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European Journal of Biology
Eur J Biol 2023; 82 (2): 132–141
DOI: 10.26650/EurJBiol.2023.1247199

RESEARCH ARTICLE

Solvation Methods Affect the Amount of Active Components in the Extract of Propolis as well as Its Anti-Inflammatory Activity in THP-1 Cells

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ABSTRACT

Objective: : Propolis has been found to have various effects, including antioxidant and anti-inflammatory properties, according to studies. In this recent research, we discovered that reducing allergenic compounds in propolis through biotransformation using specific *Lactobacillus plantarum* strains enhanced its anti-inflammatory qualities. The study aimed to identify the extraction methods and solvents that had the most significant anti-inflammatory effects and assess how *L. plantarum* strains biotransformation of propolis affected these qualities in THP-1 cell line cultures.

Materials and Methods: Propolis samples were biotransformed with different concentrations (1.5%, 2.5%, 3.5%) of several *L. plantarum* strains (ISLG-2, ATCC®8014, visbyvac) before extraction using various solvents (ethanol, polyethylene glycol-PEG, water) and ultrasound treatments (300 W/40 Hz for 5, 10, 15 min). Liquid chromatography-mass spectrometer/mass spectrometry was used for phenolic analysis of the samples. ELISA test kits were employed to assess NF-kβ, IL-1α, IL-1β, IL-6, IL-10, TNF-α, IFN-γ , COX-1 in the cell culture supernatant.

Results: : Results showed that, except for NF-kβ, all cytokine levels decreased in four separate propolis samples. Caffeic acid, kaempferol, ferulic acid, quercetin, pelargonin, and naringenin were the key physiologically active components associated with the anti-inflammatory activity of propolis. The biotransformation process to reduce allergen compounds did not alter propolis's anti-inflammatory properties.

Conclusion: In samples that were dissolved in water, dissolved in ethanol+biotransformed with *L. plantarum* ATCC®8014, dissolved in water+biotransformed with *L. plantarum* ATCC®8014, and dissolved in water+sonicated for 15 min and biotransformed with *L. plantarum* ATCC®8014, the maximum anti-inflammatory effect of propolis was assessed.

Keywords: LPS, inflammation, propolis, biotransformation, extraction

INTRODUCTION

The inflammatory process is expressed with the migration of phagocytes, accumulation of neutrophils, monocytes, and macrophages, and, subsequently, loss of tissue function. At the beginning of the inflammatory process, macrophages are activated by different stimuli, such as cytokines from T and natural killer cells, and lipopolysaccharides (LPS) from G-negative bacteria cell wall.[1] Upon activation, macrophages differentiate into two phenotypes. Classical activation by LPS results in the M1 phenotype, which is involved in phagocytosis, secretion of inflammatory cytokines, reactive oxygen species, and nitric oxide enzymes.[1,2] During the inflammatory process, proinflammatory cytokines, such as interleukin (IL)-6, IL-1 and tumor necrosis factor-alpha (TNF-α) were released by M1 macrophages [3] M2 phenotype produces regulatory cytokines (such as IL-10) and get involved in tissue repair and regeneration.[4] While the inflammatory process is protective against pathogens, the resulting inflammatory activity can also contribute to the development of various diseases, including cardiovascular diseases, diabetes, arthritis, and inflammatory bowel disease.[5]

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Submitted: 03.02.2023 • **Revision Requested:** 24.05.2023 • **Last Revision Received:** 04.08.2023 • **Accepted:** 08.08.2023 • **Published Online:** 15.09.2023

Honeybees create propolis as a means of safeguarding their hives against bacterial, viral, and fungal invasions. Propolis contains over 300 active components.[6] Many active molecules such as flavonoids, flavones, flavanones, and flavonols have been identified in propolis. Some molecules from these groups have anti-cancer, anti-oxidant and anti-inflammatory effects.[6–10] It was observed that the anti-inflammatory effect of propolis in cell culture is closely associated with its phenolic content and solvation methods. [1,10–12]

As an alternative, it has been noted that about 10% of people have propolis sensitivity.[11,13] Caffeic acid esters, 1,1-dimethyl allyl caffeic acid ester (DMEA), benzyl caffeates, geranyl caffeate, similar compounds, and cinnamic acid esters are among the main allergens in propolis.[11–16] 1,1-dimethylallyl caffeic acid ester and its isomers make up the majority of the propolis (87%) and are the most prevalent molecules. 63% of these isomers were shown to be connected to DMEA.[15] Caffeic acid phenethyl esters (phenethyl caffeate; CAPE), a second allergen molecule, exhibit allergic effects comparable to those of DMEA.[15]

It was stated that the less allergenic propolis form might be produced by biotransformation and using lactic acid bacteria. Previous research has demonstrated that specific strains of *Lactobacillus helveticus* and *Lactobacillus plantarum* with cinnamoyl esterase activity can reduce the allergenic molecules in propolis (Patent No: TR2015 16914B, dated 2018/07/23).[16,17] It was proven that employing *L. plantarum* to properly extract and biotransform propolis lowered the concentration of allergenic compounds, specifically DMEA and CAPE molecules.[18,19]

Our aim in this study was to identify the extraction methods and solvents that had the largest anti-inflammatory effects, as well as to assess how *L. plantarum* strains biotransformation of propolis in the human monocyte cell line (THP-1 cells) culture affected those anti-inflammatory qualities. In this research, THP-1 cells were used as an inflammatory model, which was induced by LPS. The main inflammatory cytokine levels were measured in the supernatant of cell culture to detect possible protective effects of propolis. The relationships between the propolis extracted by different solvents/extraction methods and biotransformed by different *L. plantarum* strains and anti-inflammatory activity were evaluated.

MATERIALS AND METHODS

Propolis Sample Preparation

We sourced the propolis from a local Turkish company as raw material. The samples were taken in July from the Sarkikaraagac neighborhood of Isparta city, which is located in Turkey's Mediterranean region (coordinates: 38.8040 N, 3182100 E).

To remove unwanted rough particles, the samples underwent a milling process using conventional machines at the beginning of the experiments. Particle size was determined as 35 mesh (0.5 mm) using sieve analysis. After this physical treatment, the treated samples were mixed in order to homogenize them.

Biotransformation was carried out using different *L. plantarum* strains (ISLG-2, ATCC®8014 and Visbyvac) at different concentrations (1.5%, 2.5%, and 3.5%). All lactic acid bacteria were taken from stock culture collections (Ege University Food Engineering Department, Prof. Dr. Hatice Kalkan Yıldırım collection) stored at –80°C and containing 20% glycerol.

The frozen cultures were reactivated by growing at MRS broth containing media (9 mL MRS broth/10 mL tubes). The prepared media was sterilized at 121°C for 15 min. After allowing the media to cool up to room temperature, the inoculation was done aseptically. The prepared double samples were incubated at 30°C for 24 h. In order to eliminate the effect of glycerol solution used during storage at –80°C, the cultures were grown, and the activation process was performed consecutively two times. In such a way, the pure cultures were obtained for the next step of inoculation used during biotransformation.[18]

Before bioconversion, the propolis samples (w/v:1/1) were subjected by to various solvents or exaction methods. During the experiment, the extraction solvents were treated with 10% ethanol, 40% polyethylene glycol (PEG), and water. Another solvation method that was applied was ultrasound treatment (40 Hz and 300 W) for 5, 10, and 15 min.

The choice of water as a solvent was for control purposes. The upper values of ultrasonication treatment time (15 min) were determined by a preliminary study (5, 10, 15, 20, 30 min with propolis) (unpublished data). According to the study, increasing the duration time more than 15 min led to heating of food and consequently to possible loss of nutritional values, especially active phenolic molecules. Each extraction solvent and method was applied in duplicate for each culture and inoculum concentration. After aseptic inoculation of propolis samples with pure culture at different concentrations (1.5%, 2.5%, and 3.5%), the incubation was carried out at anaerobic conditions of 30 °C for 72 h.

Following the biotransformation process, the resulting bioproducts (biologically converted propolis) were treated with 70 mL of ethyl acetate and incubated at room temperature for 10 min. The mixture was then centrifuged at 1500 x g for 5 min. The solid particles were separated from the mixture and another 70 mL of ethyl acetate was added. The procedure was repeated under similar conditions (centrifugation at 1500 × g for 5 min). The solid extracts obtained were dried and dissolved in 100 mL methanol. The supernatants obtained after the final centrifugation at 4000 x g for 1 min were used for analyses.[19]

Analysis of Phenolic Content

A Waters Xevo ACQUITYTM TQD tandem quadrupole UPLCMS/MS instrument was used to evaluate the phenolic content of all propolis samples. This study was conducted with reference to the method we previously applied.[20]

Cell Culture Experiments

Cell culture experiments were conducted using the human monocyte cell line THP-1, which was cultured in Roswell Park Memorial Institute 1640 medium RPMI 1640) supplemented with 10% heat-inactivated fetal bovine serum, and 100 unit/mL penicillin, 100 µg/mL streptomycin at 37°C with 5% CO_2. The cells were seeded at a density of $4x10^4$ cells/well in 96-well plates and treated with propolis samples at concentrations ranging from 25–1500 µg/mL for 24, 48, and 72 h. Propolis samples were applied to these cells three times. The WST-8 assay was used to assess cell viability and any potential cytotoxicity.

For this purpose, after the end of 24, 48, and 72 h, 10 µL of water-soluble tetrazolium salt (WST) solution was added into the suspension containing 100 µL of cells and propolis. Then, absorbance values were measured at 450 nm with a reference wavelength at 620 nm using a microplate reader between 30 min and 4 h. In line with the results obtained by the WST-8 assay, the dose-effect graphs were drawn and IC_{50} values were calculated using CalcuSyn v2.1 software. In the experiments performed in this study, the IC_{50} doses and the time interval determined in the cytotoxicity study, were used. For the induction experiments with LPS, the THP-1 cells were seeded into 24-well plates so as to be $2x10^5$ cells per well.

The propolis samples for which the IC_{50} values were determined in the previous cytotoxicity assay (as shown in Table 2) were added to the cell culture and incubated for 1 h. Subsequently, 2000 ng/mL LPS was added to induce inflammation following a previously established protocol.[21] The aim of this study was to examine the potential protective effect of propolis against inflammation. To achieve this, these cells were treated with propolis extract for 1 h, followed by the induction of inflammation by adding LPS at a concentration of 2000 ng/mL. After 2 h incubation at 37°C, the supernatants were collected and centrifuged at 2000 x g for 5 min to measure cytokine levels. The experiment was performed independently three times to ensure reproducibility and reliability of the results.

Biochemical Analysis

In order to determine the appropriate concentration and incubation time that will yield the highest induction, LPS samples in different concentrations (0.1, 0.5, 1 and 2 µg/mL) were added into THP-1 cells and the TNF-α levels were determined in different time intervals (1, 2, 3, 4, 6, 18 and 24 h).[22]

Before induction with LPS, THP-1 cells were treated with propolis in a dose which was previously determined. The LPS-induced cell without propolis was used as the positive control and THP-1 cells treated with PBS (without LPS) was used as the negative control. After 24 h of incubation, the cell culture medium was removed and fresh LPS-containing medium (2 µg/mL) was added for 2 h.

Nuclear factor kappa B (NF-kβ) , IL-1α, IL-1β, IL-6, IL-10, TNF-α, interferon-gamma (IFN-γ) and cyclooxygenase (COX)-1 assays in a supernatant of cell culture were performed using a commercially available ELISA assay kit.

Statistical Analysis

Graphs of % cytotoxicity values in cell culture were plotted in the Graphpad v5 program. The results were analyzed using a two-way ANOVA test and a Bonferroni test as post test. IC_{50} values were calculated using CalcuSyn v2.0 (Biosoft) software.

The results were evaluated using the SPSS 22.0 statistical program. Phenolic content quantification, *in vitro* antioxidant, anti-inflammatory activity, and cytokine analysis tests were performed at least 3 times for each propolis sample. Results were calculated as mean and standard deviation. The least significant differences (LSD) test was used for significance between means. In addition, a Kruskall Wallis test was used for multiple comparisons and an LSD test was used as a post hoc test.

Spearman's Rho test was used for correlations between parameters.

RESULTS

Phenolic Content

Table 1 shows the quantities of phenolic compounds present in the propolis extracts added to cell culture. In general, the propolis extracts contained the highest amounts of trans-cinnamic acid, followed by ferulic acid, kaempferol, and quercetin as determined by the quantitative analysis of phenolic content. According to solvents, extracted amounts of phenolic molecules including the allergens (DMEA caffeic acid, Caffeic acid phenyl ester) were higher in extracts of propolis dissolved in water plus treated with ultrasound for 10 to 15 min, compared to those dissolved in other solvents. The biotransformation process depleted the allergen molecule content in all samples.

Cell Viability

The IC_{50} levels of propolis extracts at 24, 48, and 72 h changed between 166 µg/mL and 1012 µg/mL (Table 2). When propolis samples dissolved in water (untransformed) and dissolved in water and inoculated with 2.5% *L. plantarum* ATCC 8014 strain (transformed) were compared, significant differences

Table 1. Phenolic compounds detected in propolis samples added to cell culture.

	Ethanol	E+L2	PEG	PEG+L2	Water	W+L2	W+L3	W-US5+L2	W-US5+L2	W-US10	W-US1D+L1	W-US10+L3	W-US15	W-US15+L2
Added Prapolis	500.00	500.00	500.00	500.00	500.00	500.00	500.00	500.00	500.00	500.00	500.00	500.00	500.00	500.00
Ferulic acid ng/mL	31.31	35.07	7.52	6.59	77.62	81.66	25.18	138.71	58.53	110.65	60.99	18.44	122.10	57.45
Caffeic add ng/mL	5.01	4.49	0.95	1.34	10.35	15.64	3.99	18.60	6.94	15.41	8.61	2.70	18.59	10.60
Kampherol ng/mL	11.33	6.32	8.03	9.04	13.85	20.62	5.48	35.82	10.54	26.33	11.63	3.54	26.84	12.04
Miricetine ng/mL	4.09	1.17	8.34	0.98	4.86	1.25	0.99	10.91	4.57	7.32	4.39	0.94	8.67	0.96
Trans-sinnamic acid ng/mL	299.63	0.00	84.15	0.00	144.21	137.96	9.72	1091.89	34.85	412.57	62.28	0.00	435.00	28.79
Ellagic acid ng/mL	6.25	0.89	10.64	7.19	4.08	17.08	0.00	22.56	4.84	13.30	0.75	0.00	12.60	5.97
CAPE ng/mL	5.44	2.00	1.87	0.87	3.73	4.83	1.49	9.17	2.95	6.84	2.61	1.45	6.92	2.70
DMEA ng/mL	15.90	0.00	0.00	0.00	7.70	15.62	0.00	46.41	0.00	26.95	2.51	0.00	27.28	0.14
Naringenin ng/mL	4.18	0.10	0.08	0.47	0.91	0.82	18.10	31.29	17.60	14.10	0.00	26.11	8.62	0.80
Quercetine ng/mL	8.91	7.31	19.82	14.38	10.88	19.02	4.00	26.41	10.15	17.19	9.69	2.54	20.66	9.47
Rutin ng/mL	0.07	0.08	0.36	0.68	0.01	0.11	0.00	0.15	0.05	0.00	0.15	0.00	0.07	0.01
4-Hydroxy Benzole acid	1.87	1.89	1.48	9.10	1.07	3.49	0.76	0.64	0.51	1.97	0.36	1.21	0.74	0.47
Salydlic add ng/mL	0.05	0.05	0.03	0.26	0.03	0.03	0.03	0.05	0.02	0.03	0.05	0.03	0.03	0.03
Gentlsic acid ng/mL	0.00	0.01	0.00	0.20	0.00	0.03	0.01	0.06	0.01	0.01	0.04	0.01	0.01	0.01
Procatechoic acid ng/mL	0.00	0.00	0.00	0.24	0.00	0.01	0.00	0.06	0.01	0.00	0.03	0.00	0.00	0.00
Paracoumaric acid ng/mL	0.00	0.00	0.00	0.00	0.06	2.42	0.00	2.51	0.00	1.62	0.18	0.00	1.65	0.00
Vanillc Acid ng/mL	0.50	0.57	0.75	4.16	0.45	0.99	0.26	0.78	0.59	0.47	0.92	0.54	1.40	0.86
Chlorogenic Acid ng/mL	0.00	1.00	1.07	54.31	1.04	1.15	0.09	0.52	2.07	0.80	0.70	0.00	0.75	0.77

Propolis samples with a weight-to-volume ratio of 1:1 were treated with different Solutions, including ethanol (10%)=E. poly-ethylene glycol (40%)=PEG and water=w, and subjected to ultrasonication= US at a frequency of 40 Hz (5, 10 and 15 min) for samples dissolved in water. Biotransformation was carried out using *L. plantarum* strains= L1 (ISLG-2), L2 (ATCC®8014), and L3 (Visbyvac).

were found at concentrations of 250, 500, and 1500 µg/mL at 24 h, 250 and 500 µg/mL at 48 h, and 500 µg/mL at 72 h. When water-dissolved propolis samples (untransformed) and water-dissolved propolis samples (transformed) inoculated with 3.5% *L. plantarum* visbyvac strain were compared at 24, 48, and 72 h, the concentrations were 250–1500 g/mL, 250–500 g/mL, and 50–500 g/mL, respectively. Significant differences were discovered when propolis samples inoculated with 2.5% *L. plantarum* ATCC®8014 strain (transformed) and 3.5% *L. plantarum* visbyvac strain (transformed) dissolved in water were examined. When 3.5% *L. plantarum* visbyvac strain-inoculated propolis samples were compared, a significant difference was reported at concentrations of 500 g/mL at 24 h, 100-500 g/mL at 48 h, and 50 g/mL at 72 h. At 24 h, the cytotoxic effect value of the propolis sample inoculated with 3.5% *L. plantarum* visbyvac strain at a concentration of 500 µg/mL was 2.59 times higher than that of the sample inoculated with 2.5% *L. plantarum* ATCC®8014 strain. At 100 and 250 µg/mL concentrations, the propolis sample inoculated with 2.5% *L. plantarum* ATCC®8014 strain was 3.12 and 1.8 times more cytotoxic than the sample inoculated with 3.5% *L. plantarum* visbyvac strain.

Anti-Inflammatory Effects of Propolis Extracts

In this study, allopurinol and gossypol were used as standard molecules to investigate anti-inflammatory response. TNF-α levels increased up to 20 times compared to control samples upon administration of LPS. While IFN-γ levels elevated 5 times compared to the controls, IL-1α, IL-1β, and IL-6 levels showed elevation two times and more than those of the control cells ($p<0.01$). IL-10 slightly increased ($p<0.05$), but NF-kβ did not show any statistically significant change upon LPS induction. None of the propolis samples affected NF-kβ levels (Figure 1).

As can be seen in the Figure 1, all propolis samples significantly decreased TNF-α levels, in particular, propolis samples solvated in "water (W)," in "water + sonicated 15 min (W+US15)," and in "water+sonicated 15 min+biotransformed with *L. plantarum* ATCC®8014 (W+US15+L2)" returned TNF-α to normal levels.

Four extract types (dissolved in water, in ethanol biotransformed with *L. plantarum* ATCC®8014 (L2), in water biotransformed with *L. plantarum* ATCC®8014 and in "water and sonicated for 15 min, biotransformed with *L. plantarum* ATCC®8014) decreased all cytokine levels except NF-kβ. Two extract types dissolved in water and sonicated 15 min and in water+sonicated for 10 min and biotransformed with *L. plantarum* visbyvac (L3) decreased all cytokine levels except IL-6 and NF-kβ. Propolis samples dissolved in ethanol and biotransformed with *L. plantarum* ATCC®8014, water and sonicated for 15 min, biotransformed with *L. plantarum* ATCC®8014, in water+ biotransformed with *L. plantarum* visbyvac and in water+biotransformed with *L. plantarum* ATCC®8014 returned IL-6 to normal levels ($p<0.01$; Figure 1)

Two extract types dissolved in PEG and biotransformed with *L. plantarum* ATCC®8014 (L2) and in water and biotransformed with *L. plantarum* visbyvac (L3) decreased all cytokine levels except IL-1β and NF-kβ (Figure 1).

While 3 extract types (dissolved in "ethanol (E)," in "water +biotransformed with *L. plantarum* ATCC®8014 (W+L2)," and in "water+sonicated for 10 min+biotransformed with *L. plantarum* ISLG-2 (W+US10+L1)") did not show any effect

Table 2. The IC_{50} levels of propolis on THP-1 cell line were determined at the 24^{th}, 48^{th}, and 72^{nd} h. Biotransformation was performed using *L. plantarum* strains, including L1 (ISLG-2), L2 (ATCC®8014) and L3 (Visbyvac).

Propolis samples	ICso values (concentration range, pg/mL)		
	24 h	48 h	72 h
Water + 40 kHz/5 min US	363.89 (250-1500 pg/mL)	290.89*** (50-1500 pg/mL)	166.49 (25-1500 pg/mL)
Dissolved in water, 40 kHz/5 min ultrasound application and 1.5% *L. plantarum* L2 strain inoculated propolis	398.98 (100-1500 pg/mL)	378.87 (100-1500 pg/mL)	402.17 (100-1500 pg/mL)
Dissolved in water and 40 kHz/15 min propolis with ultrasound application	427.96 (100-1500 pg/mL)	337.14 (100-1500 pg/mL)	347.65 (100-1500 pg/mL)
Dissolved in water, 40 kHz/15 min ultrasound application and 2.5% *L. plantarum* L2 strain inoculated propolis	598.69 (100-1500 pg/mL)	304.63*** (25-1500 pg/mL)	426.77 (100-1500 pg/mL)
Dissolved in water and 40 kHz/10 min propolis with ultrasound application	309.47 (25-1500 pg/mL)	417.92 (250-1500 pg/mL)	264.19 (25-1500 pg/mL)
Dissolved in water, 40 kHz/10 min ultrasound application and 2.5% of *L. plantarum* L1 strain inoculated propolis	262.59** (50-1500 pg/mL)	272.19* (50-1500 pg/mL)	205.45*** (25-1500 pg/mL)
Dissolved in water, 40 kHz/10 min ultrasound application and 3.5% L. *plantarum* L3 strain inoculated propolis	1012.26 (50-1500 pg/mL)	577.98 (50-1500 pg/mL)	947.66 (250-1500 pg/mL)
Propolis dissolved in water	429.91 (100-1500 pg/mL)	298.36*** (25-1500 pg/mL)	216.88 (100-1500 pg/mL)
Propolis dissolved in water and inoculated with 2.5% *L. plantarum* L2 strain	673.77 (50-1500 pg/mL)	352.13** (25-1500 pg/mL)	519.31 (100-1500 pg/mL)
Propolis dissolved in water and inoculated with 3.5% *L. plantarum* L3 strain	668.83 (100-1500 pg/mL)	480.62* (25-1500 pg/mL)	452.46 (100-1500 pg/mL)
Propolis dissolved in 10% ethyl alcohol	446.31 (25-1500 pg/mL)	468.68 (100-1500 pg/mL)	276.24 (25-1500 pg/mL)
Dissolved in 10% ethyl alcohol, 2.5% *L. plantarum* Culture-1 inoculated propolis	675.49 (50-1500 pg/mL)	526.85 (25-1500 pg/mL)	618.68 (25-1500 pg/mL)
Propolis dissolved in 50% PEG	303.14** (100-1500 pg/mL)	358.41 (100-1500 pg/mL)	405.82 (100-1500 pg/mL)
Dissolved in 50% PEG and 3.5% *L. plantarum* Culture-2 inoculated propolis	731.34 (100-1500 pg/mL)	356.59 (25-1500 pg/mL)	491.38 (25-1500 pg/mL)
Gossypol	5.98 pM	2.77 pM***	3.6 pM
Allopurinol	---	---	374.1 pM

*p<0.05, *p<0.01 and *p<0.001 according to control.

on IL-10 levels, all other propolis samples decreased IL-10 levels.

While two extract types of propolis (dissolved in "PEG" and in "water+sonicated 5 min") did not show any effect on IFN-γ levels, all other propolis samples decreased IFN-γ levels.

Ferulic acid and caffeic acid content of propolis extracts were inversely correlated with TNF-α levels (r=-0.479, p=0.001 and r=-0.485, p=0.001, respectively) of LPS induced THP-1 cells. The highest levels of ferulic acid and caffeic acid were found in propolis samples dissolved in "water+sonicated 5/10/15 min" and in propolis samples dissolved in "water+biotransformed with *L. plantarum* ATCC®8014".

COX-1 levels in LPS induced THP-1 cells were inversely correlated with pelargonin (r=-0.734, p=0.003) and naringenin (r=-0.483, p=0.08) levels in propolis samples.

NF-kβ levels in the propolis-added THP-1 cells (with LPS) were inversely correlated with ferulic acid (r=-0.295, p=0.005), trans-cinnamic acid (r=-0.395, p=0.01), and CAPE (r=-0.354, p=0.021). TNF-α levels showed negative correlations with trans-cinnamic acid (r=-0.395, p=0.01), CAPE (r=-0.415, p=0.006), kaempferol (r=-0.426, p=0.005), and DMEA

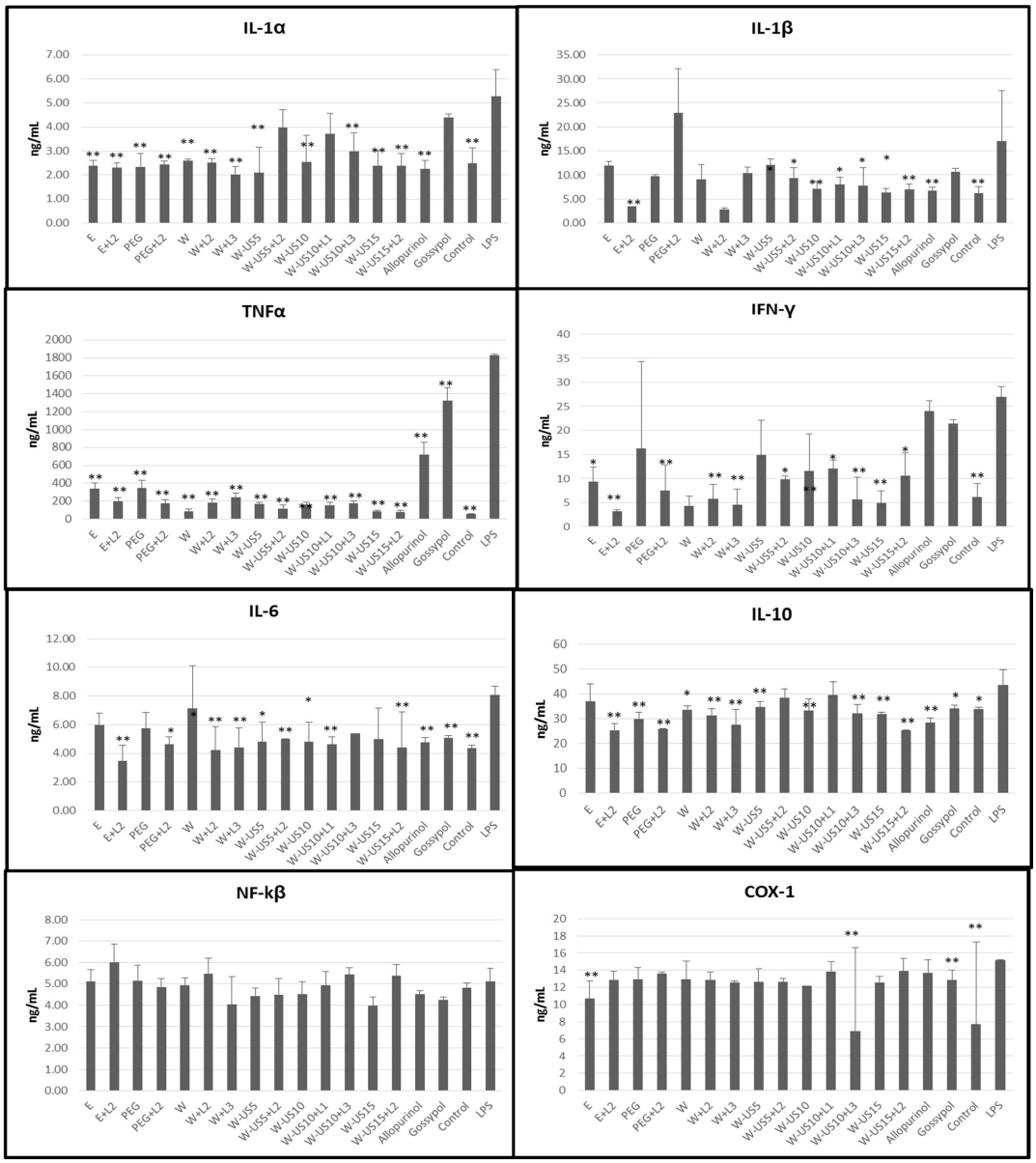

Figure 1. NF-kβ, IL-1α, IL-1β, IL-6, IL-10, TNF-α, IFN-γ, and COX-1 levels in supernatant of propolis-added THP-1 cells. (*p<0.05 and **p<0.01) *Comparisons were made versus only LPS-added cell-line. Propolis (w/v:1/1) were extracted with different solutions (ethanol (10%)=E, poly-ethylene glycol (40%)=PEG, and water=w) and ultrasonication= US was applied at 40 Hz (5, 10, and 15 min) for samples that were dissolved in water. *L. plantarum* strains= L1, L2, L3 (ISLG-2, ATCC®8014, and Visbyvac, respectively) were used for biotransformation.

caffeic acid (r=-0.338, p=0.029) levels. There is an inverse correlation between IL-1α levels and ferulic acid (r=-0.428, p=0.005), caffeic acid (r=-0.391, p=0.01), and CAPE (r=-0.349, p=0.023) levels. In the present work, the first two principal components with eigenvalues 7.1 and 2.5 accounted for 44.4% and 15.7% of the dataset. The first principal component (PC1) is correlated positively with the phenols (kaempferol, DMEA-CA, cafeic acid, CAPE, trans-cinnamic acid, ferulic acid, myricetin, quercetin, and naringenin) and PC2 is correlated with TNF-α, IFN-γ, IL-6, IL1α, IL-1β, and COX-1.

As can be seen from Figure 2, kaempferol, DMEA-CA, Cafeic acid, CAPE, trans-cinnamic acid, ferulic acid, myricetin, quercetin, and naringenin are presented on the positive side of Figure-2. TNF-α, IL-1α, IL-1β and NF-kβ are on the left side of the Figure 2. Similarly to our correlation analysis, PCA showed that TNF-α, IL-1α, NF-kβ, and IL-1β were inversely correlated with ferulic acid, CAPE, DMEACA, cafeic acid, kaemferol, and trans-cinnamic acid. It was also shown that IFN-γ, COX-1, and IL-6 were clustered in the same area and inversely correlated with naringenin and pelargonium.

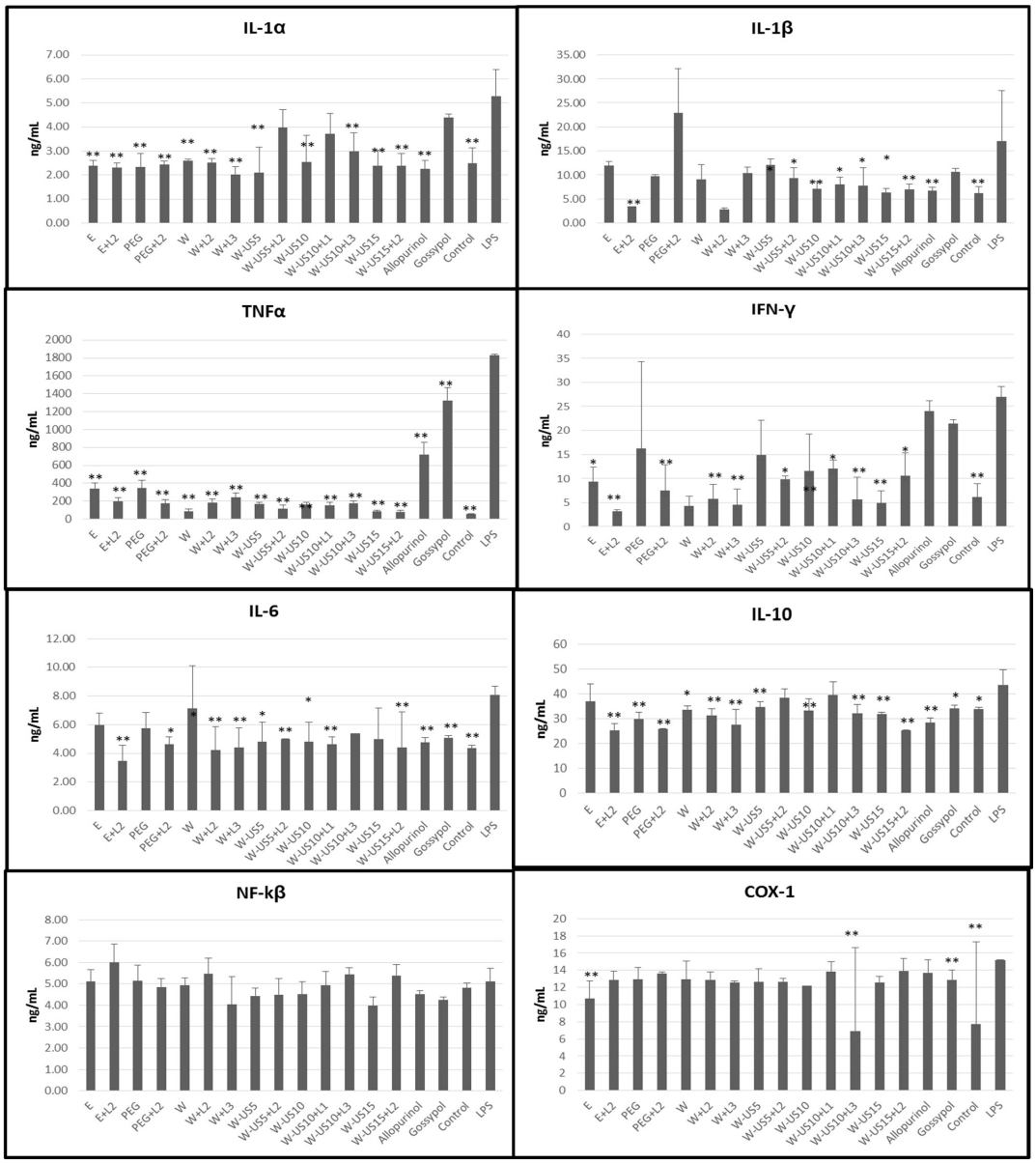

Figure 2. PC1 and PC2 correlation between phenolic compounds of propolis and inflammation markers *The first Principal component (PC1) is correlated positively with the phenols (kaempferol, DMEA-CA, cafeic acid, CAPE, trans-cinnamic acid, ferulic acid, miricetin, quercetin, and naringenin) and PC2 correlated is correlated with TNF-α, IFN-γ, IL-6, IL-1α, IL-1β, and COX-1.

DISCUSSION

When we evaluated the phenolic content, we observed that phenolic content of propolis from lowest to highest are as follows: Water+US10min+*L.plantarum* visbyvac < Ethanol+*L.plantarum* ATCC®8014 < Water+*L.plantarum* visbyvac < PEG+*L. plantarum* ATCC®8014 < Water+US15min+*L.plantarum* ATCC®8014. The highest phenolic content was in W+US5min, W+US10min, and W+US15min. Biologically transformed samples have had slightly lower total phenolic content due to a decrease in caffeic acid and DMEA by transformation process. Biologically transformed samples have had slightly lower total phenolic content due to a decrease in caffeic acid and DMEA by the transformation process.

Propolis is one the most studied natural compounds due to its antioxidant, anti-inflammatory, and cytotoxic effects in many cell lines.[8,12,23–25] The authors observed that the phenolic content, antioxidant, and anti-cancer effects of propolis samples varied according to different factors including solvents used, preliminary extraction process, and collected regions.[26] In this study, we investigated the effect of the solvation procedure and biotransformation on the anti-inflammatory effect

of propolis in THP-1 cells. Generally, extraction procedures, i.e., solvents used, culture strains, and inoculum concentrations of *L. plantarum* affect the extracted amount of phenolic content and its anti-inflammatory activity, which has been shown by determining the levels of inflammatory cytokines in cell supernatants.

The IC_{50} levels of propolis samples changed between 160 µg/mL and 1000 µg/mL in the 24^{th} h. The THP-1 cell line is commonly used as *in vitro* model in investigations on inflammatory processes.[3] It is well known that monocytes in a lesion area differentiate into macrophages, which release inflammatory cytokines. *In vitro* studies showed that THP-1 cells become activated by LPS and/or pro-inflammatory molecules. Chanput et al. observed that LPS-induced THP-1 cells released the cytokines such as IL-1β, IL-6, IL-8, IL-10, and TNF-α associated with inflammation.[3,27] THP-1 monocytes are transformed to macrophages in response to LPS induction, which is more effective than PMA induction.[27] It has been observed that propolis-treated LPS-activated macrophages were polarized to M1 phenotype, which has a role in producing pro-inflammatory cytokines and mediators (TNF-β, IL-1β, IL-6, IL-8, and IL-12) for immune defense against microorganisms.[1,3] In accordance with this data, elevation in different ratios of TNF-α, IL-1α, IL-1β, and IL-6 levels upon induction with LPS were determined in our study. All extracts of propolis decreased TNF-α levels to normal levels. Bueno-Silva et al. showed that Brazilian Propolis reduced the production of proinflammatory factors such as NO, IL-12, IL-1β, and GM-CSF, compared to only LPS-treated control macrophages.[28]

We observed a negative correlation between the TNF-α levels in cell line and the caffeic acid and ferulic acid content of propolis. Touzani et al. showed that propolis in a similar dose (250 µg/mL) to our study inhibited the TNF-α and IL-6 secretion down to control levels.[12] Girgin, et al. also assessed that propolis inhibited production of proinflammatory cytokines (IFN-γ and TNF-β).[29] Ethanolic extract of propolis suppressed production of IL-1α, IL-1β, IL-6, IL-12, and TNF-α in the J774A.1 macrophage cell line.[30,31] In our study, 4 extracts of propolis (dissolved in "water," in "ethanol+biotransformed with *L. plantarum* ATCC®8014," in "water+biotransformed with *L. plantarum* ATCC®8014," and in "water+sonicated 15 min+ biotransformed with *L. plantarum* ATCC®8014") were found to be the most effective in reducing all cytokine levels. Because of their phenolic content in terms of trans-cinnamic acid, kaempferol, ferulic acid, quercetin, and caffeic acid levels. These 4 extracts of propolis were found to be higher than those of other propolis samples (Figure 1).

Many authors have suggested that the main phenolic molecules, such as caffeic acid, caffeic acid phenyl ester, chrysin, kaempferol, quercetin, cinnamic acid, ferulic acid, and chlorogenic acid, in propolis are responsible for its anti-inflammatory activity.[15,25,32,33] According to our results, these molecules might inhibit NFk-β pathway, NO signaling pathway, and COX-1/COX-2 expression. Although CAPE and phenyl esters of caffeic acid were asserted as the main anti-inflammatory molecules in propolis, they might lead to allergic reactions in susceptible subjects. Our data evidenced that propolis samples with lowered CAPE and/DMEA-caffeic acid levels by biological transformation showed anti-inflammatory activity in the same ratio with non-transformed propolis samples.

Bueno-Silva et al. proposed that propolis inhibited different pathways in inflammatory process: a) down regulation of IL-1β resulting in inhibition of IL-1β pathway and subsequent inhibition of NF-kβ pathway; b) reduced activation of MAPK pathway resulting in the reduction of IL-12 levels c) depletion of activation of PI3K/AKT pathway; d) inhibition of NO pathway resulting in low NO production and e) down-regulation of the expression of genes related to Toll-like receptor (TLR) response (Cd14, Elk1, Pik3cg, Tirap, and Tlr4).[28] In our study, propolis samples decreased the IL-1β levels but had no effect on NF-kβ, which has some the following logical explanations.[1] Exposure time to LPS and propolis and timing of sample collection might be important, because NF-kβ is produced in the early stages of inflammation and the studies showed that the peak of degradation occurred in the 30^{th} min [9,31], so its level in the 2^{nd} h of induction might decreased.[2] Nakayama et al. showed that TNF-like weak inducer of apoptosis (TWEAK) expression, which induces signaling cascades including the NF-kβ, MAPK, and AKT pathways, did not increase upon stimulation with LPS but increased with IFN-γ.[33] This observation might explain the non-effectiveness of LPS on NF-kβ levels in our study. It has been shown that ethanolic extract of propolis suppressed production and transcription of IL-1β and IL-6 by depletion in mRNA levels of IL-1β and IL-6 induced by LPS in RAW 264.7 cells and J744A.1 cells.[34,35] These effects of propolis are dependent on time and dose. It has been shown that pro-IL-1β levels increased 90 min after stimulation by LPS and ATP, and were released from cells in the 2nd h.[33] IL-1β induced production of IL-8, IL-6, TNF-α, and prostaglandin E2. In this study, IL1β levels increased up to 2 times accompanied by increases in TNF-α and IL-6 levels.

In our study, almost all propolis samples decreased IL-6 and IL-1β in the 2^{nd} h, however, 4 of the propolis extracts (1. dissolved in Ethanol+biotransformed with *L. plantarum* ATCC®8014;" 2. "dissolved in water+sonicated 15 min and biotransformed with *L. plantarum* ATCC®8014;" 3. "dissolved in water and biotransformed with *L. plantarum* visbyvac;" and 4. "Dissolved in water and biotransformed with *L. plantarum* ATCC®8014") that returned IL-6 to normal levels were proposed as the most effective samples. It has been noted that IL-10 has an anti-inflammatory effect by inhibiting pro-inflammatory cytokines and by blocking NF-kβ activity.[33] The effect of propolis on IL-10 levels were conflicting in the literature due to the dose used and duration of treatment.[33,36]

In some studies, an increase was observed upon stimulation by LPS.[12,25] In accordance with these observations, we found a slight increase in IL-10 levels upon stimulation by LPS. The three extracts (1. dissolved in ethanol, 2. dissolved in water+biotransformed with *L. plantarum* ATCC®8014, and 3. dissolved in water+sonicated 10 min+biotransformed with *L. plantarum* ISLG-2 did not show any effect on IL-10 levels. All of these extracts of propolis have had a low concentration of naringenin followed by pelargonin, ellagic acid, vanillic acid, and myricetin. IL-10 levels showed negative correlation with trans-cinnamic acid (r=-0.495, p=0.072) and DMEA caffeic acid (r=-0.538, p=0.047) levels. Although there is no clear evidence on the suppressive effect of DMEA caffeic acid on IL-10, this correlation might suggest an inhibitory effect of DMEA caffeic acid. In regard to this explanation, low levels of DMEA caffeic acid in biologically transformed propolis samples might be an advantage to protect IL-10 levels while pro-inflammatory cytokine levels are decreasing. Touzani et al. also showed that treatment with propolis at concentrations of 125 µg/mL and 250 µg/mL increased the secretion of IL-10 compared to LPS-stimulated cells and suggested that the effect mechanism of propolis on IL-6 and TNF-α is mediated through by induction of IL-10 production.[12]

Two extracts of propolis (dissolved in PEG and dissolved in water+sonicated 5 min) did not show any effect on IFN-γ levels. All of these propolis samples have had a low concentration of naringenin followed by caffeic acid and ellagic acid. The negative correlation between naringenin levels and IFN-γ levels supported the inhibitory effect of naringenin during inflammation. In accordance with this, propolis dissolved in PEG (with or without biotransformation) has the lowest anti-inflammatory effect due to their low phenolic content, especially caffeic acid, pelargonidin, and naringenin.

Our study and the others showed that the anti-inflammatory activity of propolis is closely associated with its biologically active constituent, especially caffeic acid, kaempferol, ferulic acid, quercetin, and naringenin, which are found in different concentrations.[30,36,37] Although concentrations and type of active molecules in propolis samples varied with solvents and extraction procedures as well as collection season and region, it has been proposed that the anti-inflammatory activity of propolis is exacerbated with the synergistic and/or antagonistic effects of all these molecules.[10] Our study has also demonstrated for the first time that, biologically, transformation of propolis did not affect its anti-inflammatory activity in LPS-induced THP-1 cells.

CONCLUSION

We proposed that extracts of propolis (1) dissolved in water, (2) dissolved in ethanol+biotransformed with *L. plantarum* ATCC®8014, (3) dissolved in water+biotransformed with *L. plantarum* ATCC®8014, (4) dissolved in water+sonicated 15 min, and biotransformed with *L. plantarum* ATCC®8014 have the highest anti-inflammatory activity. Since the biotransformation process did not affect propolis's anti-inflammatory activity, it can be used as a reliable agent for allergic subjects. It should be noted that it is crucially important to determine the biologically active molecule content to get the beneficial effect on health.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.Y.S.; Data Acquisition- E.Y.S., N.S.G., H.M., L.M.O..; Data Analysis/Interpretation- E.Y.H., H.K.Y.; Drafting Manuscript- E.Y.S., B.D.; Critical Revision of Manuscript- E.Y.S., B.D.; Final Approval and Accountability- B.D., L.M.O., H.M., N.S.G., H.K.Y., E.Y.S.

Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: Authors declared no financial support.

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How cite this article

Durmaz B, Oktay LM, Memmedov H, Gunel NS, Kalkan Yildirim H, Yildirim Sozmen E. Solvation Methods Affect the Amount of Active Components in the Extract of Propolis as well as Its Anti-Inflammatory Activity in THP-1 Cells. Eur J Biol 2023; 82(2): 132–141. DOI: 10.26650/EurJBiol.2023.1247199