Review Article

Immunomodulatory Effects of Propolis and its Components on Basic Immune Cell Functions

K. WOLSKA*, A. GÓRSKA, K. ANTOSIK AND K. ŁUGOWSKA

Department of Dietetics and Food Assessment, Institute of Health Sciences, University of Natural Sciences and Humanities, Prusa 14, 08-110 Siedlce, Poland

Wolska *et al.*: Immunomodulatory action of propolis and its components

Propolis (bee glue) is a resinous hive product collected by honey bees from many plant sources in temperate and tropical climates. It's fairly complex chemical composition includes polyphenols, phenolic aldehydes, sequiterpenes, quinins, coumarins, amino acids, steroids and inorganic compounds. The contents of propolis depended especially on its location and plant sources. Consequently, the biological activity of propolis gathered from different phytogeographical areas can vary. Propolis is known to have a broad spectrum of biological properties, including antimicrobial, antioxidant, antiinflammatory, antiallergic, dermatoprotective, laxative, antidiabetic, antitumor and immunomodulatory activity. The immunomodulatory activity of propolis has been well-researched. This activity is attributed to flavonoids and some phenolic acids, mainly caffeic acid (cinnamic acid) phenethyl esters and artepillin C (3,5-diprenyl-4-hydroxycinnamic acid). Propolis and these components exhibited immunomodulatory effects on a wide spectrum of immune cells, including cells of lymphoid or monocytic lineages, mediated by the extracellular signal-regulated kinase 2 and mitogen-activated protein-kinase signalling pathway and by eukaryotic transcription factors: nuclear factor of activated T cells and nuclear factor-kappa B. *In vitro* and *in vivo* assays have demonstrated that propolis activated monocytes/macrophages and neutrophils, increasing their microbicidal activity. It enhanced the lytic activity of natural killer cells against tumour cells. It also exhibited antiallergic effects, in part by inhibiting degranulation of mast cells or basophils. Propolis stimulated greater antibody production, suggesting that it could be used as an adjuvant in vaccines. Its inhibitory effects on lymphoproliferation might be linked to its antiinflammatory properties. However, this effect appeared to occur in the presence of high concentrations of propolis, while at low concentrations the effect is reversed, causing stimulation of lymphocyte proliferation.

Key words: Propolis, components of propolis, immunomodulatory action, immune cells, antiinflammatory action

Propolis is a product of the vital activity of the honey bee *Apis mellifera*. Bees produce propolis by mixing substances gathered from budding plants, flower buds and resinous exudates. They thus produce a material suitable for closing gaps, embalming dead insects within the beehive and protection from invasion by microorganisms and insects. This activity of propolis results from its composition. Raw propolis is typically composed of 50 % plant resins, 30 % waxes, 10 % essential and aromatic oils, 5 % pollens and 5 % other organic substances[1].

Propolis possessed a variety of biological and pharmacological effects, such as antibacterial, antioxidant, antitumour, antiinflammatory and immunomodulatory. However, propolis could not be used as a raw material, so it must be purified by extraction to remove the inert material and preserve the polyphenolic fraction, which is primarily responsible for its activity[1,2]. Generally, ethanol is the best solvent for propolis preparation, but other solvents such as ethyl ether, water, methanol and chloroform could also be used for extraction and identification of propolis constituents[3]. More than 300 different compounds have been identified in propolis, including phenols, tannins, polysaccharides, terpenes, aliphatic acids, esters, aromatic acids, fatty acids, aldehydes, amino

***Address for correspondence**
E-mail: kwolska@uph.edu.pl

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acids, ketones, chalcones, dihydrochalcones, vitamins and inorganic substances. Among these, flavonoids and phenolic acids are of greatest interest to researchers[3,4].

The chemical characteristics of propolis have been found to be linked to the diversity of geographical location, plant sources and bee species. Poplar (*Populus nigra* L. and *P. alba* L) is common in the temperate zone, especially in Europe, and gives its name to a type of propolis that is rich in flavonoids and phenylpropanoids. However, flavonoids are not restricted to poplar; furthermore, in areas where poplars are not native plants, such as Australia and the equatorial regions of South America, bees would seek other plants to produce propolis, which contain flavonoids of poplar-type propolis[5]. The characteristic constituents of propolis of temperate regions are flavonoids without B-ring substituents, such as chrysin, galangin, pinocembrin, pinobanksin, and aromatic acids and their esters. Caffeic acid phenethyl ester (CAPE) is a major constituent of temperate propolis with broad biological activity. Parts of the world outside of Europe in which propolis exhibits chemical profiles similar to those of poplar-type propolis included China[6], North America[3], various regions of Taiwan[7,8], New Zealand[9] and Africa[10].

Significant amounts of phenolic glycerides (23.1 mg/g), i.e. dicoumaroyl acetyl glycerol, diferuloyl acetyl glycerol, feruloyl coumaroyl acetyl glycerol, and caffeoyl coumaroyl acetyl glycerol were isolated by Popravko *et al*.[11] from North-Russian propolis and the exudate of *P. tremula* was found to be their plant source. However, the primary plant source of propolis in Russia is *Betula verrucosa* Ehrh., and its main biologically active substances are flavones and flavonols (but not the same as those in poplar propolis)[12].

The propolis of tropical regions, especially the green propolis of southern Brazil, which has *Baccharis dracunculifolia* as its primary plant source, contains dihydrocinnamic acid, *p*-coumaric acid, prenyl- and diprenyl-*p*-coumaric acids and flavonoids[13]. Typical constituents of this propolis are kaempferide (3,5,7-trihydroxy-4'-methoxyflavone) and cinnamic acid derivatives, *p*-coumaric acid (4-hydroxycinnamic acid), artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), baccharin (3-prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid) and drupanin (3-prenyl-4-hydroxycinnamic acid)[14,15]. The other common-type of propolis in the tropical region is Brazilian red propolis (BRP), which is collected in northern Brazil from the surface of *Dalbergia ecastophyllum*. This propolis is rich in flavonoids, mainly liquiritigenin, daidzein, dalbergin, isoliquiritigenin, formononetin and biochanin A. Three of these (daidzein, formononetin and biochanin A) are isoflavonoids[16]. Propolis is produced by the honey bee *Apis mellifera*, which is wide spread in Europe, the Ural mountains, Africa, and Asia. The European honey bee *A. mellifera* is the most common species of honey bee. Brazilian green and red propolis originate from the Africanized *A. mellifera*[4].

The immunomodulatory activity of propolis (fig. 1) and its antiinflammmatory properties have been particularly well researched. The eukaryotic transcription factors nuclear factor of activated T cells (NFAT) and nuclear factor-κ-B (NF-κB) play a central role in immune and inflammatory responses. Activation of NFAT and NF-κB proteins is induced by many factors, such as inflammatory cytokines (interleukine-1, IL-1 and tumour necrosis factor, TNF), bacterial products, and oxidative stress. These two major transcription factors are involved in expression of the IL-2 and IL-2R genes, which are necessary for T cell activation and proliferation[17]. The immunomodulatory properties of propolis are contributed by its flavonoids and phenolic acids[18-21]. Yuan *et al*.[21] reported that these phenolic compounds might be important components activating innate and adaptive immune cells. Although the precise mechanism of action of propolis on immune cells remains unknown, it is possible that the flavonoids stimulate macrophages and lymphocytes to produce IL-1 and IL-2, respectively, which have mitogenic action on B and T lymphocytes[22]. For example, artepillin C, found in large quantities in green propolis samples, acts on macrophages by stimulating the production of IL-12, which stimulates the Th1 lymphocyte response and inhibits the Th2 response. Other phenolic compounds found in the propolis samples, such as cinnamic acid, also induce the production and release of cytokines, such as IL-1, IL-6 and IL-8 by activated macrophages, stimulating antibody production by B lymphocytes and acting chemotactically on neutrophils. Such mechanisms may explain the increment in innate and adaptive (humoral and cellular) immunity[22].

Some authors[23,24] suggested that the immuno-modulatory activity of propolis is strictly dependent on its concentration. A low concentration of propolis has a stimulating effect on the cellular immune response, whereas a high propolis concentration exerts an inhibitory effect. In particular, flavonoids have an immunosuppressive effect in the lymphoproliferative

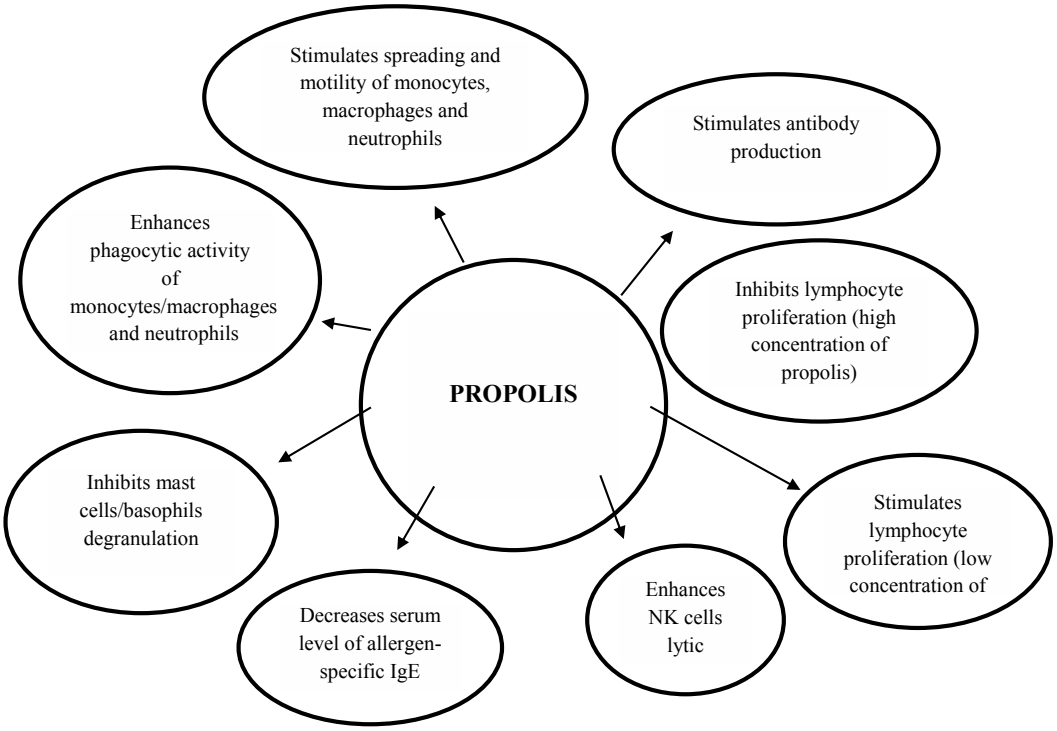

Fig. 1: Propolis immunomodulatory action

response. These natural components block activation of DNA binding of nuclear factors (NFAT and NF-κB)[17].

Immunomodulatory effects on neutrophils, monocytes and macrophages:

Propolis and some of its constituents, such as CAPE, cinnamic acids, and artepillin C, affect nonspecific (innate) immunity via modulation of neutrophile and monocytes/macrophages activity[25-28]. The neutrophil polymorphonuclear leukocytes are an important component in nonspecific immunity and are the main phagocytic cells found in the peripheral blood[24,29]. Monocytes/macrophages are the other effector cells of the immune system that protect against microbial infection. Macrophages are mononuclear phagocytic cells derived from peripheral blood monocytes and are resident in most tissues. They act as a bridge between the innate and the adaptive immune systems by differentiating into cells to exert diverse functions after being activated by various stimuli, such as interferon-γ (IFN-γ). Monocytes/macrophages have three important functions, phagocytosis, antigen presentation to effective T cells, and synthesis of various inflammatory mediators[30,31].

When bacteria as an antigen enter the body they will be eliminated by neutrophils and monocytes/ macrophages. Phagocytosis is the main mechanism for destruction of microorganisms within phagocytic cells in the innate immune system. This complex process has the following phases, phagocyte chemotaxis, adhesion of microorganisms to the surface of phagocytes, endocytosis, and killing of the microorganisms. Microbes are destroyed within phagocytic cells through two mechanisms; one oxygen-dependent (production of reactive oxygen intermediates (ROI) and nitrogen intermediates) and the other oxygen-independent (including the release of lysosomal enzymes such as lysozyme and proteinases). It should be added that immune phagocytosis with non-specific opsonins, e.g. complement components (C3b) or specific opsonins (antibodies IgG) is more efficient than what is known as non-immune phagocytosis without opsonins[24,29,30]. The secretion of ROI is the main mechanism for the destruction of microorganisms. However, ROI may also destroy important biomolecules and are involved in tissue injury associated with inflammatory diseases[32]. As professional phagocytes, neutrophils are responsible for killing extracellular microorganisms, whereas monocytes/macrophages destroy cells infected with pathogens, including intracellular bacteria (e.g. *Salmonella*), yeasts and parasites.

Several studies have demonstrated that the phagocytic activity of these cells and their motility can be enhanced

by immunomodulatory substances such as propolis and its components (Table 1). As early as the 1970s, Scheller *et al*.[33] suggested that immunostimulatory activity of propolis is associated with macrophage activation and enhancement of macrophage phagocytic capacity. Orsi *et al*.[34] demonstrated that after propolis treatment (2.5 and 5 mg/kg) of mice for 3 consecutive days, peritoneal macrophages were activated *in vitro* with IFN-γ, and production of H_2O_2 and NO was increased in comparison to non-activated cells (control). This suggested that propolis treatment leads macrophages to a higher responsiveness to stimuli such as IFN-γ. However, depending on its concentration (10, 30 and 60 mg/kg), macrophages from propolis-treated animals, stimulated *in vitro* with IFN-γ, inhibitied H_2O_2 and NO generation.

Further research by the same author[35] showed that both Brazilian and Bulgarian propolis samples increased the bactericidal activity of macrophages against *Salmonella typhimurium* - the causative agent of typhoid fever in humans, depending on its concentration (3, 10, 30 and 100 µg/ml). However, no differences were seen between these samples, although they were produced in different geographic regions. The data confirmed previous research findings that the bactericidal activity of macrophages, with different macrophage/bacteria ratios and different propolis concentrations, might have occurred through oxygen and nitrogen intermediates.

Peritoneal macrophages from Sprague Dawley rats infected with *Staphylococcus aureus* and treated with propolis of *Trigona* spp. exhibited improved phagocytosis. A significant increase in phagocytic activity (number of macrophages with phagocytic latex particles per one hundred macrophages) was observed with different concentrations of propolis (0.16-80.7 %; 0.46-88.4 %; 1.44-90.1 %) similar to that observed for CAPE (0.5-87.2 %). This study also found that propolis increased NO production in macrophages[36]. The compound in propolis of *Trigona* sp. from Indonesia, which activated the macrophages was probably limonene[37].

Similarly, Possamai *et al*.[38] found that mononuclear phagocytes in human blood exposed to 50 µg/ml of Brazilian propolis adsorbed onto polyethylene glycol microspheres presented high levels of superoxide release, phagocytosis of pathogenic yeast *Candida albicans*, and microbicidal activity. In this study the main chemical constituents of this propolis were tannins, phenols, flavonoids and xanthones, and to a lesser degree, flavanones and resins. The study by Murad *et al*.[39] suggested an increase in the fungicidal activity of macrophages by propolis stimulation, independently of its geographic origin. Peritoneal macrophages from BALB/C mice were stimulated with Brazilian or Bulgarian propolis and subsequently challenged with *Paracoccidioides brasiliensis*, which causes paracoccidioidomycosis, the most important systemic mycosis in Latin America.

Dantas *et al*.[40] investigated the effects of Bulgarian propolis (25 and 50 mg/kg) in an experimental model of *Trypanosoma cruzi*-infected Swiss mice, verifying that this bee product led to a decrease in parasitaemia and showed no hepatic or renal toxic effect. Syamsudin *et al*.[41] demonstrated that Indonesian propolis extract displayed more immunostimulatory activity as shown by the increase in IgG against surface antigens of *Plasmodium berghei*. The antiplasmodial activity of propolis hydroalcoholic solution (PHS) was due to the increased immunity in the mice, owing to which they lived longer. However, PHS (25, 50 and 100 mg/kg) showed weaker antiplasmodial activity than chloroquine (10 mg/kg).

TABLE 1: EFFECT OF PROPOLIS ON PHAGOCYTIC ACTIVITY OF MACROPHAGES AND NEUTROPHILS

Cells	Effect	Propolis dose	*In vivo*/*in vitro*	Main groups or active components	Reference
Macrophages	[1]↑	0.46% [2]bw	*In vivo* male rats Spraque Dowley	Limonene, 1-heptacosanol, heptacosane, 1-hexadecanol, dioctyladipote, hexadecane	37
	↑	157.4 mg/kg bw	*In vivo* aged Kunning mice	Polyphenols, flavonoids, cinnamic acid, artepillin C	24
Neutrophils	↑	40 µg	*In vitro* [3]neutrophils	Pinocembrin, galangin	42

[1]↑ – stimulant action; [2]bw – body weight; [3]neutrophils – nutrophils isolated from periphenal venous blood of adult male

Other authors[24] demonstrated that administration of Brazilian green propolis promoted phagocytosis of chicken red blood cells by peritoneal macrophages in aged mice at a dose of 157.4 mg/kg, and significantly increased the phagocytic index (the average number of chicken red blood cells phagocytized per peritoneal macrophage cell) at 352.9 mg/kg (83.20±10.25 and 2.53±0.87 %, respectively) in comparison to the control group (without propolis; 70.71±13.70 and 1.80±0.48 %, respectively). They concluded that artepillin C might be one of the most important constituents of Brazilian green propolis responsible for activating macrophage phagocytosis. The content of this component in the analysed sample of propolis was 23 mg/g.

The immunomodulatory activity of partially purified propolis extracts (PPPEs) from northern Argentina and flavonoids (galangin and pinocembrin) was investigated by testing their *in vitro* effect on neutrophil chemotaxis and phagocytic activity[42]. PPPEs were more effective (about 45 and 50 % for a concentration of 40 µg of PPPEs/ml of active substance) as chemotactic agents than galangin and pinocembrin (around 20 and 25 %, respectively) and showed higher neutrophil phagocytic activity (270±10 %) than galangin and pinocembrin (180±10 and 135±9 %, respectively). The greater effect of PPPEs could be attributed to a synergistic effect among components of PPPEs. The results of this study indicated that PPPEs and flavonoids (galangin and pinocembrin) stimulate neutrophil chemotaxis and phagocytic activity. A study by Rindt *et al*.[43] also confirmed the effect of propolis from Romania on the phagocytic activity of neutrophils. They investigated the immunological activity of ethanol extracts of propolis on *in vitro* phagocytic activity of blood leukocytes from cows with subclinical mastitis using the carbon particle inclusion test. Phagocytosis in the control without propolis was significantly decreased as compared to the test sample, especially during the second half of the incubation period ($p<0.001$).

A study by Fischer *et al*.[44] showed that the polyphenol compounds extracted from Brazilian green propolis could act as vaccine adjuvants, improving innate and adaptive (humoral and cellular) immune responses in mice inoculated with inactivated vaccines. However, propolis flavonoids do not easily dissolve in water. Therefore, when propolis flavonoids are encapsulated with liposome, not only their solubility increased, but also the immunological adjuvant is synergistic. The results showed that propolis flavonoids liposome can significantly enhance the phagocytic function of macrophages *in vitro* and the release of IL-1β, IL-6, and IFN-γ. Other research has demonstrated that propolis flavonoids liposome, like pathogen-associated molecular patterns, can be recognized by the innate immune system through interaction with macrophages, and the results could be linked to the activation of toll-like receptor (TLR) signal pathways[28].

Neutrophils, monocytes and macrophages additionally play an important role in inflammation. During inflammation, upon stimulation by LPS from Gram-negative bacteria and IFN-γ or TNF-α from host immune cells, neutrophils or monocytes/macrophages release large amounts of reactive oxygen species (ROS) and nitrogen species (RNS), nitric oxide (NO), and numerous cytokines, and generate important inflammatory mediators such as prostaglandins (PG) and leukotrienes[45]. Overexpression of these mediators causes pathological acute or chronic inflammatory responses[31]. Numerous findings have shown antioxidant and antiinflammatory effects of propolis extract[15,18,25,45-48]. The biologically active molecules in propolis are phenolic acids and flavonoids, which act as scavengers of free radicals and inhibitors of NO and inflammatory cytokine production by monocytes/macrophages and neutrophils.

Significant linear correlations were observed between total phenolic content of propolis from different regions of Poland and antiradical activity measured using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•; 1.92-2.69 mM TE/g) and 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS•$^+$; 3.96-4.98 mM TE/g)[49]. The dominant phenolic acid was *p*-coumaric acid, while chrysin and galangin were dominant among flavonoids. Similarly, Brazilian green propolis and two of its components, kaempferide and artepillin C, exerted strong free-radical scavenging activity based on reduction of DPPH• and ABTS•+[15,50]. The effect of Brazilian and Polish propolis in ROS scavenging has also been determined by a chemiluminescence assay. ROS and RNS release by PMA-activated J774A.1 murine macrophage cells was significantly inhibited by EEP-B in a dose-dependent manner[51]. Others have demonstrated that extracts of Polish and Brazilian green propolis and kaempferide decrease the chemiluminescence produced by stimulated neutrophils[45,47,48].

Błońska *et al*.[46] observed inhibition of NO synthesis and iNOS mRNA expression in LPS-stimulated J774A.1 macrophages by Polish propolis extract

and its phenolic components: chrysin, galangin, kaempferol, and quercetin. Similarly, data obtained by Szliszka *et al*.[51] have confirmed the effect of Brazilian propolis on NO generation in J774A.1 cells stimulated with LPS+IFN-γ. Another study[52] demonstrated that artepillin C decreases NO concentration and has an inhibitory effect on PG E2 by modulating NF-κB in RAW264.7 murine macrophage lines incubated with LPS. In an experiment by Hu *et al*.[53], treatment of ICR mice and Wistar rats with ethanol (EEP) and water (WEP) propolis extracts inhibited the increase of PGE_2 and also had a significant inhibitory effect on NO in carrageenan-induced pleurisy.

According to Mirzoeva and Calder[54] dietary propolis markedly affected the inflammatory response. Mice fed a diet containing 0.2 % propolis produced decreased amounts of LTB_4, LTC_4 and PGE_2 in response to inflammatory challenge *in vivo*. Some substances found in propolis, such as caffeic acid, quercetin, naringenin, and caffeic acid phenethyl ester, contribute to the suppression of PG and leukotriene synthesis by peritoneal macrophages stimulated with A23187 or LPS. Similarly, the antiinflammatory activity observed in green propolis seems to be due to the presence of prenylated flavonoids and cinnamic acid. These compounds exhibited inhibitory activity against cyclooxygenase (known as PG-endoperoxide synthase) and lipoxygenase, which produces several, chemically different leukotrienes[45].

Cytokines secreted by immune cells play an important role in immune and inflammatory responses *in vivo*. Many cytokines secreted by macrophages were analysed by Szliszka *et al*.[51], who demonstrated that Brazilian green propolis significantly downregulated the production of IL-1α, IL-1β, IL-6, IL-12p40 and TNF-α in LPS+IFN-γ-treated J774A.1 cells. The secretion of interleukins IL-4 and IL-13 and colony-stimulating factors G-CSF, and GM-CSF were also reduced by propolis, whereas the concentrations of IL-3, IL-5, IL-9, IL-10, IL-17 and IFN-γ were only slightly affected by incubation with EEP-B, or not at all. No significant increase in serum IL-1β, IFN-γ and IL-4 levels was found after administration of Brazilian green propolis at varying doses (83.3, 157.4 and 352.9 mg/kg) to aged mice[24]. Hu *et al*.[53] showed that EEP and WEP had significant inhibitory effects on IL-6 levels in rats with FCA-induced arthritis, but not on IFN-γ and IL-2 levels. Others[55,56] have reported that extracts from Chinese propolis suppressed mRNA and protein expression of IL-1β and IL-6 induced by LPS in RAW264.7 cells. A decrease in TNF-α production in monocytes after LPS stimulation by Brazilian green propolis was described by Chan *et al*.[25].

Results reported by Szliszka *et al*.[51] suggested a crucial contribution of Brazilian green propolis in the modulation of chemokine-mediated inflammation. EEP-B downregulated the expression of MCP-1, MIP-1α, MIP-1β, and RANTES in LPS+IFN-γ stimulated J774A.1 cells. An ethanol extract of BRP at 10 mg/kg downregulated the expression of TNF-α. This was linked to neutrophils transmigration and IL-1β, which increased inflammatory cells recruitment in various inflammatory models, including the peritoneal cavity[57]. Bueno-Silva *et al*.[57] have also suggested that BRP inhibition of neutrophil migration might be linked to a reduction in CXCL1/KC and CXCL2/MIP-2 release and consequently a decrease in neutrophil chemotaxis, as well as bearing, adhesion and transmigration into the inflammatory focus. The researchers identified formononetin from BRP as the major compound, present in the amount of 24.45 mg/g. In addition, daidzein and biochanin A were identified as minor compounds. Another EEP (1 µg/ml) mechanism observed in this study was the direct blockage of calcium influx in neutrophils, which in turn decreased neutrophil chemotaxis, since calcium is necessary for chemotaxis. The study suggested that propolis and its components (CAPE) are potent and specific inhibitors of NF-κB activation. CAPE not only inhibited transcription factors, but also reduced production of IL-8 (a cytokine that increases neutrophil chemotaxis) and monocyte chemotactic protein (MCP)[58].

Immunomodulatory effects on the function of T and B lymphocytes:

T and B cells are another major population of peripheral blood mononuclear cells (PBMC), which play an important role in adaptive immune defence. These cells' ability to distinguish self from nonself allows them to clear exogenous antigens. In this manner the immune system plays a pivotal role in the human body's defence against infection. Numerous harmful influences of endogenous or exogenous origin cause pathological alterations in the human body. The immune system is activated by these stimuli and switches on various molecular mechanisms of innate and adaptive immunity. Individual types of antigens bind and activate different populations of T cells and/or B cells, which have different immune functions. Previously, however, inside macrophages,

bacteria will be phagocytized and then recognized by major histocompatibility complex II (MHC II) and presented in the form of peptide antigen. Furthermore, MHC II binds to T lymphocytes. T lymphocytes are known to have several surface molecules or clusters of differentiation (CD). Antigen peptides that have been presented by MHC II bind to T helper lymphocytes (CD4) on the T cell receptor (TCR). Ligands between the antigen-MHC complex with TCR-CD3 activate inositol triphosphate and glycerol compounds in the T cell membrane. Inositol triphosphate will increase calcium ion in the cytoplasm, whereas diacylglycerol activates the enzyme protein kinase-C. Both are a signal to activate T cells[59].

The activation of T lymphocytes leads to the expression of cytokine genes. Cytokines bound to specific receptors on the surface of target cells regulate the growth or/ and differentiation of the cells and thus optimize the immune response. The development of humoral and cellular immunity depends on cytokine production mainly by $CD4^+$ cells[60]. For examples, type I helper T cells produce IL-2 and IFN-γ to activate CD8 cytotoxic T cells[61] and type II helper T cells secrete IL-4, IL-5, IL-6 and IL-13 to facilitate activation of B cells[62].

The immunomodulatory effect of propolis in resting human PBMC is mainly due to their action on monocytes. However, T and B lymphocytes are another major component of PBMC that may be affected by propolis. Some authors have shown that propolis stimulates lymphocyte proliferation, while others, claim that propolis and its components, especially flavonoids are active immunosuppressants that inhibit lymphocyte proliferation. In fact both statements are correct, but the effect of propolis depends on its concentration. Marquez *et al*.[17] have evaluated the immunosuppressive activity of CAPE in human T cells, discovering that this phenolic compound is a potent inhibitor of early and late events in receptor-mediated T cell activation. Moreover, they found that CAPE at a concentration of 10 μM specifically inhibited both IL-2 gene transcription and IL-2R (CD25) expression in stimulated human T cells. They proved that CAPE inhibited T-lymphocyte activation by targeting both NFAT and NF-κB transcription factors. Another explanation for the inhibitory effects of propolis on lymphoproliferation derives from observations by Ansorge *et al*.[63]. They studied the mitogen-activated protein (MAP) kinase signalling pathway, measuring the induction of mRNA expression of extracellular-signal-regulated kinase (Erk-2), which is capable of regulating several transcription factors. These in turn control the regulation of critical genes of lymphocytes, including the IL-2 gene. Erk-2 was strongly suppressed in propolis-stimulated PBMC, which clearly suggests that one way of signalling triggered by propolis is mediated by MAP kinase Erk-2.

Draganova-Filipova *et al*.[23] studied the *in vitro* participation of Bulgarian propolis and CAPE in propolis in the modulation of both cellular and humoral immunity (Table 2). The percentages of T helper (h)/inducer (i) ($CD4^+CD3^+$), T cytotoxic (c) ($CD8^+CD3^+$) and B ($CD19^+CD3^-$) lymphocyte subsets were determined by flow cytometry after cultivation with EEP and CAPE at different concentrations. The low concentration of propolis had a stimulating effect on the T cell immune response. On the other hand, the positive effect of the low concentration on B-lymphocytes determined its potential to stimulate antibody production. The highest propolis concentration (10 mg/l) had a toxic effect and caused cell death. After CAPE treatment of the cells the tendencies observed were similar to those determined after propolis treatment, but the effect was more intense. The results might be explained by the fact that CAPE was a chemically synthesized product and had a stronger effect at a lower concentration than the complex product.

Similarly, an inhibitory effect of propolis (5-100 mg/ml) on splenocyte proliferation was observed *in vitro* in a study by Sá-Nunes *et al*.[64]. Park *et al*.[65] have also reported that treatment with CAPE directly or indirectly causes immune cells to decrease in number, especially T cells.

Previous research by Draganova-Filipova *et al*.[23] found that propolis-treated PBMC had the ability to produce IL-2. The stimulating effect of IL-2 was probably directed to T cells. The authors supposed that as a result of intermolecular interactions propolis activates Th cells and stimulates cellular and humoral immunity *in vitro*. The same authors[23] demonstrated that the alteration in expression of CD69 (a cell surface protein synthesized rapidly about two hours after activation) after CAPE treatment was greater in $CD4^+/CD69^+$ cells than $CD8^+/CD69^+$ cells. The effect was more pronounced at the lowest concentration, i.e. 2 mg/l.

In vivo experiments have demonstrated that propolis stimulated T lymphocyte proliferation and the secretion of IL-2, IL-4 and IFN-γ in Balb/c mice,

TABLE 2: EFFECT OF PROPOLIS ON LYMPHOCYTES ACTIVITY

Immunological assay	Effect	Propolis dose	*In vivo*/*in vitro*	Main groups or active components	Reference
Proliferation of B cells	[1]↑	0.01; 1; 2.5; 5 mg/l	*In vitro*	Phenolic acids; aromatic esters (CAPE); flavonoids (chrysin, pinocembrin, galangin)	23
	[3]↓	10 mg/l	[2]PBMC		
Production of IgG	↑	3 g/kg	*In vivo* White Leghorn laying hens	Flavonoids; aliphatic acids, aromatic acids, esters, alkohol, terpene, kinons, benzene	71
	↑	157.4 mg/kg [4]bw	*In vivo* aged Kunning mice	Polyphenols, flavonoids, cinnamic acid, artepillin C	24
Production of IgM	↑	3 g/kg	*In vivo* As above	As above	71
	No effect	157.4 mg/kg bw	*In vivo* As above	As above	24
T lymphocyte proliferation	↓	3 g/kg	*In vivo* As above	As above	71
	↑	157.4 mg/kg bw	*In vivo* As above	As above	24
Proliferation of T helper/induce cells	↑	0.01; 1; 2.5; 5 mg/l	*In vitro*	As above	23
	↓	10 mg/l			
Proliferation of T cytotoxic cells	↑	0.01; 1; 2.5; 5 mg/l	As above		
	↓	10 mg/l			

[1]↑ – stimulant action; [2]PBMC – peripheral blood mononuclear cells from heparinized venous blood of healthy donors; [3]↓– inhibitory action; [4]bw – body weight

but had no effect on B lymphocyte blastogenesis induced by lipopolysaccharide. This led to a decrease in their number in relation to the total number of lymphocytes[65]. However, in other *in vivo* studies 10 % propolis stimulated humoral immunity, causing antibody production in bovine serum albumin-immunized rats. Two propolis components used in this experiment, caffeic acid and quercetin, had no effect on antibody production, which can be explained by the synergetic effect of the propolis components as a whole[66]. Similarly, Kalsum *et al*.[36] showed that extracts of propolis of *Trigona* sp. increase the potential of the humoral immune response as compared to rats that received antigen (bacteria *Staphylococcus aureus*) without propolis (negative control, 8.19 ng/ml IgG). The content of serum IgG was increased significantly in rats that received 0.46 % propolis of *Trigona* sp. (13.73 ng/ml IgG) as compared with other dosages of propolis (0.16 %-10.72 ng/ml and 1.44 %-11.85 ng/ml). Treatment with 0.46 % propolis was not significantly different from that observed for CAPE (0.5 %-13.46 ng/ml - a positive control). Kalsum *et al*.[37] considered the possibility that limonene, one of the most important constituents in propolis of *Trigona* sp. from Indonesia may play a role in increasing antibody production. Brazilian green propolis increased serum IgG levels and SRBC-specific antibody production in aged mice receiving 83.3 and 157.4 mg/kg (34.48±5.94 and 32.35±9.08 g/l, respectively), as compared to control mice (23.03±8.30 g/l), suggesting improved IgG production and an enhanced specific antibody response[24]. It is worth adding that aging is associated with a decline in B cell function and substantial impairment of the humoral response[67]. The composition analysis indicated that the Brazilian green propolis used in this study was rich in polyphenols, flavonoids, cinnamic acid and artepillin-C (189.12, 98.46, 1.95 and 23 mg/g, respectively)[24]. Furthermore, Scheller *et al*.[68] reported that an ethanol extract of propolis (500 µg/mouse) increases antibody production in SRBC-immunized mice. However, a further increase in the propolis dose or in the frequency of its administration had an inhibitory effect on antibody production in immunized cells.

Similar results have been reported in laying hens and broiler chickens[69,70]. Results obtained by Cetin *et al*.[69] showed that serum IgG and IgM levels (141.77±35.71

and 261.00±44.4 mg/dl, respectively) in a group of laying hens receiving 3 g/kg of Turkey propolis were significantly higher than in the control (79.70±16.4 and 172.65±29.2 mg/dl, respectively), suggesting that 3 g/kg of propolis could promote humoral immunity in birds. However, 6 g/kg of propolis did not produce a significant elevation of the serum IgG and IgM level (98.50±28.27 and 211.00±39.7 mg/dl, respectively), which could be attributed to the main constituents of the Turkey propolis preparation such as benzene and flavonoids. Cetin`s *et al*.[69] findings were supported by Ziaren *et al*.[70], who reported that humoral immunity was modulated by various levels of propolis in the diet of broilers. They observed that dietary propolis increased the antibody titre at low levels but decreased at high levels, thereby exhibiting a bell-shaped dose-response relation.

A new line of research involving propolis involves its potential application as a vaccination adjuvant, although most commercial vaccines use aluminum salts to this end. A sample of green Brazilian propolis was tested, together with other adjuvant compounds, to immunize mice against inactivated swine herpesvirus. When administered together with aluminium hydroxide, the propolis extract increased both cellular and humoral responses[60]. According to Sforcin *et al*.[66], the ability of propolis to modulate antibody synthesis is part of its adjuvant action. The immunostimulatory capacity of propolis, through increased immunoglobulin level has already been reported in patients with fibrosing alveolitis. Similarly, data presented by Fischer *et al*.[60] showed that the use of an ethanol extract of green propolis can contribute to the efficacy of inactivated vaccines, acting as an immunostimulant. The ethanol extract of green propolis (40 mg/dose) increased the potency of the humoral immune response in cattle when associated with an inactivated oil vaccine against bovine herpesvirus-type 5, showing adjuvant action. Phenolic compounds such as artepillin C and cinnamic acid derivatives, besides other flavonoid substances, were abundant in this propolis extract. These substances are known to have mitogenic action for B and T lymphocytes[22].

Cheung *et al*.[71] postulated that propolis and artepillin C could serve as a novel immunosuppressive drug for the treatment of graft-versus-host disease (GVHD). There is a delicate balance in the immune system so that immune defence does not target self-antigens. This self-preservation characteristic becomes a barrier during allogeneic bone marrow transplants. Unwanted T cell activation in the recipient can lead to graft rejection. However, this response is always inhibited by the immunosuppressants targeted at treating GVHD. Hence, a good immunosuppressant for the treatment of GVHD should be able to inhibit T cell activation and at the same time suppress cancer cells[56].

Graft-versus-leukaemia is an important response to help eradicate leukaemia after haematopoietic stem cell transplantation. Propolis and artepillin C at concentrations that can suppress T cell proliferation showed a significant inhibitory effect on leukemic cell growth, especially for T lineage leukaemia, while having no effect on normal blood cells[56].

Granzyme is a cytolytic enzyme produced cytotoxic T cells that play a role in lysing target cells, including cancer cells. Results of Kusnul *et al*.[72] showed that the increased level of $CD8^+$ T cells-expressing granzyme and a decreased level in $CD4^+CD25^+$ T cells-expressing granzyme by propolis extract were both provide positive benefits in cancer therapy.

Immunomodulatory effects on the function of natural killer (NK) cells:

NK cells are characterized as a lymphocyte subpopulation different from T and B cells, non-adherent and non-phagocytic, showing lytic activity mainly towards several types of tumour and virus-infected cells. NK cells are considered the host primary defence mechanism.

In vitro research has demonstrated that NK cells display considerably higher resistance to Bulgarian propolis in comparison with Th and Tc cells. Low concentrations of propolis (0.01; 1; 2.5; 5 mg/l) had a proliferative effect and almost no pro-apoptotic effect on NK cells. After CAPE treatment the resistance of NK cells was lower than after propolis treatment. At 4 mg/l of CAPE the vitality of NK cells decreased to 46.77 %. The 8 mg/l concentration increased the percentage of apoptotic NK cells, but the values were lower than those of the apoptotic $CD3^+$ cells –53.5 % (Tc) and 61.48 % (Th)[23].

The effect of propolis on NK cells was investigated *in vivo*. Results reported by Sforcin *et al*.[73] indicated that NK activity was increased in spleen cells from propolis-treated rats. On the other hand, no effect was demonstrated on splenic NK cell activity in aged mice after treatment with Brazilian green propolis[24].

Recent reports have suggested that the antitumour effects of propolis could be attributed to its modulatory effect on the immune system, which include macrophage

activation; modulation of B and T lymphocytes and NK cells; antibody proliferation, and production of cytokines (IL-2, IL-10 and IFN-γ); downregulation of TLR-2 and human leukocyte antigen (HLA-DR) expression; induction of H_2O_2 release; inhibition of NO, PG and leukotriene generation; suppression of the lipoxygenase pathway of arachidonic acid metabolism and suppression of activity of myeloperoxidase, NADPH-oxidase, ornithine decarboxylase, tyrosine-protein-kinase, and hyaluronidase; and downregulation of transcription factors[74].

Several authors have studied the antitumour properties of propolis and its chemical constituents, artepillin C, baccharin and drupanin, CAPE and chrysin in *in vivo* and *in vitro* models[75-78]. Resistance to spontaneous tumour development has been associated with the cytotoxic activity of NK cells, found both in humans and experimental animals. Scheller *et al*.[79] observed the cytotoxic effect of ethanol extract of propolis in mice-bearing Ehrlich carcinoma *in vivo*. The NK activity of non-adherent spleen cells was evaluated by the ^{51}Cr-release cytotoxicity assay against murine lymphoma (Yac-1) target cells[72]. Furthermore, an inhibitory effect of aqueous and ethanol extracts of propolis on colon carcinogenesis was reported in rats[80,81]. Tripp *et al*.[82] suggested that propolis-activated macrophages could produce cytokines such as TNF-α and IL-12, which act on NK cells, increasing their cytotoxic activity against tumour cells. The findings of Kimoto *et al*.[27] indicated that artepillin C possesses direct antitumour activity. When artepillin C was applied to human and murine malignant tumour cells *in vitro* and *in vivo*, the growth of tumour cells was clearly inhibited. In addition to suppression of tumour growth, there was an increase in the ratio of CD4 to CD8 T cells and in the total number of helper cells.

IMMUNOMODULATORY EFFECT OF PROPOLIS AND ITS COMPONENTS ON BASOPHILE/MASTOCYTE FUNCTION

Basophiles are present in the peripheral blood in trace amounts, accounting for 0-1 % of total white blood cells and mast cells are widely distributed throughout vascularized tissues and certain epithelia. Both types of cells play a major role in allergic diseases, such as asthma[83].

Sensitivity to individual antigens (allergens) depends on the production of IgE antibodies. This process requires the presence of Th2 cells to induce the switching of antigen-specific antibody classes produced by B lymphocytes and the production and secretion of IL-4 needed for the growth and differentiation of B lymphocytes. IgE antibodies produced after prior contact with a specific antigen bind to IgE receptors on mast cells or basophils. Cross-linking of allergen with IgE and high affinity receptors (FcεR) and calcium influx induces rapid cell degranulation and release of inflammatory mediators such as histamine, PGs, leukotrienes and platelet activating factor (PAF) and several proinflammatory and chemotactic cytokines, such as TNF-α, interleukins IL-6, IL-8, IL-4, and IL-13, and transforming growth factor (TGF)-β1[84].

The literature reported a few clinical studies on the antiallergic activity of propolis. Khayyala *et al*.[85] administered an aqueous extract of 13 % propolis daily for 2 mo to patients with mild-to-moderate asthma. The propolis-treated patients showed reduced incidence and severity of nocturnal attacks and improvement in ventilatory functions, which was associated with decreased PGs, leukotrienes, and proinflammatory cytokines such as TNF-α, IL-6, and IL-8. The findings of Hirota *et al*.[86] suggested that the ethanol extract of Brazilian green propolis could be potentially beneficial as a prophylactic and therapeutic agent for asthma. Ten percent propolis treatment (1 mg/kg, once a day) inhibited the production of IgE, representative Th-2 cytokines, chemokines MCP-1 and TGF-β1, allergen-specific IgE, which resulted in inhibition of AHR (airway hyper-responsiveness) and reduced collagen deposition and the influx of inflammatory cells into the lungs of BALB/c mice challenged with allergens such as *Dermatophagoides farine* and diesel exhaust particles. In an experimental study by Shinmei *et al*.[87], propolis was given to rats for itchy nose and sneezing. The authors reported that symptoms were improved through decreased histamine release and that the long-term effects were favourable.

Likewise, Sy *et al*.[88] and Ammar *et al*.[89] used a model of pulmonary inflammation induced by ovalbumin (OVA) and demonstrated that treatment with propolis inhibits pulmonary inflammation and decreases serum level of IgE. Results reported by Nagai *et al*.[90] indicated that EEP modulates the proliferation of lung Th cells from Th2-cell-dominant to Th1-cell-dominant in an airway inflammation model of mice. This suggested that EEP has an effect on allergic asthma that developed in an IgE-dependent manner.

Ethanol and water extracts of Brazilian and Chinese propolis have been shown to possess antiallergic action,

inhibiting histamine release in rat peritoneal mast cells induced by Compound 48/80 and Concanavalin A[91]. These results were in agreement with Nakamura's study evaluating the effects of Brazilian and Chinese propolis on β-hexosaminidase release from antigen-stimulated rat basophilic leukaemia (RBL-2H3) cells. Both water and ethanol extracts of Chinese propolis inhibited degranulation more potently than Brazilian propolis extracts[92].

Nakamura *et al*.[92] identified chrysin and kaempferol as the major antiallergic components of ethanol extracts of Chinese propolis. Chrysin was also revealed to inhibit IL-4 and MCP-1 (monocyte-chemoattractant protein-1) production by these cells. Park *et al*.[93] showed that kaempferol suppressed elevation of intracellular calcium as well as histamine release from activated RBL-2H3 cells. Hirose *et al*.[94] made an interesting suggestion of the involvement of haeme oxygenase-1 in inhibition of mast cell activation by kaempferol. CAPE, the main component of Chinese propolis extract, reportedly inhibited NF-κB activation and inhibited PAF release, thereby reducing anaphylaxis in mice with OVA-induced asthma[95]. Those findings were in agreement with those of Jung *et al*.[96], who found that caffeic acid phenethyl ester exerted inhibitory effects on the inflammatory cells in BAL fluid in a murine model of asthma. CAPE significantly decreased the total leukocyte, eosinophil, lymphocyte and macrophage counts. Mice treated with caffeic acid phenethyl ester also showed a marked reduction in the infiltration of inflammatory cells within the peribronchiolar and perivascular regions. Mirzoeva and Calder[54] showed that propolis and CAPE were very potent inhibitors of PG and leukotriene synthesis by murine peritoneal macrophages *in vitro* and during zymosan-induced acute peritoneal inflammation *in vivo*. Orsi *et al*.[97] demonstrated that propolis at higher concentrations (300 mg/ml) directly activated mast cells, promoting the release of inflammatory mediators, which could be linked to allergic processes in propolis-sensitive individuals.

Current *in vitro* and some preliminary *in vivo* data suggested that propolis has immunomodulatory and antiinflammatory properties. Propolis from different geographic locations might have different active constituents. CAPE is one of the major components of many varieties of propolis found in the temperate zone, while artepillin C is one of the major compounds uniquely found in Brazilian green propolis in the tropical region. Propolis and these components have been shown to have immunomodulatory effects on a wide spectrum of immune cells, including those of lymphoid or monocytic lineage. It exhibited an immunoregulatory effect on the basic functional properties of immune cells, which is mediated by the Erk2/MAP-kinase (extracellular signal-regulated kinase 2 and MAP-kinase) signalling pathway and eukaryotic transcription factors: NFAT and NF-κB. Some authors have shown that propolis activated immune cells, while others claimed that propolis and its components, especially flavonoids, were the active immunosuppressants. In fact both statements appear to be correct, but the effect of propolis depended on its concentration.

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