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Research Article

Polyphenolic composition and biological activities of Bulgarian propolis samples with potential pharmaceutical applications

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Abstract

Propolis (bee glue) is a complex biological substance produced by honeybee (*Apis mellifera* L.) and used as a natural remedy due to its therapeutic properties. The present research aimed to investigate the phenolic content, antioxidant, acetylcholinesterase inhibitory, antihemolytic, and *ex vivo* anti-inflammatory activities of DMSO extracts of six propolis samples (P1-P6) from three regions of Bulgaria. The DMSO extracts exhibited TPC values from 189.30 to 290.80 mg GAE.g⁻¹, TFC values from 64.41 to 151.60 mg QE.g⁻¹ and significant antioxidant activity (863.73 - 1799.22 mM TE.g⁻¹ by the DPPH assay, and 796.86 - 1168.02 mM TE.g⁻¹ by the FRAP assay). The values of AChE inhibitory activity at concentration of 1 mg.ml⁻¹ ranged between 40.12 and 46.44% (IC₅₀ = 1.07 - 1.39 mg.ml⁻¹). All extracts demonstrated high antihemolytic activity, which at concentration of 0.5 mg.ml⁻¹ showed erythrocyte protection of 76.28 - 89.32% (IC₅₀ = 0.06 - 0.14 mg.ml⁻¹). The results of the immunohistochemical analysis showed that extracts P1, P2, P3, P4 and P6 suppressed the expression of IL-1 β and NOS3 in varying degree. The samples P1 and P2 completely suppressed NOS3 expression and strongly reduced the intensity of IL-1 β expression; consequently, they had the highest *ex vivo* anti-inflammatory activity. Based on the results, we can conclude that the investigated propolis samples possessed promising antioxidant and anti-inflammatory potential. Therefore, they can find application as prospective therapeutic agents.

Keywords

propolis, anti-acetylcholinesterase activity, antihemolytic activity, anti-inflammatory activity

Abbreviations

ACh – acetylcholine; AChE – acetylcholinesterase; DMSO – dimethylsulphoxide; DPPH – 2,2-diphenyl-1-picrylhydrazyl (assay); FRAP – Ferric-reducing antioxidant power (assay); IC₅₀ – half maximal inhibitory concentration value; IL-1 β – interleukin 1 β ; NOS3 – nitric oxide synthase; TFC – total flavonoid content; TPC – total phenolic content

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Introduction

Propolis, popular also as bee glue, is a complex mixture of biological substances produced by honeybees (*Apis mellifera* L.). It is used mainly as a building material (to repair damaged cells of honeycomb, fill cracks, smooth out the inner beehive walls, reduce the size of the entrance, thus regulating the temperature during cold seasons), but it also helps prevent infections in the bee colony by mummifying the invaders that have penetrated and died inside the hive, thus avoiding their decomposition and maintaining aseptic conditions (Przybyłek and Karpiński 2019; Kasote et al. 2022). Production of propolis is a complex physiological and biochemical process that begins with the collection of various plant exudates by worker bees, which subsequently enrich the collected material with secretions and beeswax, conferring its specific physicochemical properties (Almuhayawi 2020).

Similar to the other bee products, the chemical composition of propolis is highly variable and can be influenced by multiple factors, such as the botanical source, food availability, bee race, bee diseases, harvesting season and environmental conditions in the certain geographical area (Mountford-McAuley et al. 2021). Generally, propolis is a biological product consisting of lipids (50%), beeswax (30%), essential oils (10%), pollen (5%) and organic compounds (5%). The organic components include carboxylic acids (20%), terpenoids (15%), steroids (12%), hydrocarbons (10%), sugars (6%), alkaloids (6%), flavonoids (4%), phenols (3%), vitamins (2%), amino acids (2%), ketones (2%), proteins (1%), and other compounds (14%). The major biologically active compounds of propolis are polyphenols presented by flavonoids (flavanones, flavones, flavonols and dihydro-flavonols), hydroquinones, phenolic acids and esters and phenolic aldehydes. It has been found that propolis contains numerous nonphenolic substances belonging to different classes such as aliphatic acids and aliphatic esters, coumarins, aliphatic and aromatic hydrocarbons, terpenoids, steroids and isoprenylated benzophenones. Propolis has also proved to be a rich source of amino acids, macro- and microelements (Mg, Ni, Ca, Fe, Zn, Cs, Mn, Ag, Cu, Al, V), and vitamins B, C and E that also contribute to its biological activities (Hossain et al. 2022; Zullkiflee et al. 2022). It should be noted here that the amounts of chemical constituents in the

propolis extracts can vary greatly depending on the type of solvent and extraction method used (Bankova et al. 2021).

The most common biologically active compounds that determine the therapeutic potential of propolis belong to the group of phenolic compounds, namely flavonoids (apigenin, chrysin, galangin, luteolin, kaempferol, pinobanksin, pinocembrin, quercetin) and phenolic acids (cinnamic acid, ferulic acid, p-coumaric acid and its derivative artepillin C, caffeic acid and its caffeic acid phenethyl ester - CAPE) (Zullkiflee et al. 2022). They contribute to the significant anti-cancer (Forma and Bryś 2021; Oršolić et al. 2006; Oršolić and Jembrek 2022; Catchpole et al. 2015), anti-inflammatory (Mendez-Encinas et al. 2023; Zuhendri et al. 2022; Funakoshi-Tago et al. 2015), antioxidant, antimicrobial (Nichitai et al. 2021; Peixoto et al. 2021; Altuntaş et al. 2023), antiviral, immunomodulatory (Wolska et al. 2019; Ripari et al. 2021; Magnavacca et al. 2022), acetyl-cholinesterase inhibitory (El-Guendouz et al. 2016; Miguel et al. 2014), anti-allergic (Liew et al. 2022), anti-diabetic (El-Guendouz et al. 2016; Shahinozzaman et al. 2018), anti-atherogenic and anti-angiogenic (Daleprane et al. 2012) activities of propolis.

The search for biologically active compounds as an alternative to conventional anti-inflammatory agents, which are known to possess negative effects on the organism, especially with prolonged use, is of special interest to the pharmaceutical industry. Inflammation is a defensive response of the organism to external and internal stimuli resulting from mechanical injury or pathogen infections. In the early phases of the inflammatory process macrophages trigger the release of pro-inflammatory cytokines, such as interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF- α). Besides being involved in the expression of pro-inflammatory and inflammatory cytokines, macrophages also stimulate the activation of nuclear factor kappa B (NF- κ B) that mediates the expression of genes implicated in the apoptosis. Nitric oxide (NO) is another mediator of inflammation that can damage tissues when produced in excess by the inflamed endothelial cells. In some cases, untreated or uncontrolled inflammation process may result in chronic inflammation diseases, including diabetes, rheumatoid arthritis, cardiovascular and respiratory

disorders, and even cancer (Mendez-Encinas et al. 2023). Different mechanisms of the anti-inflammatory activity of propolis and its components (phenolic compounds, terpenoids, steroids and amino acids) have been reported, the main one being the inhibition of mediators of inflammation (Araujo et al. 2012).

Presently, acetylcholinesterase inhibitory activity is not among the most studied therapeutic activities of propolis. Acetylcholinesterase (AChE) is a key enzyme, involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) in the synaptic gaps of the central and peripheral nervous system. By reducing the ACh level in the synapses, AChE terminates the nerve impulses. The abnormal hydrolysis of ACh, leading to its progressive decline, is associated with the development of some neurodegenerative conditions with loss of cognitive functions, such as Alzheimer's disease (AD). Therefore, the application of AChE inhibitors in the treatment of AD is crucial for slowing and stabilizing ACh hydrolysis (Necip et al. 2024; Shahinozzaman et al. 2018). *Myasthenia gravis* (MG) is an autoimmune disorder of neuromuscular transmission characterized by the production of antibodies against the acetylcholine receptors and clinically manifested with pronounced weakness and fatigue of the voluntary muscles (Sieb 2014). The application of AChE inhibitors in the treatment of MG does not modify the pathological immune process, but enhances the neuromuscular transmission by slowing the hydrolysis of ACh in the synapses, thus prolonging the interaction of ACh with its muscle membrane receptor. Therefore, AChE inhibitors are recommended in addition to the basic immunosuppressive therapy in patients with MG (Maggi and Mantegazza 2011). As a source of bioactive compounds with significant AChE inhibitory potential, propolis can be successfully used as a natural substitute for synthetic AChE inhibitors, thereby overcoming their adverse health effects (toxicity).

As a continuation of our previous research on six selected Bulgarian propolis samples, this study aimed to investigate polyphenolic content and anti-oxidant activity of their dimethylsulphoxide (DMSO) extracts and to compare the results with those for the ethanolic extracts the same propolis (Tumbariski et al. 2025) as well as to extend the knowledge about their potential therapeutic effects,

such as anti-acetylcholinesterase, antihemolytic and *ex vivo* anti-inflammatory activities.

Materials and Methods

Materials

Raw propolis material. The propolis samples (n=6) (Table 1) were investigated in our previous research (Tumbariski et al. 2025). The raw propolis material was collected by beekeepers from six different locations in Northern Bulgaria in 2022, thereafter the samples were delivered to the laboratory by a courier. The samples were labelled and stored in plastic containers at room temperature in darkness until analysis.

Table 1. Origin of the propolis samples

Propolis sample*	Village	District	GPS Coordinates
P1	Gamzovo	Vidin	44°05' N 22°45' E
P2	Parsha	Gabrovo	42°57' N 25°29' E
P3	Ritya	Gabrovo	42°59' N 25°25' E
P4	Kozi rog	Gabrovo	42°57' N 25°16' E
P5	Burya	Gabrovo	43°02' N 25°19' E
P6	Malinovo	Lovech	42°90' N 24°90' E

*Coding of the propolis samples corresponds to that in our previous study (Tumbariski et al. 2025)

Animals. Male Wistar rats (n=6) (age of 10-12 weeks) with body weight in the range of 250-280 g were used in the experiment. All animals were housed in the vivarium under standard conditions: living space 350 cm², temperature 22 ± 2°C, relative humidity 55 ± 10%, and 12/12 h light/dark cycle. The rats (2 to 4 animals per cage) had free access to standard rodent food and water. For the purposes of the experiment, animals were anesthetized with ketamine (90 mg.kg⁻¹) and xylazine (10 mg.kg⁻¹) intramuscularly, and then euthanized by cervical decapitation. All animals involved in the experiment were provided by the vivarium of the Medical University of Plovdiv, Bulgaria.

Preparation of propolis extracts. In order to prepare the extracts (20 mg.ml⁻¹), the raw propolis samples (n=6) were finely chopped and macerated with dimethylsulphoxide - DMSO (Carlo Erba Reagents SAS, Val de Reuil, France) as previously described (Tumbariski et al. 2025). To conduct the

extraction, the samples were left for 3 days at room temperature in darkness. Afterwards, the extracts were filtered through a filter paper and stored at identical conditions prior to analyses.

Methods

Total phenolic content. The total phenolic content (TPC) of DMSO propolis extracts was assessed according to the standard method using the Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, USA). The absorbance was measured at 765 nm and the results were expressed as mg equivalents of gallic acid (GAE).g⁻¹ of extract (Ivanov et al. 2014).

Total flavonoid content. The total flavonoid content (TFC) of DMSO propolis extracts was evaluated following the method described by Ivanov et al. (2014). The absorbance was measured at 415 nm and the results were expressed as mg of quercetin equivalents (QE).g⁻¹ of extract.

Total caffeic acid derivatives. The total caffeic acid derivatives (TCADs) of DMSO propolis extracts were determined by the method of Ivanov (2014) using Arnov's reagent. The absorbance was measured at 525 nm and the results were expressed as mg of caffeic acid equivalents (CAE).g⁻¹ of extract.

High-performance liquid chromatography (HPLC) analysis. The phenolic compounds in DMSO propolis extracts were determined using an HPLC unit Elite LaChrome (VWR™ Hitachi, Tokyo, Japan) equipped with a diode array detector (DAD) as previously described (Tumbariski et al. 2025). The results were expressed as mg.g⁻¹ of extract.

Antioxidant activity

DPPH radical scavenging assay. The DPPH assay was performed using DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent (Sigma-Aldrich, St. Louis, USA). The absorbance was measured at 517 nm and the results were expressed as mM Trolox equivalents (TE).g⁻¹ of extract. The half maximal inhibitory concentration (IC₅₀) values were expressed as mg.ml⁻¹ of extract (Ivanov et al. 2014).

Ferric-reducing antioxidant power (FRAP) assay. The FRAP assay was performed using 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich, St. Louis, USA). The absorbance was measured at 593 nm and the results were expressed as mM

Trolox equivalents (TE).g⁻¹ of extract (Ivanov et al. 2014).

Acetylcholinesterase inhibitory activity. Acetylcholinesterase (AChE) inhibitory activity was determined using the colorimetric method of López et al. (2002) with slight modifications by Ivanov et al. (2019). The absorbance was measured at 405 nm and the results were expressed as % inhibition of acetylcholinesterase.

Red blood cell (RBC) membrane stabilization (antihemolytic activity). The red blood cell (RBC) membrane stabilization assay or antihemolytic activity test was performed according to Alamgeer (2019) with minor modifications.

Preparation of solutions. Alsever's solution was prepared by dissolving 0.8 g sodium citrate, 2 g dextrose, 0.05 g citric acid and 0.42 g sodium chloride in 100 ml of distilled water. Hypotonic saline (0.36%) was prepared by dissolving 0.36 g of sodium chloride in 100 ml of distilled water. Isotonic saline (0.85%) was prepared by dissolving 0.85 g of sodium chloride in 100 ml of distilled water. Phosphate buffer saline (PBS) was prepared by dissolving 8 g sodium chloride, 1.44 g disodium hydrogen phosphate, 0.24 g of potassium dihydrogen phosphate and 0.2 g potassium chloride in 1 L of distilled water. The pH of PBS was adjusted to 7.2 - 7.4 using 0.1 N HCl. All solutions were sterilized by autoclaving at 121°C for 20 min (liquid cycle).

Preparation of RBC suspension. Bovine blood sample was taken from a healthy animal and gently mixed during the taking with an equal volume of sterilized Alsever's solution. Thereafter, the blood sample was centrifuged at 3000 rpm for 10 min. The packed blood cells were collected, washed with isotonic saline solution, centrifuged at identical conditions, and then 10% RBC suspension with isosaline was prepared for further analysis.

Experimental procedure. To perform the test, 1 ml of PBS, 2 ml of hypotonic saline, 0.5 ml of DMSO propolis extract at various concentrations (0.5, 0.25, 0.1 and 0.05 mg.ml⁻¹) and 0.5 ml of 10% RBC suspension were mixed. 1 ml of PBS, 2 ml of hypotonic saline, 0.5 ml of DMSO and 0.5 ml of 10% RBC suspension in isosaline was used as a control. 1 ml of PBS, 2 ml of hypotonic saline, 0.5 ml of standard non-steroid and steroid drug

solutions (Aspirin and Prednisolone Cortico) in DMSO at the same concentrations and 0.5 ml of 10% RBC suspension served as additional controls. Next, the assay mixtures were incubated at 37°C for 30 min, centrifuged at 3000 rpm for 10 min, the supernatant was poured in a cuvette and hemoglobin content was estimated spectrophotometrically at 560 nm. Percentage protection of RBC against hemolysis was estimated using the following equation (1):

$$\% \text{ Protection of RBC} = 100 - \frac{OD_{\text{sample}}}{OD_{\text{control}}} \times 100 \quad (1)$$

Ex vivo anti-inflammatory activity. *Ex vivo* anti-inflammatory activity assay was performed according to Milusheva et al. (2023).

Immunohistochemical analysis. In order to obtain smooth muscle preparations (SMPs), the animals were sacrificed, and their stomachs were washed with a physiological serum and cut immediately into stripes (12-13 mm long and 1.0-1.1 mm wide). The SMPs were incubated in 20 ml of Krebs solution containing 50 µL of the tested DMSO propolis extract (20 mg.ml⁻¹) for 1 h. Next, the SMPs were fixed with 10% neutral formalin. After the conventional paraffin wax embedding, serial sections (4 µm thick) were cut to observe the circular and longitudinal layer of the smooth muscle (SM) cells as well as the myenteric plexus of the stomach. They were used for the immunohistochemistry tests.

The sections described above were deparaffinised, and then subjected to the following procedures: detection of antigenic epitopes with citrate buffer, blocking endogenous peroxidase with 3% hydrogen peroxide, blocking endogenous biotin using a kit (ref. No BBK 120, Scy Tek, Lab. Inc., Logan, UT, USA), blocking non-specific binding using a reagent (Superblock, Scy Tek, Lab. Inc., Logan, UT, USA), followed by incubation with interleukin-1β (IL-1β) (E-AB-52153) and nitric oxide synthase (NOS3) (E-AB-70065) (Elabscience Biotechnology Inc., Houston, TX, USA), after which a second 10 min incubation followed, with a biotinylated secondary antibody (ref. No AGL015, Scy Tek Lab. Inc., Logan, UT, USA). The reaction was visualized by 3,3'-diaminobenzidine tetrachloride (DAB, Scy Tek Lab. Inc., Logan, UT, USA), and the slices were counterstained with Mayer's hematoxylin. All microphotographs were taken using a Leica

DM1000 LED microscope (Leica Microsystems GmbH, Oxford, UK), combined with Leica ICC50 W digital camera (Leica Microsystems GmbH, Oxford, UK).

Morphometric analysis. The intensity of the immune reaction in the stomach was measured in arbitrary units (AU) on the slices immunostained for IL-1β and NOS3. Using software, the average intensity of pixels was recorded in arbitrary units in the range 0-256 on microphotographs of the stomach, 0 being black, and 256 being white. A minimum of 50 points were measured in the stomach at magnification ×400. All measurements involved five slices per animal and an examination of all cross-sections of the stomach. The measurements were performed using the LAS X software (Leica Microsystems GmbH, Oxford, UK).

Statistical analysis. The results from triplicate experiments were expressed as mean values ± standard deviation (±SEM). One-way analysis of variance (ANOVA) was performed using the Statgraphics Centurion statistical program version XVI, 2009 (Stat Point Technologies, Inc., Warrenton, VA, USA). The mean differences were established by Fisher's least significant difference test for paired comparison with a significance level $p < 0.05$.

One-sample t-test and Wilcoxon test were used for the immunohistochemical analysis. Quantitative data were analyzed using the GraphPad Prism software (GraphPad Software 8.0.1 version, Inc., La Jolla, CA, USA). Asterisk indicates significant differences between groups - *** $p < 0.001$.

Results and Discussion

Total phenolic content (TPC), total flavonoid content (TFC), total caffeic acid derivatives content (TCADC), antioxidant activity and phenolic profile of DMSO propolis extracts. As seen from the results in Table 2, propolis extract P4 exhibited the highest TPC, TFC and TCADC values. Propolis extract P3 showed the lowest TPC and TCADC, while extract P1 had the lowest TFC values. TPC values of DMSO extracts were similar compared to the 70% ethanolic extracts of the same propolis samples at the same concentration (190.4 - 317.0 mg GAE.g⁻¹) tested in our previous research (Tumbariski et al. 2025). It is noteworthy that the

TFC and TCADC values of DMSO extracts were significantly higher than those obtained by ethanolic extraction (53.4 - 79.3 mg QE.g⁻¹ and 5.9 - 12.1 mg CAE.g⁻¹, respectively) at the same concentration as we reported earlier (Tumbariski et al. 2025).

Consequently, the type of solvent used for propolis extraction is an important factor determining the amount of biologically active compounds in the extracts.

Table 2. Total phenolic content (TPC), total flavonoid content (TFC) and total caffeic acid derivatives content (TCADC) of DMSO propolis extracts (20 mg.ml⁻¹)

Propolis sample	Total phenols, mg GAE.g ⁻¹ of extract	Total flavonoids, mg QE.g ⁻¹ of extract	Total caffeic acid derivatives, mg CAE.g ⁻¹ of extract
P1	213.75 ± 0.30 ^a	64.41 ± 0.26 ^a	15.85 ± 0.69 ^a
P2	275.09 ± 0.60 ^b	136.70 ± 0.13 ^b	18.77 ± 0.30 ^b
P3	189.30 ± 0.10 ^c	95.75 ± 0.55 ^c	11.75 ± 0.85 ^c
P4	290.80 ± 0.23 ^d	151.60 ± 0.66 ^d	21.79 ± 0.31 ^d
P5	256.03 ± 0.50 ^e	125.24 ± 0.19 ^e	19.06 ± 0.27 ^b
P6	226.01 ± 0.95 ^f	114.94 ± 0.52 ^f	15.84 ± 0.21 ^a

^{a-f} Means in the column differ significantly (p < 0.05)

Table 3. Antioxidant activity of DMSO propolis extracts (20 mg.ml⁻¹)

Propolis sample	DPPH, mM TE.g ⁻¹ of extract	IC ₅₀ , mg.ml ⁻¹ of extract	FRAP, mM TE.g ⁻¹ of extract
P1	1257.50 ± 2.12 ^a	0.34 ± 0.02 ^a	976.77 ± 2.06 ^a
P2	1522.55 ± 2.24 ^b	0.29 ± 0.01 ^b	1168.02 ± 2.43 ^b
P3	863.73 ± 1.56 ^c	0.48 ± 0.03 ^c	796.86 ± 0.59 ^c
P4	1799.22 ± 3.02 ^d	0.24 ± 0.01 ^d	1145.24 ± 0.99 ^d
P5	1479.69 ± 1.58 ^e	0.28 ± 0.01 ^b	1080.10 ± 0.67 ^e
P6	1260.21 ± 1.42 ^a	0.32 ± 0.02 ^{ab}	968.07 ± 1.52 ^f

^{a-f} Means in the column differ significantly (p < 0.05)

Antioxidant activity is a biological characteristic that primarily relies on the polyphenolic content. The results presented in Table 3 show that DMSO propolis extracts P4 and P2 had the highest values of the antioxidant capacity determined by the DPPH and FRAP assays, which correlated with the highest TPC and TFC values. Propolis extract P3 exhibited the lowest antioxidant activity by the two methods, which corresponded to the lowest TPC and TCADC values. It should be noted here that the antioxidant activity values of DMSO extracts by the DPPH and FRAP methods were identical to those of ethanolic ones of the same propolis samples at the same concentration (DPPH values from 1000.3 to 1606.0 mM TE.g⁻¹ and FRAP values from 634.1 to 1134.5 mM TE.g⁻¹), as we previously reported (Tumbariski et al. 2025).

The results from the HPLC analysis of DMSO propolis extracts showed the presence of five phenolic acids and four flavonoids in various concentrations (Table 4), which were consistent with those obtained for the 70% ethanolic extracts of the same propolis samples. In addition, DMSO extracts exhibited higher flavonoid concentrations (isorhamnetin, pinocembrin, chrysin and pinobanksin-3-O-propionate) and higher caffeic acid and caffeic acid benzyl ester (CABE) contents compared to the ethanolic extracts of the same propolis samples examined in our previous research (Tumbariski et al. 2025). Consequently, as a polar solvent, DMSO led to a better extraction of certain bioactive compounds compared to 70% ethanol.

Table 4. Phenolic compounds identified in DMSO propolis extracts by HPLC analysis

Phenolic compounds, mg.g ⁻¹ of extract	DMSO propolis extracts					
	P1	P2	P3	P4	P5	P6
<i>Phenolic acids</i>						
Caffeic	5.18 ± 0.12 ^a	6.60 ± 0.13 ^b	4.21 ± 0.04 ^c	6.85 ± 0.14 ^b	5.17 ± 0.09 ^a	4.98 ± 0.03 ^a
p-coumaric	3.00 ± 0.09 ^a	5.42 ± 0.11 ^b	2.27 ± 0.02 ^c	2.99 ± 0.03 ^a	3.54 ± 0.03 ^c	3.20 ± 0.04 ^d
Sinapic	1.47 ± 0.02 ^a	1.27 ± 0.05 ^b	3.12 ± 0.07 ^c	3.43 ± 0.01 ^d	1.76 ± 0.01 ^e	2.76 ± 0.07 ^f
Cinnamic	0.26 ± 0.02 ^a	1.96 ± 0.01 ^b	0.18 ± 0.00 ^c	0.48 ± 0.00 ^d	0.59 ± 0.01 ^e	1.25 ± 0.02 ^f
CABE*	4.25 ± 0.05 ^a	5.06 ± 0.08 ^b	4.71 ± 0.09 ^c	9.43 ± 0.15 ^d	7.83 ± 0.05 ^e	5.19 ± 0.11 ^f
<i>Flavonoids</i>						
Isorhamnetin	1.91 ± 0.07 ^a	3.51 ± 0.06 ^b	1.84 ± 0.01 ^c	2.85 ± 0.02 ^d	1.98 ± 0.04 ^a	2.21 ± 0.08 ^e
Pinocembrin	11.48 ± 0.15 ^a	36.32 ± 0.17 ^b	11.90 ± 0.10 ^a	18.61 ± 0.13 ^c	25.75 ± 0.26 ^d	22.11 ± 0.23 ^e
Chrysin	31.42 ± 0.55 ^a	53.98 ± 0.36 ^b	59.62 ± 0.44 ^c	81.50 ± 0.62 ^d	82.21 ± 0.57 ^d	81.90 ± 0.39 ^d
Pinobanksin-3-O-propionate	35.26 ± 0.28 ^a	56.85 ± 0.51 ^b	34.27 ± 0.18 ^a	75.28 ± 0.46 ^c	63.74 ± 0.42 ^d	54.28 ± 0.21 ^e

^{a-f} Means in the row differ significantly (p < 0.05)

*CABE - Caffeic acid benzyl ester

Propolis is known as a rich source of polyphenolic compounds, which determine its antioxidant potential and other biological activities. It is important to note that the chemical composition of propolis, especially the polyphenolic content, depends on the plant source, collection site and environmental conditions of the certain geographical region. It has been established that propolis from Central and Eastern Europe, which is collected from the buds of black poplar (*Populus nigra* L.), birch (*Betula pendula* Roth), alder (*Alnus glutinosa* L.), pine (*Pinus sylvestris* L.) and willow species (*Salix* sp. L.) contains mainly phenolic compounds (flavonoids, phenolic acids, and their esters) (Kurek-Górecka et al. 2014), whereas in propolis from the Mediterranean region, where the predominant flora is represented by coniferous species, diterpenes are considered to be the main bioactive constituents (El-Guendouz et al. 2019).

Presently, the literature is deficient in information regarding the polyphenolic content and antioxidant activity of DMSO propolis extracts. Consistent with our results were obtained by Bozkuş and Değer (2022) who determined that DMSO extracts showed

the highest TPC values (141.2 mg GAE.g⁻¹), TFC values (55.3 mg QE.g⁻¹) and antioxidant activity (AOA) by the FRAP method (273.8 mg TE.g⁻¹) compared to the ethanolic (TPC 122.7 mg GAE.g⁻¹, TFC 47.8 mg QE.g⁻¹, AOA 236.9 mg TE.g⁻¹), acetone (TPC 100.0 mg GAE.g⁻¹, TFC 47.3 mg QE.g⁻¹, AOA 221.3 mg TE.g⁻¹), glycerol (TPC 88.0 mg GAE.g⁻¹, TFC 23.3 mg QE.g⁻¹, AOA 141.8 mg TE.g⁻¹) and aqueous (TPC 19.7 mg GAE.g⁻¹, TFC 1.3 mg QE.g⁻¹, AOA 26.2 mg TE.g⁻¹) extracts of the same propolis sample. High values of TPC and TFC of a mixed Turkish propolis sample were reported by Ozdal et al. (2018). The authors found that the TPC of the ethanolic extract was 314.36 mg GAE.g⁻¹, which agreed with our results from the present and the previous study (Tumbariski et al. 2025); however, the TFC value was 522.71 mg QE.g⁻¹, which was significantly higher than our results. The values of antioxidant activity measured by the DPPH and FRAP assays were 391.73 mg TE.g⁻¹ and 156.59 mg TE.g⁻¹, respectively.

In contrast, lower than our results for the total phenolic and flavonoid contents were obtained by Belmehti et al. (2021). The authors investigated

four Moroccan ethanolic propolis extracts and established that TPC values varied from 27.80 to 91.46 mg GAE.g⁻¹, whereas the TFC ranged between 6.14 and 29.79 mg QE.g⁻¹. Significantly lower values compared to our results for DMSO and ethanolic propolis extracts were also reported by [Shehata et al. \(2020\)](#), who examined propolis from Egypt, Saudi Arabia, Oman, China, Bulgaria and Brazil, and determined that TPC values of ethanolic extracts varied between 210.17 and 313.67 mg GAE.100 g⁻¹, while TFC values were from 96.30 and 162.03 mg catechol equivalents (CAT).100 g⁻¹. [Altuntaş et al. \(2023\)](#), who evaluated 24 propolis samples from different regions of Turkey, also reported lower TPC but higher TFC values compared to our results. The TPC values of 70% ethanolic propolis extracts ranged between 16.73 and 125.83 mg GAE.g⁻¹, while the TFC varied from 57.98 to 327.38 mg QE.g⁻¹. In terms of antioxidant activity, the authors stated that values varied from 46.72 to 228.23 mg TE.g⁻¹ according to the DPPH assay and between 61.55 and 378.93 mg TE.g⁻¹

according to the CUPRAC method. A study on 12 ethanolic propolis extracts from Iran conducted by [Fathi Hafshejani et al. \(2023\)](#) revealed that the TPC values varied between 26.59 and 221.38 mg GAE.g⁻¹, which correlated with the lowest (4.8 mg QE.g⁻¹) and the highest (100.03 mg QE.g⁻¹) TFC values, respectively, as well as with the lowest (IC₅₀ = 1031.57 µg.ml⁻¹) and the highest (IC₅₀ = 4.62 µg.ml⁻¹) ability of the same samples to inhibit the DPPH-free radicals.

Acetylcholinesterase inhibitory activity. The results in Table 5 indicate that all DMSO propolis extracts possessed the ability to inhibit acetylcholinesterase (AChE) activity in a dose-dependent manner. As seen from the results, at the highest concentration of propolis extracts evaluated (1 mg.ml⁻¹), the AChE inhibitory activity values ranged between 40.12% (P3) and 46.44% (P5). Regarding the individual IC₅₀ values, they varied from 1.07 mg.ml⁻¹ (P5) to 1.39 mg.ml⁻¹ (P3).

Table 5. Acetylcholinesterase inhibitory activity of DMSO propolis extracts

Propolis sample	Inhibition, %				IC ₅₀ , mg.ml ⁻¹
	1 mg.ml ⁻¹	0.5 mg.ml ⁻¹	0.25 mg.ml ⁻¹	0.1 mg.ml ⁻¹	
P1	40.99 ± 0.28 ^a	39.76 ± 0.52 ^a	37.64 ± 0.61 ^a	23.13 ± 0.83 ^a	1.23 ± 0.03 ^a
P2	43.58 ± 0.44 ^b	41.40 ± 0.08 ^a	35.00 ± 0.96 ^b	26.73 ± 0.57 ^b	1.25 ± 0.02 ^a
P3	40.12 ± 0.09 ^a	31.85 ± 0.82 ^b	24.74 ± 0.04 ^c	18.82 ± 0.29 ^c	1.39 ± 0.03 ^b
P4	43.58 ± 0.44 ^b	34.57 ± 0.56 ^c	27.14 ± 0.18 ^d	18.14 ± 0.11 ^c	1.18 ± 0.02 ^c
P5	46.44 ± 0.17 ^c	38.84 ± 0.29 ^d	23.93 ± 0.28 ^c	17.33 ± 0.04 ^d	1.07 ± 0.03 ^d
P6	41.00 ± 0.07 ^a	35.06 ± 0.88 ^e	29.12 ± 0.25 ^e	12.75 ± 0.89 ^e	1.36 ± 0.24 ^e

^{a-e} Means in the column differ significantly (p < 0.05)

According to the literature, phenolic compounds, especially flavonoids, can act as inhibitors of AChE. For example, the flavonoid derivative quercetin at a concentration of 1 mg.ml⁻¹ was found to be active against AChE with inhibition rate of 76.2% ([Orhan et al. 2007](#)). The existing data from the studies on anti-acetylcholinesterase activity of propolis have shown different inhibitory activity depending on the origin of the samples and their polyphenolic content. Variable IC₅₀ (0.081 - 1.353 mg.ml⁻¹) of ethanolic propolis extracts from Turkey, Azerbaijan and Brazil were reported by [Baltas et al. \(2016\)](#) as three of them originating from Turkey showed values close to our results. [El-Hady et al. \(2016\)](#)

studied the AChE inhibitory activity of three propolis samples from Sudan and determined that the inhibition values varied from 25.5 to 91.7%, compared to the conventional drug Distigmine bromide (72.4%). In another research, the same authors evaluated the AChE inhibitory activity of two propolis samples from Egypt and determined IC₅₀ values of 360 and 600 µg.ml⁻¹ ([El-Hady et al. 2015](#)). AChE inhibitory activity ranging between 78% and 95% of 13 propolis samples (10 mg.ml⁻¹) originating from different parts of Europe and America was reported by [Osés et al. \(2020\)](#). Likewise, the authors team stated that anti-acetylcholinesterase activity correlated with the

catechin and p-coumaric acid content of the extracts. The analysis of 12 propolis samples from different regions of Iran showed that they were capable to inhibit AChE in varying degree with IC_{50} values from 14.37 to 239 $\mu\text{g.ml}^{-1}$ compared to the conventional cholinesterase inhibitor Neostigmine ($IC_{50} = 0.023 \mu\text{g.ml}^{-1}$) (Fathi Hafshejani et al. 2023).

Some researchers have investigated the anti-acetylcholinesterase potential of the individual phenolic compounds of propolis. In this regard, Shahinozzaman et al. (2018) purified and characterized five prenylated flavonoids from Okinawa propolis, and reported that isonymphaeol-B and nymphaeol-A exhibited higher AChE inhibitory activity ($IC_{50} = 7.23 \mu\text{M}$ and $7.77 \mu\text{M}$, respectively) compared to the conventional drug Donepezil ($IC_{50} = 8.13 \mu\text{M}$). In contrast, 3'-geranylnaringenin, nymphaeol-B and nymphaeol-C also inhibited AChE, but to a lesser extent than the standard drug used as a control, with IC_{50} values of 12.34 μM , 15.09 μM , and 15.70 μM , respectively. Based on the findings of this study and the relevant literature, we can conclude that propolis is a natural product with promising AChE inhibitory potential, which can be administered to relieve symptoms and improve cognitive abilities in patients with various cholinergic dysfunctions such as Alzheimer's disease, *Myasthenia gravis*.

Red blood cell (RBC) membrane stabilization (antihemolytic activity). As seen from the results in Table 6, all propolis extracts demonstrated

remarkable ability to protect bovine erythrocytes from hypotonicity-induced hemolysis in a dose-dependent manner. At the highest concentration of propolis extracts evaluated (0.5 mg.ml^{-1}), the antihemolytic activity ranged between 76.28% (P3) and 89.32% (P5). These values (except P3) were found to be very close to that of the steroid anti-inflammatory drug Prednisolone Cortico (94.97%) and significantly higher as compared to that of the non-steroid anti-inflammatory drug Aspirin (60.12%) used as controls at the same concentrations. With respect to the individual IC_{50} values, they varied between 0.06 mg.ml^{-1} (P4 and P6) and 0.14 mg.ml^{-1} (P2). IC_{50} values of the controls were 0.06 mg.ml^{-1} (Prednisolone Cortico) and 0.19 mg.ml^{-1} (Aspirin).

The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization hypothesizes that the propolis extracts may also stabilize the lysosomal membrane. Stabilization of the lysosomal membrane is of great importance in limiting inflammatory processes by preventing the release of lysosomal constituents (enzymes) from the activated neutrophils, leading to further inflammation and tissue damage. Most of the anti-inflammatory drugs act by membrane stabilization or by inhibiting of hydrolytic enzymes. Due to the structural similarities between the erythrocyte and lysosomal membranes, prevention of erythrocyte hemolysis is considered as a measure of anti-inflammatory activity (De et al. 2017).

Table 6. Red blood cell membrane stabilization activity of DMSO propolis extracts

Propolis sample	Protection, %				IC_{50} , mg.ml^{-1}
	0.5 mg.ml^{-1}	0.25 mg.ml^{-1}	0.1 mg.ml^{-1}	0.05 mg.ml^{-1}	
P1	86.59 $\pm$ 0.39 ^a	72.97 $\pm$ 0.44 ^a	53.78 $\pm$ 0.23 ^a	34.03 $\pm$ 0.63 ^a	0.11 $\pm$ 0.01 ^a
P2	85.29 $\pm$ 0.06 ^a	73.32 $\pm$ 0.23 ^a	48.56 $\pm$ 0.74 ^b	21.30 $\pm$ 0.59 ^b	0.14 $\pm$ 0.02 ^a
P3	76.28 $\pm$ 0.54 ^b	68.53 $\pm$ 0.87 ^b	45.47 $\pm$ 0.55 ^c	39.61 $\pm$ 0.68 ^c	0.13 $\pm$ 0.02 ^a
P4	86.40 $\pm$ 0.17 ^a	77.09 $\pm$ 0.08 ^c	66.69 $\pm$ 0.67 ^d	41.65 $\pm$ 0.89 ^d	0.06 $\pm$ 0.01 ^b
P5	89.32 $\pm$ 0.20 ^c	78.94 $\pm$ 0.49 ^d	55.93 $\pm$ 0.69 ^e	41.71 $\pm$ 0.59 ^d	0.08 $\pm$ 0.01 ^b
P6	84.28 $\pm$ 0.45 ^d	72.74 $\pm$ 0.84 ^a	64.74 $\pm$ 0.40 ^f	42.31 $\pm$ 0.25 ^d	0.06 $\pm$ 0.01 ^b
Aspirin	60.12 $\pm$ 0.36 ^e	55.22 $\pm$ 0.26 ^e	52.18 $\pm$ 0.10 ^g	22.68 $\pm$ 0.38 ^d	0.19 $\pm$ 0.02 ^c
Prednisolone	94.97 $\pm$ 0.86 ^f	88.26 $\pm$ 0.48 ^f	74.74 $\pm$ 0.69 ^h	35.71 $\pm$ 0.63 ^a	0.06 $\pm$ 0.01 ^b

a-h Means in the column differ significantly ($p < 0.05$)

Currently, the literature is lacking of sufficient information on the antihemolytic activity of propolis. Research conducted by Humaira et al. (2024) showed that propolis produced by *Trigona* sp. bees at concentrations from 10% to 30% protected human erythrocytes from hypotonicity-induced hemolysis with values ranging between 57.92% and 84.45%. The results obtained by Mendez-Encinas et al. (2023) revealed that Sonoran propolis extracts were capable of stabilizing erythrocyte membrane against hypotonicity-induced hemolysis. The highest concentration of extracts evaluated ($1111 \mu\text{g} \cdot \text{ml}^{-1}$) showed protection values from 54% to 97%, while the control drug Diclofenac sodium at the same concentration showed protection of only 28%. Some studies emphasize the key role of flavonoids as natural antihemolytic agents. In this regard, Veiko et al. (2023) reported the efficacy of quercetin in preventing hemolysis of sheep erythrocytes by α -hemolysin from *Staphylococcus aureus*.

Ex vivo anti-inflammatory activity. The SMPs obtained from the stomach wall of rats were incubated in 20 ml of Krebs solution containing 50 μL of the tested DMSO propolis extract ($20 \text{ mg} \cdot \text{ml}^{-1}$) for 1 h. The activity of pro-inflammatory cytokine interleukin- 1β (IL- 1β) and nitric oxide synthase (NOS3) was evaluated using immuno-histochemical methods. The results obtained are illustrated in Fig. 1 and Fig. 2 A, B.

The data for IL- 1β expression intensity and density in SMPs incubated with sample P1 ($125.7 \pm 9.3 \text{ AU}$) were comparable to those incubated with samples P2 ($145 \pm 8.8 \text{ AU}$) and P3 ($138.5 \pm 6.6 \text{ AU}$). In these SMPs, the intensity of the immune response was lower than the control ($166.9 \pm 6.03 \text{ AU}$, $p < 0.001$) (Fig. 1 C, E, G and Fig. 2 A). Samples P4 ($152.2 \pm 10.8 \text{ AU}$) and P6 ($149 \pm 4.9 \text{ AU}$) also exhibited lower values compared to the control, but without statistical significance (Fig. 1 I, M and Fig. 2 A). Immunohistochemical analysis revealed that DMSO propolis extracts (except P5) significantly reduced the expression of inflammatory marker IL-

1β . The results also indicated a significant reduction in NOS3 expression in the samples treated with propolis ex-tracts (except P5). These findings are aligned with some recent studies demonstrating that propolis reduced the levels of hydroxyarginine and citrulline - products of NO formation (El-Guendouz et al. 2019).

Samples P4 ($152.2 \pm 10.8 \text{ AU}$) and P6 ($149 \pm 4.9 \text{ AU}$) also exhibited lower values compared to the control, but without statistical significance (Fig. 1 I, M and Fig. 2 A). The reduced expression of IL- 1β indicated a stronger *ex vivo* anti-inflammatory effect. Only sample P5 ($175.5 \pm 7.3 \text{ AU}$) exhibited a higher intensity of IL- 1β expression compared to the control (Fig. 1 K and Fig. 2 A), indicating a weaker *ex vivo* anti-inflammatory effect.

Regarding NOS3, we found a weak to absent expression in the cells and neurons of myenteric plexus in SMPs incubated with samples P1 ($95.9 \pm 6.6 \text{ AU}$) and P2 ($107.6 \pm 6.9 \text{ AU}$) compared to the control ($152.5 \pm 11 \text{ AU}$, $p < 0.001$) (Fig. 1 D, F, Fig. 2 B). In SMPs incubated with samples P3 ($116.3 \pm 4.07 \text{ AU}$), P4 ($129.3 \pm 4.5 \text{ AU}$) and P6 ($142.1 \pm 4.9 \text{ AU}$), we also found a weaker expression of NOS3 (Fig. 1, H, J, N, Fig. 2 B) in comparison with the control. In SMPs incubated with sample P5 ($160.5 \pm 5.5 \text{ AU}$), we found a higher intensity of NOS3 expression compared to the control but without statistical significance (Fig. 1 L, Fig. 2 B). The analysis of the experimental data showed that samples P1, P2, P3, P4, and P6 suppressed the expression of IL- 1β and NOS3 in varying degrees. The most pronounced reduction in NOS3 expression was observed in samples P1 and P2 compared to the control ($p < 0.001$), indicating a stronger *ex vivo* anti-inflammatory effect. In our previous research (Tumbariski et al. 2025), the same propolis samples exhibited remarkable *in vitro* anti-inflammatory potential by inhibition of albumin denaturation ranging from 73.59% to 78.44%, which was higher than that of the commercially available anti-inflammatory drugs Aspirin (58.44%) and Prednisolone Cortico (57.34%).

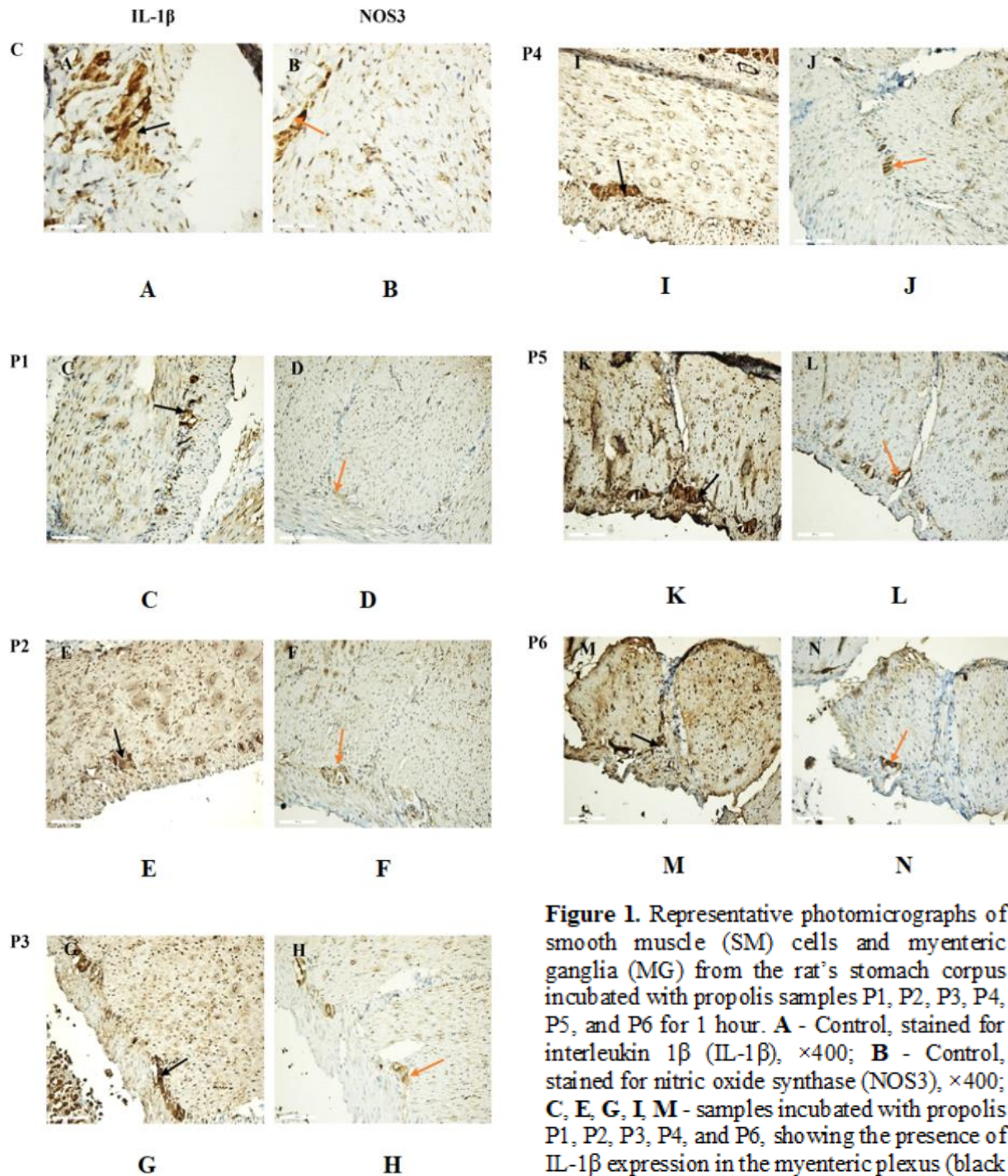
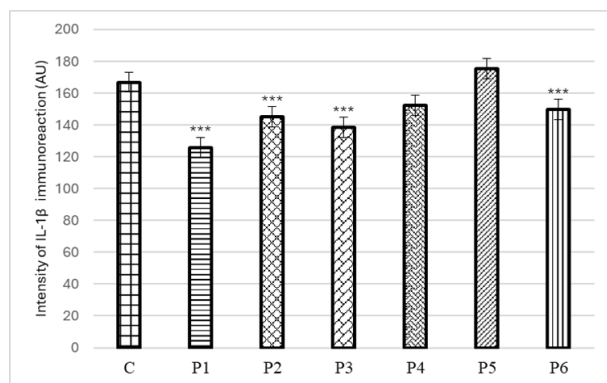
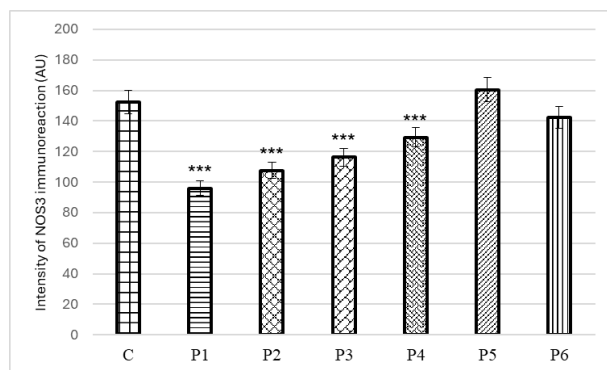


Figure 1. Representative photomicrographs of smooth muscle (SM) cells and myenteric ganglia (MG) from the rat's stomach corpus incubated with propolis samples P1, P2, P3, P4, P5, and P6 for 1 hour. **A** - Control, stained for interleukin 1 β (IL-1 β), $\times 400$; **B** - Control, stained for nitric oxide synthase (NOS3), $\times 400$; **C, E, G, I, M** - samples incubated with propolis P1, P2, P3, P4, and P6, showing the presence of IL-1 β expression in the myenteric plexus (black arrow), $\times 200$; **K** - samples incubated with propolis P5, showing strong IL-1 β expression in the myenteric plexus (black arrow), $\times 200$; **D, F, H** - samples incubated with propolis P1, P2, and P3, showing weakly stained cells for NOS3 in the myenteric plexus (orange arrow), $\times 200$; **J, L, N** - samples incubated with propolis P4, P5, and P6, showing NOS3 expression in the myenteric plexus (orange arrow), $\times 200$.



A



B

Figure 2. Intensity of interleukin 1 β (IL-1 β) (A) and nitric oxide synthase (NOS3) (B) immunoreaction, expressed in arbitrary units (AU) in smooth muscle preparations (SMPs) of rat's stomach. ***, $p < 0.001$

The anti-inflammatory activity of propolis is attributed to its polyphenolic content, especially the presence of caffeic acid phenethyl ester (CAPE) and flavonoids, such as quercetin, chrysin, galangin, kaempferol and others (Valenzuela-Barra et al. 2015). Among these, CAPE, its analogues and chrysin were reported as potent inhibitors of IL-1 β expression and NO production (Nagaoka et al. 2003; Blonska et al. 2004). It was established that flavonoids pinobanksin and its derivatives (Elangovan 2024) and pinocembrin (Xool-Tamayo et al. 2020) also contribute to *in vitro* and *in vivo* anti-inflammatory activity of propolis. In line with our results, the anti-inflammatory potential may be related to the high flavonoid concentrations of the six propolis samples, particularly chrysin (31.42 - 82.21 mg.g⁻¹), pinocembrin (11.48 - 36.32 mg.g⁻¹) and pinobanksin-3-O-propionate (34.27 - 75.28 mg.g⁻¹) (DMSO extracts). Other authors as

Demestre et al. (2009) found that flavonoids in propolis can inhibit inducible cyclooxygenase and iNOS by binding to the PPAR- γ receptor on macrophages, thus contributing to their anti-inflammatory properties. As primary bioactive components of propolis, phenolic acids and their esters are reported to inhibit the expression of inflammatory cytokines and increase the production of anti-inflammatory cytokines, including IL-10 and IL-4 (Shinde et al. 1999; Bueno-Silva et al. 2015). Based on the results obtained in the present and previous study, the propolis extracts investigated can be utilized as effective anti-inflammatory agents and substitutes for the conventional ones. Further research is needed to identify the key bioactive components responsible for the anti-inflammatory potential and their mechanism of action.

Conclusions

The studied DMSO extracts of six propolis samples from three different regions of Bulgaria exhibited high total phenolic, total flavonoid and total caffeic acid derivatives contents, resulting in significant antioxidant activity, which coincided with the values of ethanolic extracts of the same samples – a subject of our previous research. For the first time Bulgarian propolis samples were evaluated for anti-acetylcholinesterase activity, antihemolytic activity and *ex vivo* anti-inflammatory activity on rat's gastric smooth muscle preparations. The propolis samples exhibited acetylcholinesterase inhibitory activity, remarkable antihemolytic potential, expressed as protection of bovine erythrocytes from hypotonicity-induced hemolysis and promising *ex vivo* anti-inflammatory activity, expressed by suppression of IL-1 β and NOS3 expression. Based on the results obtained, we can conclude that investigated Bulgarian propolis is a high-quality beekeeping product, which can find successful therapeutic application as an anti-AChE, anti-hemolytic and anti-inflammatory agent to be used as an alternative to conventional drugs, thus reducing their adverse health effects.

Author Contributions

Conceptualization, Y.T. and I.I.; methodology, Y.T., I.I., and M.P.; formal analysis, Y.T., I.I., and M.P.; investigation, Y.T., I.I., and M.P.; resources, Y.T., I.I., M.P., N.P. and K.N.; data curation, Y.T., I.I., M.P., N.P. and K.N.; writing - original draft

preparation, Y.T., M.P. and N.P.; writing - review and editing, Y.T.; visualization, M.P.; supervision, Y.T. and K.N.; project administration, K.N.; funding acquisition, K.N. All authors have read and agreed to the published version of the manuscript.

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Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki. Animal experiments were approved by the Ethics Committee of the Bulgarian Food Safety Agency (BFSA) (resolution # 391/09.05.2024) and the ethical standards of the Ethics Committee of the Medical University of Plovdiv, Bulgaria (resolution # P 395/20.02.2023).

Informed Consent Statement

Not applicable.

Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare no conflicts of interest.

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