, 31.2%, and 39.6%, respectively, at the concentrations of 25 µg/mL, 100 µg/mL, and 500 µg/mL at the 48th hour, it did not show the cytotoxic effects on CCD-841 CoN healthy colon cells (Figure-5a, 5b).

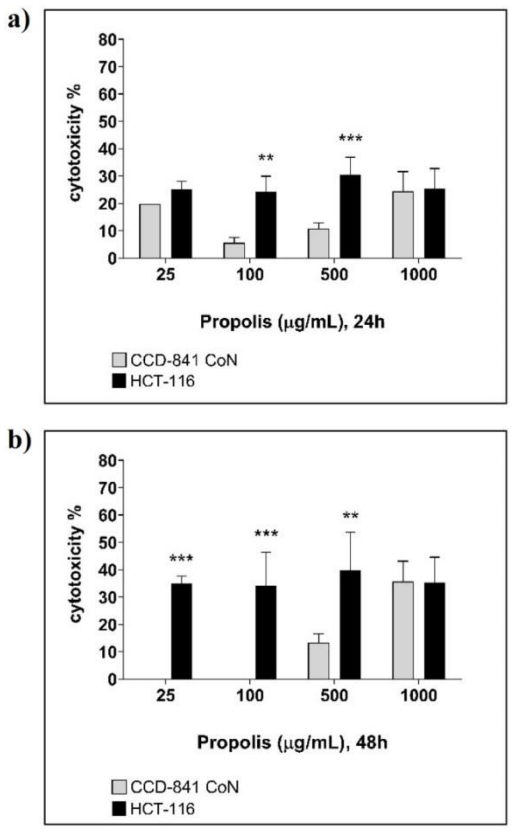

Figure-5a, 5b: Cytotoxic effects of the propolis sample, dissolved in water, treated with 40 kHz ultrasound for 15 min, and which was inoculated with 2.5% *L. plantarum* ATCC® 8014 strain, on colon cancer, and healthy colon epithelium cells at the 24th (5a), and 48th (5b) hours (**p<0.01, ***p<0.001*).

DISCUSSION

According to the results we obtained from the cytotoxicity studies, the sample dissolved in water, sonicated at 40 kHz for 5 min, and inoculated with 1.5% *L. plantarum* ATCC 8014 and the sample dissolved in water, sonicated at 40 kHz for 15 min, and inoculated with 2.5% *L. plantarum* ATCC 8014 strains have shown more cytotoxic effect on the colon cancer cells than the healthy colon cell line. Similarly, the sample dissolved in ethanol and the sample dissolved in PEG were also found to have higher cytotoxic effects in the colon cancer cells than in the healthy colon cell lines.

The samples dissolved in water, treated by ultrasound at 40 kHz for 10 min, and inoculated with 2.5% *L. plantarum* ATCC 8014 strain have shown cytotoxic effects on the colon cancer cells, but they have not shown a cytotoxic effect on the healthy colon cell line.

The same amount of propolis samples used in these experiments caused significantly more cancer cell line death when compared to that of the healthy colon cell line.

The other remaining normal and the transformed samples showed more cytotoxic effects in the healthy cells compared to the cancerous cell lines.

The extract of propolis in ethanol has been used in the study of Masashi Ishihara et al. and its effects on $CaCO_2$, HCT116, HT29, and SW480 colon cancer cell lines have been compared with the effect on healthy colon epithelial cell lines. While the propolis sample at the same concentration prevents the development of colon cancer cell lines, it does not cause a significant change in the healthy cell line (24). Calhella et al. have investigated the cytotoxic effect of ethanolic extract of Portuguese propolis on MCF7-breast adenocarcinoma, NCI-H460-non-small cell lung carcinoma, HCT15-colon carcinoma, HeLa-cervical carcinoma, HepG2-hepatocellular carcinoma, and non-tumor primary cells (PLP2). While the samples that were used at the same concentrations have shown cytotoxic effects on all cell lines (cancer and healthy), a lower IC_{50} value was obtained in the cancer cell lines than that of the healthy cell line (25). In our study, we found that the extract of propolis in ethanol has a higher cytotoxic effect in the cancer cell line than in the healthy cell line.

The findings of our study align with and extend upon previous research on the cytotoxic effects of propolis in cancer cell lines. For example, Ishihara et al. and Calhella et al. demonstrated that ethanolic extracts of propolis exhibit significant cytotoxic effects on colon cancer cells, often attributed to the high solubility and polyphenolic content of ethanol-based preparations (24,25). However, our results highlight the added value of biotransformation via Lactobacillus plantarum inoculation and ultrasonic treatment. Specifically, biotransformation increased the selectivity of propolis by enhancing its cytotoxic effects against colon cancer cells while reducing its impact on healthy colon epithelial cells, addressing a critical limitation in previous studies (26).

Since propolis is easily soluble in ethanol, the cytotoxicity values of propolis ethanol extracts on the cancerous, and the healthy cell lines were found to be closer each other with respect to that of its extract in water. Propolis dissolves in water

at a ratio of 1% at most, but it dissolves in ethanol up to 20%. In this study, being much higher the polyphenolic content of the ethanolic extract of propolis is much higher. Some research done on the same cancer cell lines may yield different results. The reason for this result is that the content of propolis collected from different geographies is different from each other. Even propolis samples collected from various regions of the same country can have different effects on the same cell lines.

In a study made with propolis provided from Greece, it has been observed that while propolis samples have shown cytotoxic effects on the fibrocarcinoma cell line, it had no effect on the fibroblast cell line (27). Tubi-bee propolis was used in another study associated with the same cell lines; it has been determined that the propolis samples show cytotoxic effects on both cancerous and healthy cell lines (28). There are two main reasons for obtaining different results from the studies associated with the same cell lines: 1. There are nearly 300 active components in the content of propolis, and these components can be extracted at different ratios in different solvents. 2. The polyphenolic content in propolis samples collected from the different regions differs in terms of both quantity and variety.

The content analysis of propolis collected from the different regions of Turkey and purchased from Azerbaijan were carried out in a study by our team.

The main polyphenols in the propolis of each region were found to be less or more in comparison with the propolis collected from the other regions. Although all propolis samples are qualitatively similar in terms of polyphenols in their contents, they can be quantitatively separated from each other (29). In a review article, it has been stated that the region where propolis is collected and climate are the main factors affecting the content of propolis. There are more subgroups of polyphenols specifically in different regions and climatic conditions. There are more polyphenol subgroups in different regions and specifically in different climatic zones. For example, propolis collected from the mild climate zone, where Turkey is also located, contains flavonoids more than those collected from other parts of the world. This result is related to the characteristics of vegetation of the region itself (30). In a study comparing the content and biological activities of propolis collected from different regions of Hungary, it has been shown that propolis has different content and different biological activities (31). According to this and our study results, it can be pointed out that propolis samples collected from different regions may have different contents and different biological activities.

Another factor that can also affect is the choice of solvent, and extraction method. In most of the studies made on biological activity up to now, only ethanolic extracts of propolis have been used. There are very few studies in which different solvents and extraction methods are tried.

In one of these studies, total polyphenol contents of propolis extracts and antioxidant capacity of propolis have been compared using different extraction methods. This research has made possible the extraction of propolis with different solvents and techniques, especially for people with alcohol intolerance (32).

Since three different solvents, various sonication, and extraction methods were tried in our study, and the samples have different concentrations, it can be accepted to be obtained different results from the studies related to the cancerous and healthy cell lines.

In a similar study, the biological activity results of propolis extract made in polyethylene glycol and those made in ethanol have been compared. It has been found that the extract in polyethylene glycol is at least as effective as the extract in ethanol (33).

In our study, the sample extract made in polyethylene glycol showed more cytotoxic effects than the sample extraction in ethanol.

Transformation affects the polyphenolic content of propolis. To the best of our knowledge, a study that investigates the effects of transformed propolis (i.e., modified propolis in terms of polyphenolic content) on cancer cell lines has not been made. Therefore, in this study, the different extraction methods that affect polyphenol concentration were also investigated in order to compare the effect of the transformed propolis on the cancerous cells.

In a study, the biological activities of propolis samples have been compared using different solvents. It has been found the propolis sample extracted in ethanol to be more effective, and shown the reason for this effect to be the higher total polyphenolic content of propolis extracted in ethanol (34).

A study has shown that induction of apoptosis, cell cycle arrest in the G2/M phase, epithelial-mesenchymal transition (EMT)-related markers, and phosphoinositide 3-kinase/protein kinase B signaling pathways are among the mechanisms of action of kaempferol (35). In a study made on human hepatic cancer cells, it has been shown kaempferol to induce autophagy through AMPK, and AKT signaling molecules, and to cause G2/M arrest through downregulation of CDK1/cyclin B (36). In a study made on SW480 colon cancer cells, it has been shown kaempferol to up-regulate TRAIL receptors, thereby inducing apoptosis (37). In a previous study, we found that kaempferol reduced the expression of BIRC7 (livin) and cIAP-2, which are from the family of apoptosis inhibitory proteins. In addition, we have shown that kaempferol causes a decrease in the expression of HSP70 protein, which is known to promote cancer cell invasiveness in prostate cancer. In our same study, we have also found that kaempferol reduced the amount of Clusterin, which is thought to have a critical role in colon cancer progression. The downregulation of Claspin, which is involved in the regulation of the cell cycle by interacting with Chk1, results in decreasing cell viability. In our previous study, we have determined that the level of Claspin decreased significantly with the application of kaempferol to HCT-116 colon cancer cells (38).

In this study, we found that propolis sample dissolved in water, ultrasonicated at 40 kHz for 5 min, and inoculated with *L. plantarum* strain ATCC 8014 (1.5%) had higher cytotoxic effects in colon cancer cells than healthy colon cells. We have seen that both the amount of allergen molecules such as, ferulic acid, DMAE, and CAPE, decreased significantly and the number of molecules such as, kaempferol, chlorogenic, vanillic, and salicylic acid, increased significantly compared to the non-ultrasonicated control. The increase in kaempferol and other phenolic substances in this sample may be the cause of the higher cytotoxic effect on cancer cells.

It has been shown that myricetin induces apoptosis of HCT-15 human colon cancer cells, and may be useful in the development of therapeutic agents for human colon cancer (39). The human flap endonuclease 1 (hFEN1) enzyme is accepted to represent an important target in the DNA damage response system for anticancer drug development. In a study made with this enzyme, it has been shown that myricetin inhibits HFEN1 in the HT-29 colon cancer cell line, and it will be able to be used as a new agent in cancer treatment (40). In a study, it has been stated that M10, which is a new myricetin derivative, alleviate endoplasmic reticulum stress and prevents ulcerative colitis, and colorectal tumor, and myricetin is an effective compound in preventing colon cancer together with using it in colon cancer treatment (41). In our study, the propolis sample dissolved in water, ultrasonicated at 40 kHz for 15 min, and inoculated with *L. plantarum* ATCC 8014 strain (2.5%) showed greater cytotoxic effect in colon cancer cells than healthy colon cells. In this sample, it was found that while the amount of allergen molecules decreased, at the same time, the amount of myricetin both was more than that of the non-transformed control and 3 times greater than that of the non-ultrasonicated control. These increments explain why this sample is more effective in colon cancer cells than healthy cells.

Anti-proliferative effects of chlorogenic acid have also been demonstrated in the studies made on HT-29 colon cancer cell line (42,43). In a study made by Ekbatan et al., it has been shown that chlorogenic acid inhibits cell proliferation by causing cell cycle arrest at the S-phase on Caco-2 colon cancer cells. In the same study, they found that apoptosis was induced by activation of caspase-3 by chlorogenic acid in colon cancer cells (26). It has been reported that chlorogenic acid was capable of arresting cell cycle at G0/G1 phase and inducing apoptosis in HL-60 acute promyelocytic leukemia cells (44). In another study, it has been found chlorogenic acid to cause the cell cycle arrest in the S-phase of HCT-116, and HT-29 human colon cancer cell lines, and inhibit cell viability through ERK inactivation (45). In an *in vivo* study made with chlorogenic acid, it has been reported to be determined antitumoral activity in murine models (46). In the studies made on the cancer and healthy cell lines, it has been found that chlorogenic acid has cytotoxic activity on colon cancer cell lines (DLD-1), endometrium cancer cells (ECC-1), and renal cell carcinoma cells (A498), whereas it has no toxic effect on human embryonic kidney cells (HEK293) (47,48). In a study made on renal cell carcinoma cells, chlorogenic acid has been shown to inhibit the PI3K/Akt/mTOR path by down-regulating the ratio of p-Akt/Akt, and p-mTOR/mTOR (48). In the present study, the propolis sample dissolved in water, ultrasonicated at 40 kHz for 10 min, and

inoculated with *L. plantarum* ISLG-2 strain (2.5%) exhibited cytotoxic effect on colon cancer cells, whereas it did not show such an effect on healthy colon epithelial cells. A 2-fold increase in the amount of chlorogenic acid compared to the non-transformed control can be the cause of this. Also, the amount of ferulic acid, DMAE, and CAPE allergen molecules in this sample decreased as a result of the transformation.

Our analysis revealed a significant reduction in allergenic compounds such as ferulic acid, CAPE, and DMAE, accompanied by increased levels of kaempferol, chlorogenic acid, and myricetin. These changes are likely responsible observed in the transformed samples.

As a result, in our study, it has been shown that the extracts of propolis in ethanol and polyethylene glycol have significantly more cytotoxic effects on the colon cancer cell line compared to the healthy cell line. Kaempferol, a flavonoid well-documented for its anti-cancer properties, induces apoptosis through TRAIL receptor upregulation, downregulation of anti-apoptotic proteins (e.g., BIRC7, cIAP-2), and suppression of cell survival pathways such as PI3K/Akt (35). Chlorogenic acid further supports this activity by inducing cell activating caspase-3 in cancer cells, as observed in studies on HT-29 and Caco-2 colon cancer cell lines. Similarly, myricetin has been shown to inhibit DNA repair enzymes, such as hFEN1, and to alleviaum stress, both of which are critical for cancer cell survival (26).

The significant enrichment of these compounds in our bio-transformed samples explains their enhanced efficacy, and our results provide additional evidence that the regional origin and extraction method significantly influence the biological activity of propolis.

It has been determined that some propolis samples dissolved in water, ultrasonicated, and transformed have also higher cytotoxic effects on colon cancer cells than healthy colon cells or have not shown cytotoxic effects on healthy cells while they have shown cytotoxic effects on cancer cell line.

In different solvents, polyphenols are transferred into extracts of propolis in different ratios; this situation determines the cytotoxic effect of propolis. The cause of having different cytotoxic effects of propolis collected from different regions on the same cell lines depends on the number of polyphenols in their content. Therefore, it is of great importance to determine the number of phenolic substances in its ingredient when the anticancer effects of propolis are investigated.

Determining the best solvent that may transfer polyphenol, which is sensitive to specific cell line, into extracts of propolis will also make treatments more specific and effective. These studies will guide propolis to be used safer, and more original in the studies that will be made in the future.

CONCLUSION

In light of the promising findings from our study, the potential clinical applications of bio-transformed propolis are highly encouraging. The enhanced selectivity and cytotoxicity of bio-transformed propolis, particularly through the modification of polyphenolic content by Lactobacillus plantarum inoculation and ultrasonic treatment, opens new avenues for its use as a targeted therapeutic agent for colon cancer. These modifications have not only improved the efficacy of propolis against cancer cells but also reduced its impact on healthy cells, addressing a critical challenge in cancer therapy. The ability to manipulate the phenolic composition of propolis through biotransformation can lead to more personalized and effective treatment strategies. Moreover, the reduced allergenic compounds and the selective cytotoxic effects suggest that bio-transformed propolis could be developed into a safer, natural adjunct in cancer treatment. Future research in clinical settings, focusing on optimizing extraction methods, identifying specific cell-line targeting phenolic compounds, and ensuring bioavailability, will be crucial in harnessing the full potential of bio-transformed propolis for cancer therapy.

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Disclosure statement

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Author Contribution Statement

E.Y.S. designed and directed the project. H.K.Y. carried out the propolis sample's biotransformation process. B.D. and H.M. carried

out the extraction of the propolis samples and content analysis with liquid chromatography/mass spectrometry. L.M.O.C. and N.S.G. carried out the cell culture and cytotoxicity. All authors participated in the analysis of the data. N.S.G., L.M.O.C. and H.M. wrote the article. All authors discussed the results and contributed to the final manuscript.

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