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Propolis and the immune system: a review

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Abbreviations: ACF, aberrant crypt foci; ADCC, antibody-dependent cellular cytotoxicity; AST, aspartate aminotransferase; BSA, bovine serum albumin; CAPE, caffeic acid phenethyl ester; Con A, concanavalin A; Erk-2, extracellular-signal-regulated kinase; GC, gas-chromatography; GC-MS, gas chromatography-mass spectrometry; H_2O_2, hydrogen peroxide; IFN-γ, gamma-interferon; IL, interleukin; KLH, keyhole limpet hemocyanin; MAP, mitogen-activated protein; NK, natural killer cells; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion; OCl^-, hypochlorite; OH^-, hydroxyl radical; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SRBC, sheep red blood cells; TLC, thin layer chromatography; TGF-β1, transforming growth factor-beta 1; TNF-α, tumor

necrosis factor-alpha; TVT, transmissible venereal tumor; WSD, water-soluble derivative.

Abstract

Propolis has been used empirically for centuries and it was always mentioned as an immunomodulatory agent. In recent years, *in vitro* and *in vivo* assays provided new information concerning its mechanisms of action, thus a review dealing with propolis and the immune system became imperative. This review compiles data from our laboratory as well as from other researchers, focusing on its chemical composition and botanical sources, the seasonal effect on its composition and biological properties, its immunomodulatory and antitumor properties, considering its effects on antibody production and on different cells of the immune system, involving the innate and adaptive immune response. *In vitro* and *in vivo* assays demonstrated the modulatory action of propolis on murine peritoneal macrophages, increasing their microbicidal activity. Its stimulant action on the lytic activity of natural killer cells against tumor cells, and on antibody production was demonstrated. Propolis inhibitory effects on lymphoproliferation may be associated to its anti-inflammatory property. In immunological assays, the best results were observed when propolis was administered over a short-term to animals. Propolis antitumor property and its anticarcinogenic and antimutagenic potential are discussed. Since humans have used propolis for different purposes and propolis-containing products have been marketed, the knowledge of its properties with scientific basis is not only of academic interest but also of those who use propolis as well. This review opens a new perspective on the investigation of propolis biological properties, mainly with respect to the immune system.

Keywords: Propolis; Immune system; Antitumor property

1. Introduction

Propolis has attracted researchers' interest in the last decades, because of several biological and pharmacological properties, such as immunomodulatory, antitumor, antimicrobial, anti-inflammatory, antioxidant, among others (Bankova et al., 2000). Besides, propolis-containing products have been intensely marketed by the pharmaceutical industry and health-food stores (Banskota et al., 2001). The ethnopharmacological approach, combined with chemical and biological methods, may provide useful pharmacological leads. Thus, this review aimed to discuss its chemical composition and plant sources, as well as to discuss some mechanisms of action of this bee product on the immune system and against tumor cells.

Propolis is in no way a new discovery. The use of propolis goes back to ancient times, at least to 300 BC, and it has been used as a medicine in local and popular medicine in many parts of the world, both internally and externally. Egyptians, Greeks and Romans reported the use of propolis for its general healing qualities and for the cure of some lesions of the skin. Propolis has always been reputed as an anti-inflammatory agent and to heal sores and ulcers. Ancient Egyptians used it to embalm their dead, and more recently it was used during the Boer War for healing wounds and tissue regeneration (Ghisalberti, 1979). However, its use continues today in remedies and personal products, and the list of preparations and uses is endless. It is still one of the most frequently used remedies in the Balkan States (Bankova, 2005a), and it has only been in the last decades that scientists have investigated its constituents and biological properties.

Propolis is a resinous material collected by bees from bud and exudates of the plants, which is transformed in the presence of bee enzymes. Its color varies from green, red to dark brown. Propolis has a characteristic smell and shows adhesive properties, because it strongly interacts with oils and proteins of the skin. In general, propolis *in natura* is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and other substances (Burdock, 1998).

Etymologically, the Greek word propolis means *pro* = for or in defence, and *polis* = the city, that is "defence of the hive". Bees use it to seal holes in their honeycombs, smooth out internal walls, as well as to cover carcasses of intruders who died inside the hive, in order to avoid their decomposition. Propolis also protects the colony from diseases, because of its antiseptic efficacy and antimicrobial properties (Salatino et al., 2005).

After its administration to mice or to humans propolis does not seem to have side effects (Kaneeda and Nishina, 1994; Sforcin et al., 1995; Sforcin et al., 2002b; Jasprica et al., 2007). According to Burdock (1998) propolis is non-toxic, and its DL50 ranges from 2 to 7.3 g/kg in mice. This author suggested that the safe concentration for humans could be 1.4 mg/kg and day, or approximately 70 mg/day. After treatment of rats with different concentrations of propolis (1, 3 and 6 mg/kg/day), different extracts (water or ethanol) and varying the time of administration (30, 90 and 150 days) no significant alterations in total lipids, triglycerides, cholesterol, HDL-cholesterol concentrations, nor in AST and LDH specific activities were observed (Mani et al., 2006). The body weight of rats was measured in all these protocols, and propolis administration did not induce alterations in their weight. Cuesta et al. (2005) have not observed either mortality or growth rate alteration after daily intake of propolis in the diet, during 6 weeks.

Although few in number, some cases of propolis allergy and contact dermatitis have been reported (Hausen et al., 1987; Hegyi et al., 1990; Silvani et al., 1997; Callejo et al., 2001), differently from the common allergy to honey, which contains allergens derived from flowers. Beekeepers usually show sensitivity to propolis (Rudeschko et al., 2004; Gulbahar et al., 2005). Ethanol and water extracts of propolis possess anti-allergic action, inhibiting histamine release in rat peritoneal mast cells (Miyataka et al., 1998). However, in higher concentrations (300 μg/ml), propolis directly activated mast cells, promoting inflammatory mediators release, what could be linked to allergic processes in propolis-sensitive individuals (Orsi et al., 2005b).

Recently, the presence of radioactive particles in propolis samples was investigated, since these particles may be concentrated in the soil, contaminating the plants, insects and its products, and, consequently, humans as well. Cesium (Cs_{137}) was not found in the samples, and only natural radioactive particles such as potassium (K_{40}) and beryllium (Be_7) were found. These data suggested that propolis may be studied as an environmental contamination indicator, in order to understand the soil-plant-bee-propolis chain (Orsi et al., 2006a).

Propolis antimicrobial property has been widely investigated, and several authors have demonstrated its antibacterial action (Grange and Davey, 1990; Kujumgiev et al., 1999; Sforcin et al., 2000; Orsi et al., 2005c; Orsi et al., 2006b; Scazzocchio et al., 2006). Fernandes Jr. et al. (2001) investigated the antibacterial action of propolis produced by Africanized honeybees, comparing with that produced by the stingless bees (subfamily Meliponinae). Propolis produced by *Partamona* sp and *Melipona* sp had a similar activity to that produced by *Apis mellifera*.

Propolis also shows antiviral (Amoros et al., 1992; Serkedjieva et al., 1992; Vynograd et al., 2000; Ito et al., 2001; Huleihel and Isanu, 2002; Gekker et al., 2005), antifungal (Dobrowolski et al., 1991; Sforcin et al., 2001) and antiparasite activities (Higashi and De Castro, 1994; De Castro and Higashi, 1995; Salomão et al., 2004; Freitas et al., 2006).

Propolis extraction methods may influence its activity, since different solvents solubilize and extract different compounds. The most common extracts used in biological assays are ethanol, in different concentrations, methanol and water (Cunha et al., 2004). Its chemical composition is very complex: more than 300 components have already been identified, and its composition is dependent upon the source plant and local flora. Moreover, propolis composition is completely variable, creating a problem for the medical use and standardization (Marcucci, 1995; De Castro, 2001).

The term "propolis" is not characterizing with respect to the chemical composition, unlike the term "bee venom" for example (Bankova, 2005a), so that the biological studies with propolis must be carried out identifying its botanical sources and chemical composition as well.

Propolis samples, collected in the Beekeeping Section of the University, UNESP, Campus of Botucatu, SP, Brazil, were analysed by gas-chromatography (GC), gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC), revealing that its main components are phenolic compounds (flavonoids, aromatic acids, benzopyranes), di- and triterpenes, essential oils, among others. Flavonoids are present in small quantities in Brazilian propolis (kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether); a prenylated *p*-coumaric acid and two benzopyranes: *E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes; essential oils (spathulenol, (2Z,6E)-farnesol, benzyl

benzoate and prenylated acetophenones); aromatic acids (dihydrocinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, which are common for poplar propolis, 3,5-diprenyl-*p*-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran); di- and triterpenes were identified, among others.

In the temperate zone of the Northern Hemisphere bees collect propolis only in summer, including late spring and early autumn. In Brazil, propolis collection proceeds throughout the entire year and seasonal variations could be expected. This aspect has a practical application: propolis could be collected during the seasons with higher concentrations of biologically active compounds. Thus, propolis produced by Africanized (*Apis mellifera* L.) and Italian (*Apis mellifera ligustica*) bees all over a year was investigated, in order to analyse its constitution, as well as to compare its activities in different biological assays. Data showed that seasonal variations in propolis composition are not significant and are predominantly quantitative. This fact indicated that bees collect propolis from the same plant group, with a predominant vegetal source. Also, no differences were seen between Africanized or Italian bees, since propolis composition was qualitatively identical (Boudourova-Krasteva et al. 1997; Bankova et al. 1998a,b).

Bud exudates of different poplar species are the main sources of propolis in temperate zone, including Europe, Asia and North America. Samples originating from these regions are characterized by similar chemical composition; the most important constituents appeared to be phenolics: flavonoids, aromatic acids and their esters. In Russia, the main plant source of propolis is *Betula verrucosa* Ehrh., and its main biologically active substances are flavones and flavonols, whereas in Cuba and Venezuela, *Clusia* spp. are its main vegetal sources and polyprenylated benzophenones are the main active components (Bankova, 2005a).

The main vegetal source of propolis samples in Botucatu, SP, Brazil, is *Baccharis dracunculifolia* DC., followed by *Eucalyptus citriodora* Hook and *Araucaria angustifolia* (Bert.) O. Kuntze. Plants visited by bees in our apiary (UNESP, Campus of Botucatu) were collected, identified in the Department of Botany of our Institute. Leaves from *Araucaria* and *Baccharis* and trunk from *Eucalyptus* (parts of the plants preferably visited by bees) were investigated using GC-MS. The main components identified in *B. dracunculifolia* and in propolis were almost the same: dihydrocinnamic acid, *p*-coumaric acid, prenyl- and diprenyl-*p*-coumaric acids and flavonoids in similar concentrations. On the other hand, some components were entirely absent in *Baccharis* exudates. Overall, the main components of *Eucalyptus citriodora* are aromatic acids, a class of compounds usually found in propolis, and sugars. *Araucaria angustifolia* exudates contained only traces of aromatic acids, consisting mainly of diterpenic acids (Bankova et al. 1999).

It is important to mention that the identification of these 3 plants does not exclude the possibility that other plants could also contribute as vegetal sources of propolis, however it has been reported that bees do not change its chemical composition in a specific geographic region, because they visit essentially the same vegetal sources. Africanized bees have a preference for *Baccharis dracunculifolia* as sources of propolis in Brazil (Teixeira et al., 2005). Volatile substances, in the resiniferous ducts or gland trichomes, trigger bee attraction.

Bankova (2005b) reported that the distinct chemistry of propolis from different origins leads to the expectation that the biological properties of different propolis types will be dissimilar. Propolis is the defence of bees against infections, and the antibacterial and antifungal activities are mainly due to flavonones, flavones, phenolic acids and their esters for European

propolis, while such activities are due to prenylated *p*-coumaric acids and diterpenes for Brazilian propolis. The fact that different chemistry leads to the same type of activity and in some cases even to activity of the same magnitude is amazing. A universal chemical standardization would be impossible, and for this reason, a detailed investigation of propolis composition, its botanical origin and biological properties are meaningful (Bankova, 2005a). The use of chemically characterized propolis samples for biological assays is the way to study its properties, and to do comparative studies. This author discussed this aspect very well, mentioning that the composition of the plant source determines propolis composition. Combined with the knowledge of active principles, it gives clues to standardization and quality control. Measurement of the concentrations of groups of active compounds instead of that of individual components would be the right approach in the case of propolis. There is still a lot of work to be done in order to achieve a reliable standardization on propolis types and formulate recommendations for practitioners, as well as to connect a particular propolis sample to a specific biological activity (Bankova, 2005a,b).

2. Propolis immunomodulatory action

2.1. Propolis action on macrophages

Before the problem of propolis standardization, the greatest problem to carry out the immunological assays was to design the experimental protocols, since researchers have used different concentrations of propolis *in vivo* and *in vitro*, as well as different extracts, intake period and routes of administration. Table 1 shows some assays dealing with propolis immunomodulatory action according to its dose, chemical composition and main components, and assay conditions.

Little was known about the immunomodulatory action of propolis until the 1990s, but in the last decade new and interesting articles were published, providing an important contribution to this research field.

In immunosupression models, administration of a water-soluble derivative (WSD) of propolis (50 mg/kg) to mice prevented the cyclophosphamide effects and enhanced the survival rate of the animals (Dimov et al., 1991). These authors also suggested that propolis modulates the non-specific immunity, via macrophage activation. Propolis (0.2-1.0 mg/ml) stimulated cytokines production, such as IL-1β and TNF-α, by peritoneal macrophages of mice (Moriyasu et al., 1994). Propolis (0.150 mg/g) was also able to modulate both *in vivo* and *in vitro* production of C1q by macrophages, as well as the complement receptor function either directly or via cytokines (Dimov et al., 1992). *In vitro* assays showed that WSD of propolis (63-1000 μg/ml) inhibited the classical and alternative pathways of the complement system (Ivanovska et al., 1995a). C3 was one of the targets of propolis action, and flavonoids and phenolic compounds were pointed out as its major anticomplementary compounds (Georgieva et al., 1997).

It was demonstrated that six isolated compounds of propolis, identified as caffeoylquinic acid derivatives, enhanced the motility and spreading of macrophages (Tatefuji et al., 1996). Exposure of macrophages to a varied number of stimuli, such as the interaction with microorganisms and their products, antibodies or complement components-opsonized antigens, phorbol miristate acetate (PMA), Con A, immune complexes, leukotrienes, chemiotactic peptide fMLP (n-formyl-methionyl-leucyl-phenylalanine), cytokines, among others, may result in further metabolic changes, such as oxygen intermediates generation. The production of such reactive species

appears to be one of the mechanisms by which macrophages become microbicidal.

NADPH oxidase catalyses the reduction of molecular oxygen to superoxide anion (O_2^-) and the burst respiratory is paralleled by a higher consumption of oxygen (Krol et al., 1995). O_2^- is the precursor of other reactive oxygen intermediates, including hydroxyl radical (OH^-), hypochlorite (OCl^-) and hydrogen peroxide (H_2O_2). Oxidants produced by phagocytes may destroy important biomolecules as well as phagocyted microorganisms, and are also involved in the tissue injury associated with inflammatory diseases (Moonis et al., 1992; Brown, 1995; Babior, 2000).

Antioxidants are classically defined as molecules that, present in lower concentrations than biomolecules, may prevent, protect or reduce the extension of oxidative damage, such as, for example, glutation peroxidase, catalase and superoxide dismutase. Other antioxidants, such as ascorbic acid (vitamin C) and tocopherol (vitamin E) are non-enzymatic antioxidants. Thus, there is a delicate balance between the generation and destruction of oxidant agents, which may be beneficial or deleterious to the organism (Novelli, 2005).

Evaluating *in vitro* propolis effects on macrophage activation, it was shown that 5, 10 and 20 μg/ml of propolis increased H_2O_2 generation by these cells (Orsi et al., 2000). Ivanovska et al. (1993), investigating the effects of complexes of cinnamic and caffeic acids with lysine, at a molar ratio of 1:2, demonstrated that cinnamic acid inhibited H_2O_2 generation by peritoneal macrophages, while caffeic acid induced its production. Krol et al. (1995) reported that flavones (10 or 100 μmol/l) inhibited luminol-dependent chemoluminescence of murine macrophages, by a mechanism involving the protein kinase C phosphorilation. Simões et al. (2004), in chemoluminescence

assays with rabbit neutrophils, also observed the inhibitory effect of propolis (2-25 μg/ml) and some of its components on superoxide anion production by these cells. These results are interesting, because the inhibition of the burst respiratory may lead to some antigens persistence in the host. However, propolis mechanism of action on free radical generation by macrophages is still unclear (Cuesta et al., 2005).

Another indicative of macrophage activation is nitric oxide (NO) generation, from L-arginine by the nitric oxide synthase (NOS) (Macfarlane et al., 1999; Novelli, 2005). NO is an important microbicidal mechanism of macrophages for inhibiting DNA synthesis, mitochondrial respiration and active transport in fungal and bacterial membrane (Chan et al., 1992; Macmicking et al., 1997). Besides, NO is also an important neurotransmissor, vasodilator and cellular mediator of tissue repair (Chakraborty et al., 2006).

Propolis (50 and 100 μg/ml) inhibited NO generation by peritoneal macrophages (Orsi et al., 2000). Moriyasu et al. (1994) also observed that propolis (0.2-1.0 mg/ml) inhibited NO production by LPS stimulated-macrophages, and Krol et al. (1996) linked this effect to flavonoids (10-50 μM). Hu et al. (2005) evaluated the action of water and ethanolic extracts of propolis (1 ml/100g) in a murine model of acute inflammation, verifying that both extracts inhibited NO generation.

The most potent known endogenous suppressor of NOS2 expression in murine macrophages is TGF-β1, which destabilized NOS2 mRNA, retarded the synthesis of NOS2 protein, and accelerated its degradation (Macmicking et al., 1997). In fact, TGF-β1 concentration is increased in the supernatant of T cell or peripheral mononuclear blood cell culture, after incubation with

propolis (Ansorge et al., 2003), and this is a possible explanation for propolis inhibitory effect on NO production.

After propolis treatment (2.5 and 5 mg/kg) of mice for 3 consecutive days, peritoneal macrophages were activated *in vitro* with gamma-interferon (IFN-γ), and the production of H_2O_2 and NO was increased in comparison to non-activated cells (control) (Orsi et al., 2000). This fact suggests 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, showed an inhibition in H_2O_2 and NO generation.

Propolis effects were analysed on macrophages of BALB/c mice submitted to immobilization stress, as well as on the histopathological analysis of the thymus, bone marrow, spleen and adrenal glands. Stressed mice showed a higher H_2O_2 generation by peritoneal macrophages, and propolis treatment (200 mg/kg) potentiated H_2O_2 generation and inhibited NO production by these cells. Histopathological analysis of stressed mice showed no alterations in the thymus, bone marrow and adrenal glands, but an increase in germinal centers in the spleen was seen. Propolis treatment counteracted the alterations found in the spleen of stressed mice (Missima and Sforcin, 2007).

Biological properties of propolis vegetal sources may be a strong argument to use them in human and veterinary medicine, in order to compare their potential with propolis activities. Thus, the effect of 3 main vegetal sources of propolis in our apiary (*Araucaria, Baccharis* and *Eucalyptus* – 5, 10 and 20 μg/ml) on macrophages activation was analysed, evaluating oxygen (H_2O_2) and nitrogen (NO) metabolites determination. Data suggested no effects related to such extracts on these metabolites production (Lopes et al., 2003). Propolis action is a consequence of plant-derived products and isolated

extracts of its vegetal sources did not have the same effect in this assay. There may be synergistic effects, which lead propolis to have different pharmacological activities.

Since *Baccharis dracunculifolia* DC is the main propolis source in our region, the effect of *B. dracunculifolia* extracts and some purified compounds on reactive oxygen intermediates (H_2O_2) production by peritoneal macrophages of male BALB/c mice was also analysed. Data revealed that the leaf (25, 50 and 100 µg/ml), leaf rinse (25 µg/ml) and the root (25 µg/ml) extracts induced an elevation in H_2O_2 release by macrophages. Among the isolated compounds, bacchari*s* oxide and friedelanol (100 µM) increased the H_2O_2 production. These results suggest a stimulant action of extracts and isolated compounds of *B. dracunculifolia* on macrophages (Missima et al., 2007). Further investigations will contribute to a better comprehension of the immunomodulatory action of this plant, as well as of its secondary metabolites.

In order to evaluate propolis effect on macrophages microbicidal action, our group carried out some works, comparing the effects of Brazilian and Bulgarian propolis. The effect of different concentrations of propolis on macrophages fungicidal action against the thermally dimorphic fungus *Paracoccidioides brasiliensis*, the etiologic agent of paracoccidioidomycosis, was analysed. This human mycosis is one of the most prevalently serious mycoses in Latin America and the great majority of the infected persons develop an asymptomatic pulmonary infection, although some individuals present clinical manifestations, leading to the dissemination of the disease. Clinical and experimental data indicate that cell-mediated immunity plays a significant role in host defence, whereas high levels of specific antibodies are associated with the most severe form of this disease. Experimental models

have shown the role of macrophages in the mechanisms of resistance against this fungus (Borges-Walmsley et al., 2002).

Macrophages were incubated with Brazilian or Bulgarian propolis (5, 10 and 20 μg/100μl), and subsequently challenged with *P. brasiliensis*. Propolis increased the fungicidal activity of macrophages, but not significantly. Bulgarian propolis also showed a non-significant increase in the fungicidal activity of macrophages, and no differences were seen with the Brazilian propolis (Murad et al., 2002). Since propolis was able to activate macrophages and enhance its fungicidal action, an indirect effect might be postulated.

In experimental works of our laboratory using human cells, adequate concentrations of TNF-α alone or in a synergistic effect with IFN-γ significantly increased the fungicidal activity of these cells (Calvi et al., 2003). The process of phagocytosis is complex and involves the binding of the target to the surface of macrophages and ingestion, which usually triggers the so-called oxidative burst. Propolis could exert its function by increasing directly the liberation of fungicidal substances by macrophages, such as oxygen and nitrogen metabolites, as well as inducing production of some pro-inflammatory cytokines.

Brazilian and Bulgarian propolis effects on the bactericidal activity of macrophages against *Salmonella* Typhimurium – causative agent of typhoid fever in humans, were also analysed. The best characterized animal model for typhoid fever is the murine one, using *Salmonella* Typhimurium. Mice infected with this serovar display a systemic infection that serves as an experimental approach to study typhoid fever (Schwan et al., 2000).

Some *Salmonella* serovars are intracellular parasites, which can survive and replicate within mononuclear or polymorphonuclear phagocytes. The

inhibition of phagosome-lysosome fusion is an important factor for *Salmonella* survival within macrophages and for its virulence (Buchmeier and Heffron, 1991). Through phagocytes, the bacteria are transported to the spleen, liver, and other target tissues during the normal disease course (Huang et al., 1998).

Salmonella infection depends on the bacterial amount, which may influence the rapidity of the bacteria to invade the intestinal epithelium, to infect macrophages and to spread in the organism (Schwan et al., 2000). Thus, different ratios of macrophage/bacteria and different periods of incubation were standardized, verifying that the highest percentage of bactericidal activity occurred at 60 minutes, with the ratios 10:1, 1:1 and 1:10 (Orsi et al., 2005a).

Both Brazilian and Bulgarian propolis samples increased the bactericidal activity of macrophages against *S*. Typhimurium, depending on its concentration (3, 10, 30 and 100 µg/100µl). No differences were seen between these samples, although they were produced in different geographic regions. Data also showed that the bactericidal activity of macrophages, using different ratios macrophage/bacteria and different propolis concentrations, might have occurred through oxygen and nitrogen intermediates (Orsi et al., 2005a). However, a possible role of other microbicidal mechanisms should be investigated further. It is important to mention that no ethanol effects (propolis solvent) were seen in all immunological assays of our group.

Table 1
Propolis immunomodulatory action according to its dose, chemical composition and main components, and assay conditions of some authors

Immunological assay	Reported outcome	Dose and route	*In vivo / in vitro*	Main groups or active components	Characterization	Authors
Antibody production	↑	500µg/ mouse (ip.)	*in vivo*	NM	NM	Scheller et al. (1988)
Classical and alternative pathways of the complement system	↓	WSD (63-1000 µg/ml)	*in vitro*	phenolic acids and their esters, phenolic alcohols, aldehydes and ketones, flavonoids, stilbenes, coumarins	HPLC, GC-MS	Ivanovska et al. (1995a)
Macrophages spreading and motility	↑	10^{-5} M	*in vitro*	5-caffeoylquinic acid, chlorogenic acid, 4-caffeoylquinic acid, 4,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid	HPLC	Tatefuji et al. (1996)
Macrophages activation	↑ H_2O_2 ↓ NO	2.5-100 µg/ml	*in vitro*	prenylated p-coumaric acid and benzopyranes, essential oils, aromatic acids, di- and triterpenes	GC, GC-MS, TLC	Orsi et al. (2000)

Lymphocyte proliferation	↓	5-100 µg/ml	*in vitro*	prenylated p-coumaric acid and benzopyranes, essential oils, aromatic acids, di- and triterpenes	GC, GC-MS, TLC	Sá-Nunes et al. (2003)
	↓	2.5, 5 and 10 mg/kg (p.o.)	*in vivo*			
Antibody production	↑	5, 10 and 20 mg/kg (p.o.)	*in vivo*	CAPE	NM	Park et al. (2004)
Antibody and IFN-γ production	↑	5 mg/dose (s.c.)	*in vivo*	phenolic compounds, cinnamic acid, flavonoids (pinobanksin, kaempferol)	HPLC	Fischer et al. (2007)

NM = not mentioned; ↑ = stimulant action; ↓ = inhibitory action; ip = intraperitoneal route; p.o. = per oral; s.c. = subcutaneous route; WSD = water-soluble derivate of propolis; GC = gas chromatography; GC-MS = gas chromatography-mass spectrometry; TLC = thin layer chromatography; HPLC = high performance liquid chromatography.

2.2. Propolis action on lymphocytes and antibody production

Propolis' immunomodulatory action was thought to be limited mainly to macrophages, with no influence on lymphocyte proliferation (Dimov et al., 1991). However, Ivanovska et al. (1995b) demonstrated that splenocytes from mice treated with cinnamic acid – a propolis constituent, possessed an enhanced ability to incorporate thymidine, in the presence of mitogens such LPS, phytohemagglutinin (PHA) or Con A, suggesting a proliferative tendency of these cell cultures in the absence of mitogens. Serum IL-1β of these animals was elevated, suggesting that cinnamic acid could activate macrophages, thus affecting the initial events of the immune response. Bratter et al. (1999) investigated propolis effect on human pro-inflammatory cytokines, after propolis capsules administration (500 mg) for 2 weeks, verifying that the plasma level of TNF-α, IL-1β, IL-6 and IL-8 did not change, although propolis led to a significantly increase of both spontaneous and LPS-induced cytokine secretion capacity of peripheral blood leukocytes.

In order to evaluate the influence of propolis on the lymphoproliferative response of mice, lymphocyte polyclonal activation of propolis-treated mice and IFN-γ production by these cells were analysed. An inhibitory effect of propolis (5-100 μg/ml) on splenocyte proliferation was observed *in vitro* (Sá-Nunes et al., 2003). Previous studies have shown that flavonoids have an immunossupressor effect on the lymphoproliferative response (You et al., 1998). Since propolis contains flavonoids (Bankova et al., 1998b), this could explain the reported effect.

Propolis strongly suppresses DNA synthesis of human peripheral blood mononuclear cells (PBMC) and purified T cells in a dose-dependent manner. These effects are at least in part mediated by some of its constituents, namely

caffeic acid phenethyl ester (CAPE), and the flavonoids quercetin and hesperidin (Ansorge et al., 2003).

Baseline proliferation of splenocytes was not affected when mice were treated for 3 days with propolis (2.5, 5 and 10 mg/kg). However, Con A-stimulated cells of propolis-treated animals had a significant proliferation inhibition, whereas control mice showed a normal proliferative response to this mitogen (Sá-Nunes et al., 2003). One explanation for these results could be the production of cytokines with antiproliferative effect on responding T cells or the induction of biochemical mediators from macrophages that could decrease proliferation.

The treatment of peritoneal macrophages with these same concentrations of propolis was able to modulate nitric oxide production (Orsi et al., 2000). NO is responsible for the inhibition of DNA synthesis in several cells (Drapier and Hibbs, 1986), promotion of cytostasis in tumor target cells (Pervin et al., 2001) and depression of T cell proliferation in different experimental models. Propolis treatment could pre-activate macrophages *in vivo* to produce NO, which in turn could be responsible for the inhibitory effect against lymphocyte proliferation.

NO production by activated macrophages *in vitro* and *in vivo* is dependent of IFN-γ. To further evaluate other biological effect of propolis treatment on lymphocyte activation, IFN-γ production was measured in cell culture supernatants as another parameter of T cell activation and as an indirect evidence of NO production. Data demonstrated that propolis alone did not induce IFN-γ release, but that Con A-stimulated spleen cells from propolis-treated mice produced significantly more IFN-γ. These results corroborate our hypothesis and indirectly suggest that NO may inhibit proliferation (Sá-Nunes et al., 2003).

Dantas et al. (2006) investigated the effects of Bulgarian propolis (25 and 50 mg/kg) in the experimental model of *Trypanosoma cruzi*-infected Swiss mice, verifying that this bee product led to a decrease in parasitemia and showed no hepatic or renal toxic effect. These authors also found that propolis inhibited partially the increase in $CD69^+$ and $CD44^+$ expression in $CD4^+$ and $CD8^+$ lymphocyte subsets in infected animals and the decrease in $CD8^+CD62L$ expression, suggesting inhibition of an effector/memory function for both subsets of T cells. It was suggested that propolis could act directly on the T cells inhibiting their differentiation and consequently the development of acquired immune response.

Propolis inhibited human IL-12, IL-2, IL-4 and IL-10 production, whereas the production of TGF-β1 by T regulatory cells was increased in the supernatant of PBMC or T cells cultures, after incubation with propolis. TGF-β1 and IL-10 may be produced by T regulatory cells. Since propolis increases TGF-β1 production, this cytokine could also influence cell division, as well as decrease the production of other cytokines. IL-12 is thought to drive differentiation of T cells toward Th1 type cell. Since propolis was shown to be able to inhibit this cytokine production, as well as IL-2 and IL-4 production, it was suggested that propolis and some of its constituents could inhibit Th1 and Th2 type cells (Ansorge et al., 2003).

In order to understand the possible molecular mechanisms responsible for the negative regulation of cellular growth by propolis, Ansorge et al. (2003) studied the MAP (mitogen-activated protein) kinase signal pathway, measuring the induction of mRNA expression of the extracellular-signal-regulated kinase (Erk-2), which is capable of regulating several transcription factors which in turn control the regulation of critical genes of lymphocytes including that of IL-2. Erk-2 was strongly suppressed in propolis-stimulated

PBMC, what clearly suggests that one way of signaling triggered by propolis is mediated by the MAP kinase Erk-2, although the potential role of propolis as immunoregulating is underscored.

Another explanation for propolis inhibitory effects on lymphoproliferation comes from the observation that CAPE has inhibitory effects both on transcription factors NF-κB and NFAT (Márquez et al., 2004). As a consequence, CAPE inhibited IL-2 gene transcription, IL-2R (CD25) expression, and proliferation of human T cells, providing new insights into the molecular mechanisms involved in the anti-inflammatory and immunomodulatory activities of this natural compound.

Several authors have reported the anti-inflammatory action of propolis (Khayyal et al., 1993; Miyataka et al., 1997; Hu et al., 2005; Paulino et al., 2006). Khayyal et al. (2003) investigated the effects of an aqueous extract of propolis 13%, administered daily for 2 months as an adjuvant to therapy to patients with mild to moderate asthma. At the end of their study, patients receiving propolis showed a marked reduction in the incidence and severity of nocturnal attacks and improvement of ventilatory functions, what was associated with decreases of prostaglandins, leukotrienes, pro-inflammatory cytokines (TNF-α, IL-6, IL-8) and increased IL-10.

Propolis administration (200 mg/kg) for 14 days to C57BL/6 mice led to inhibition of IL-1β, IL-6, IFN-γ, IL-2 and IL-10 production by spleen cells, suggesting its anti-inflammatory activity once it is well established that cytokines orchestrate and perpetuate the chronic inflammatory features of several diseases (unpublished data of our group).

With regards to the humoral immune response, the ethanolic extract of propolis (500 μg/mouse) increases the antibody production in sheep red blood cells (SRBC)-immunized mice (Scheller et al., 1988), associating this

stimulatory activity to macrophages activation, which lead to cytokines production, regulating the functions of B and T cells. These authors observed higher antibody levels when propolis was administered in a short-term to the animals. Orsolic and Basic (2003) suggested that the increased IL-1β production by macrophages from propolis-treated mice might be associated with enhanced T and B cell proliferation.

Still to the adaptive immunity, propolis effect on antibody production by bovine serum albumin (BSA)-immunized rats was investigated. In this work, it was also analysed the seasonal effect on propolis action, Brazilian and Bulgarian propolis samples were compared, and the action of some active compounds and *Baccharis* extract on antibody production was investigated.

Propolis 10% administration to rats increases antibody production after 15 days of immunization (Sforcin et al., 2005). Propolis ability to modulate antibody synthesis is a part of its adjuvant activity, since it has been shown recently that propolis has a potent effect on different cells of innate immune response (Sforcin et al., 2002a; Orsi et al., 2005a).

No differences were seen between the samples from each season, what is in accordance with previous works of our laboratory (Sforcin et al., 2000; Sforcin et al., 2001; Sforcin et al., 2002a; Sforcin et al, 2002b). Brazilian and Bulgarian propolis stimulated antibody production in the same magnitude, and no differences were detected between their activities. This is in agreement with other results of our laboratory, using different experimental models (Murad et al., 2002; Orsi et al., 2005a).

Caffeic acid and quercetin (100 mg/kg) had no effects on antibody production (Sforcin et al., 2005). These compounds are responsible for several biological properties, such as antimicrobial effect (Mirzoeva et al., 1997). Caffeic acid esters possess significant cytotoxicity towards various tumor cells

(Lee et al., 2000), although other phenolic compounds and diterpenoids isolated from propolis also have antitumor properties (Banskota et al., 2001). Besides the effect of individual constituents, there may be synergistic effects of several compounds, thus conferring different pharmacological activities to propolis. Kujumgiev et al. (1999) suggested that general biological properties of propolis are due to a natural mixture of its components, and a single propolis constituent does not have an activity greater than that of the total extract. *Baccharis* extract 10% did not increase antibody production significantly when compared to control, but efficiently when compared to propolis-treated rats (Sforcin et al., 2005).

CAPE administration (5, 10 and 20 mg/kg) to female BALB/c mice for 14 days increased antibody production to SRBC and to keyhole limpet hemocyanin (KLH), what was attributed to the increased T lymphocyte proliferation, as well to the secretion of IL-4 and IL-2 by splenocytes (Park et al., 2004).

The adjuvant capacity of propolis (5 mg/dose) associated to inactivated Suid herpesvirus type 1 (SuHV-1) vaccine was evaluated, verifying that mice inoculated with SuHV-1 vaccine plus aluminium hydroxide and propolis extract presented higher levels of antibodies. The use of SuHV-1 vaccine plus propolis alone did not induce significant levels of antibodies, however, the combination was able to increase the cellular immune response, evidenced by the increase in the expression of mRNA to IFN-γ. Besides, propolis increased the percentage of protected animals against challenge with a lethal dose of SuHV-1, suggesting its use in vaccines as an adjuvant (Fischer et al., 2007).

Propolis adjuvant property in combination with the inactivated vaccine against *Aeromonas hydrophila* was analysed in carps. The phagocytic activity of these fishes and their serum antibodies against *A. hydrophila* were higher

comparing to non-adjuvant vaccinated fishes (Chu, 2006). Immunostimulants could activate antigen presenting cells (e.g. macrophages) and stimulate these cells to produce cytokines, which in turn activate T and B lymphocytes, suggesting its potential use as an adjuvant or immunostimulant in fish vaccines.

2.3. Propolis' antitumoral activity

Several researchers have reported the antitumoral property of propolis *in vivo* and *in vitro*. Propolis antiproliferative activity on tumor cells has been demonstrated and some responsible compounds were isolated (Rao et al., 1995; Huang et al., 1996; Banskota et al., 2001). Table 2 shows some assays dealing with propolis' antitumoral action according to its dose, chemical composition and main components, and assay conditions.

Matsuno (1995) isolated an active substance from Brazilian propolis and characterized it as a new clerodane diterpenoid (namely PMS-1), which inhibited the growth of hepatoma cells and arrested the tumor cells at S phase. Matsuno et al. (1997a) isolated a compound (PRF-1) from a water extract of propolis, which showed antioxidant activity and was cytotoxic to human hepatocellular carcinoma, HeLa and human lung carcinoma HLC-2 cells. Their group also isolated a tumoricidal compound identical to artepillin C, described as a constituent from *Baccharis* species, and its cytoxicity seemed to be partly attributable to apoptosis-like DNA fragmentation (Matsuno et al., 1997b). Kimoto et al. (1998) investigated the effects of artepillin C *in vitro*, verifying suppression of tumor growth, and *in vivo* there was an increase in the ratio of CD4/CD8 T cells, indicating that this compound activated the immune system.

Liao et al. (2003) demonstrated the inhibitory effect of CAPE on angiogenesis, tumor invasion and pulmonary metastatic capacity of CT26 cells. CAPE also prolonged the survival of mice implanted with CT26 cells, suggesting its potential as an antimetastatic agent.

CAPE (10-400 μM) had a dose-dependent effect on the cytotoxicity of C6 glioma cells, reducing the viability to 42% in relation to control, and increasing the proportion of hypodiploid DNA, as indication of apoptosis (Lee et al., 2003). The tumor suppressor protein p53 is a nuclear phosphoprotein that can potently regulate the growth of mammalian cells (Vogelstein and Kinzler, 1992). Activation of p53 results in altered transcription of a wide variety of genes that are involved in many aspects of cell metabolism, cell cycle regulation and apoptosis. CAPE increases the phosphorilation and expression of p53 and Bax, which can form heterodimers with Bcl-2 in mitochondrial membrane and accelerate apoptosis (Lee et al., 2003). Aso et al. (2004) reported that the antitumor activity of propolis occurs through the induction of apoptosis via caspase pathways.

CAPE also interferes in cell cycle arrest. After incubation with CAPE for 24 h, the cell number percentage of C6 glioma cells in the G0/G1 phase increased to 85%, due to the inhibition of pRB phosphorylation. The phosphorylation of pRB by the CDKs/cyclins is believed to be a crucial event in the regulation of S-phase entry, and appears to define the restriction point in the late G1 phase. An *in vivo* study demonstrated that CAPE decreased the growth of the xenografts of C6 glioma cells in nude mice by inhibiting cell proliferation. Histochemical and immunohistochemical analysis revealed that CAPE treatment significantly reduced the number of mitotic cells and proliferating cell nuclear antigen (PCNA)-positive cells in C6 glioma (Kuo et al., 2005).

CAPE derivatives (50-200 μM) were investigated on oral cancer using a cultured cancer cell line (squamous cell carcinoma = SAS; oral epidermoid carcinoma-Meng 1 = OEC-M1) and normal human oral fibroblast (NHOF), examining their effects on cell growth pattern, their cytotoxicity and changes in the cell cycle. Caffeic acid phenethyl esters showed cytotoxic effects on tumor cells but not on NHOF cell line. Flow cytometric analysis showed OEC-M1 cell arrest at G2/M phase. Such differential effects on cancer and normal cells suggested these compounds might be useful in oral cancer chemotherapy (Lee et al., 2005). The chemical structure of CAPE is shown in figure 1.

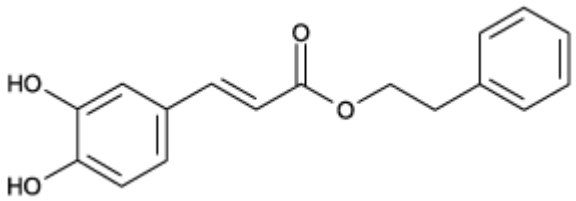

Figure 1. Chemical structure of caffeic acid phenethyl ester (CAPE).

Although direct carcinostatic effects of propolis or its isolated components have been demonstrated, an important question is whether propolis acts on the immunocompetent cells to help tumor cell destruction.

Resistance to spontaneous tumor development has been associated with the cytotoxic activity of natural killer (NK) cells, found both in humans and experimental animals. NK cells are characterized as a lymphocyte subpopulation different from T and B cells, and non-adherent and non-phagocytic cells, showing lytic activity mainly towards several types of tumor and virus-infected cells (Kaneno, 2005).

In contrast to T and B lymphocytes that require a proliferation phase that selects a population of effectors, NK cells are charging immediately on finding their targets. Although in many cases NK cells activity against tumor cells correlates with decreased levels of MHC molecules, recent observations indicate that insufficient expression of MHC might not always be necessary for effective target cells killing. Activation of NK cells is not only a result of a loss of MHC class I alleles but also results from the direct recognition of target cell structures. NK cells cooperate with adaptive immunity, secreting cytokines that regulate the function of T cells (Jakóbisiak et al., 2003).

Propolis 10% treatment for 3 days increased the cytotoxic activity of NK cells against murine lymphoma (Sforcin et al., 2002a). This finding confirmed a previous observation that propolis administration over a short-term leads to better results concerning the immune system, increasing the immunological response (Scheller et al., 1988). The lack of seasonal effect of propolis activity was also observed in NK assays (Sforcin et al., 2002a). NK cells are under cytokines action, such as IFN (α, β, γ), TNF-α, TGF-β1, IL-1β, IL-2, IL-4, IL-10, IL-12, IL-13, IL-15, IL-21, IL-23 (Kaneno, 2005), but the activation mechanism of these cells by propolis still remains obscure. One may suggest that propolis-activated macrophages could produce cytokines, such as TNF-α and IL-12, which act on NK cells, increasing its cytotoxic activity.

Macrophages play an important role in antitumor response, through antibody-dependent cellular cytotoxicity (ADCC), secretion of inhibitory cytokines for tumor growth, and production of reactive oxygen and nitrogen intermediates. Treatment of mice with water extract of propolis (50 mg/kg) modified macrophages tumoricidal activity, with a higher production of lymphocytes activating factors, thus inhibiting the human cervical carcinoma

cell line (HeLa) and Chinese hamster lung fibroblast (V79). Propolis-treated mice also showed an elevated splenocyte response to polyclonal mitogens (Orsolic and Basic, 2003).

Propolis (50 and 150 mg/kg) and some isolated polyphenolic compounds (caffeic acid, CAPE and quercetin) decreased the number of tumor nodules in the lung. The antimetastatic effectiveness of propolis was of higher degree than that achieved by its constituents. Upon activation, macrophages release mediators such as TNF-α, H_2O_2 and NO, which are involved in the inhibition of DNA synthesis and tumor cells destruction. Treatment of mice with propolis or CAPE increased NO production, which corresponded with reduction of DNA synthesis of tumor cells. However, caffeic acid did not show any effect on NO production, suggesting that another mechanism different from the one of propolis or CAPE should be considered, such as H_2O_2 generation, since caffeic acid may act as a pro-oxidant agent (Orsolic et al., 2004). Propolis, caffeic acid and CAPE (50 mg/kg) could be useful tools in the control of tumor growth, and propolis antitumor action could be the result of synergistic activities of its polyphenolic compounds (Orsolic et al., 2005).

A repair in the subsets of $CD4^+$ and $CD8^+$ lymphocytes in the spleen of metastases bearing mice was observed after propolis treatment, which led to a reverse relation between $CD4^+$ and $CD8^+$ cells in favour of $CD8^+$ population, suggesting the effects of propolis on cytolytic T cells and their effect on antitumor specific immunity and metastases containment (Orsolic and Basic, 2003). $CD8^+$ cells are able to recognize peptides in association with major histocompatibility complex-class I, and to eliminate neoplastic cells by secreting cytotoxic granules and/or inducing apoptosis of the target cell. Although $CD4^+$ cells do not promote the direct lysis of tumor cells, they

produce cytokines that stimulate or inhibit the activation, proliferation and differentiation of different cells of the immune system, regulating antibody production and other cytokines release (Ossendorp et al., 2000).

Based on studies in mice, Orsolic et al. (2006) suggested that the antitumor activity of propolis and some of its constituents is associated with their immunomodulatory action, mainly due to the augmentation of non-specific antitumor immunity, via macrophages activation, which in turn could produce soluble factors and interfere directly in the tumor cells or in the functions of other immune cells.

Propolis potential in carcinogenesis and mutagenesis assays was also investigated. Colorectal cancer is a prevalent cause of death by cancer around the world and the fifth cause of this type of death in Brazil. Since carcinogenesis is a multi-step process, the knowledge of the events occurring in each step can direct the actions to prevent and inhibit the development of cancer. In this context, the use of methodologies that allow for the evaluation of some biomarkers in each step of carcinogenesis can be useful.

The aberrant crypt foci (ACF) assay has been used to evaluate the initiation and promotion steps in chemical carcinogenesis. ACF are morphologic lesions, observed as large and elevated crypts with thickened epithelia, altered luminal openings, and clearly circumscribed by the surrounding normal crypts (Fenoglio-Preiser and Noffsinger, 1999).

Cytogenetic and molecular methods have been used to detect DNA damage, such as the comet assay or single-cell gel (SCG) test (Ostling and Johanson, 1984). Propolis effect on the process of colon carcinogenesis and DNA damage in Wistar rats was evaluated, using the ACF and comet test, respectively. Animals were treated with the carcinogen 1,2 dimethylhydrazine (DMH) and treated with ethanolic extract of propolis (10, 30 and 90 mg/kg)

simultaneously or after DMH administration. Propolis given simultaneously to DMH did not suppress the development of ACF. These results indicated that propolis was not able to block or minimize the initiation step of DMH-induced colonic carcinogenesis. Since DMH is an indirect carcinogen, which has to be metabolized to exert its carcinogenic effect, it could be postulated that propolis has no interference on DMH metabolic pathways. On the other hand, when propolis was administered during 2 weeks after DMH treatment, there was a significant reduction in the number of ACF, which could reflect a suppression of the clonal expansion of the initiated cells that characterizes the promotion step of carcinogenesis. No antigenotoxicity of propolis was observed in the comet assay, and DNA damage was seen in he peripheral blood cells (Bazo et al., 2002).

However, because of propolis solvent effect, new investigations were carried out with an aqueous extract of propolis (15, 50, 150 and 450 mg/kg), verifying its protective effect on DMH-induced genotoxicity, as evidenced in the comet test, but it did not suppress the development of ACF in the distal colon (Alves de Lima et al., 2005).

Qualitative and quantitative variations in the composition of ethanolic or aqueous extract of propolis could explain these distinct responses. In 2002, propolis samples collected in the University apiary (UNESP, Botucatu, São Paulo State, Brazil) were used, which were rich in phenolic compounds (flavonoids, aromatic acids, benzopyranes), di- and triterpenes, essential oils, among others (Bazo et al., 2002). In 2005, samples from the state of Minas Gerais, Brazil, were used, which were rich in cinnamic acid derivatives, more precisely the prenylated *p*-coumaric acid derivatives, *p*-coumaric acid and caffeic acid.

Although the mechanisms involved in the chemoprevention by propolis are not understood, interference by one or more propolis components in mutagenic/carcinogenic metabolic pathways, or its putative antioxidant activity could explain its effects on DMH genotoxicity.

Another approach to verify propolis antitumor action was to analyse its effects on canine transmissible venereal tumor (TVT). TVT is a contagious and sexually transmissible neoplasm with an unclear origin and affecting only canines. It has a worldwide distribution, although it is detected mainly in tropical and subtropical zones. Studies on TVT of natural origin do not show any predisposition of gender or breed, and it is found mainly in adult animals during reproductive age. TVT may also have extra-genital location (Albanese et al., 2002; Silva et al., 2003).

Differences between cellular lineages were seen in morphological characteristics of TVT, influencing its biological behavior (Varaschin et al*.,* 2001). According to cell characteristics, a new terminology for TVT has been suggested by our group and tumors were classified into lymphocyte-like TVT, plasma cell-like TVT and lympho-plasma cell-like TVT forms (Amaral et al*.,* 2005). This morphology classification shows TVT malignancy: plasma cell-like TVT shows a higher frequency of nuclear abnormalities associated with a larger expression of P-glycoprotein, an elevated rate of metastasis and cellular proliferation in comparison to lymphocyte-like TVT or lympho-plasma cell-like TVT forms. Plasma cell-like TVT is the most injurious and also the most malignant form.

Thus, TVT cell cultures from dogs seen in the Veterinary Hospital, FMVZ, UNESP, Campus of Botucatu, of either gender, any breed or age, with a cytological diagnosis of TVT were assayed. Anamnesis and the clinical history of these dogs were registered, including previous antitumor treatments.

Propolis (10, 25, 50 and 100 μg/100 μl) showed a time-concentration effect on TVT. With regards to TVT morphology, plasma cell-like TVT was more resistant to propolis action. The literature reports immune suppression during TVT growth, allowing metastasis. The absence of propolis solvent effect suggests that the results were exclusively due to propolis components.

In order to reduce the side effects of chemotherapy and considering that propolis possesses antitumor, anti-metastatic and immunomodulatory activities, its introduction as a therapeutic procedure *in vivo* could provide a new contribution to TVT treatment, as well as to other neoplasia treatments. There are no works dealing with propolis and TVT, demonstrating the originality of our research and its contribution to this field (BASSANI-SILVA et al., in press).

The large amount of works dealing with the antitumor action of propolis and its constituents indicates their promising usefulness, and claims for new investigations, in order to explore propolis potential as an antitumor agent.

Table 2
Propolis antitumor action according to its dose, effects, chemical composition and main components, and assay conditions of some authors

Tumor cell	Reported outcome	Dose and route	*In vivo / in vitro*	Main groups or active components	Characterization	Authors
HL-60 (human leukemia)	apoptosis-like DNA fragmentation	1-200 μg/ml	*in vitro*	artepillin C (3,5-diprenyl-4-hydroxycinammic acid)	HPLC	Matsuno et al. (1997b)
HLC (lung cancer) HCG (gastric cancer) Hepatoma Melanoma	suppression of tumor growth; ↑ ratio of CD4/CD8 T cells	500 μg (it.)	*in vivo*	artepillin C	NM	Kimoto et al. (1998)
Yac-1 cells (murine lymphoma)	↑ natural killer activity	10% (p.o.)	*in vivo*	prenylated p-coumaric acid and benzopyranes, essential oils, aromatic acids, di- and triterpenes	GC, GC-MS, TLC	Sforcin et al. (2002a)
Colon carcinogenesis	↓ aberrant crypt foci	10, 30 and 90 mg/kg (p.o.)	*in vivo*	prenylated p-coumaric acid and benzopyranes, essential oils, aromatic acids, di- and triterpenes	GC, GC-MS, TLC	Bazo et al. (2002)
C6 glioma cells	↓ viability ↑ apoptosis	(10-400 μM)	*in vitro*	CAPE	NM	Lee et al. (2003)

MCa (transplantable mammary carcinoma)	↓ tumor nodules ↑ apoptosis or necrosis	WSDP (50 and 150 mg/kg) caffeic acid and CAPE (50 mg/kg) quercetin (1200 mg/kg) (p.o.)	*in vivo*	NM	NM	Orsolic et al. (2004)
Canine transmissible venereal tumor	↑ cytotoxicity	10-100 µg/100µl	*in vitro*	prenylated p-coumaric acid and benzopyranes, essential oils, aromatic acids, di- and triterpenes	GC, GC-MS, TLC	Bassani-Silva et al. (in press)

NM = not mentioned; ↑ = stimulant action; ↓ = inhibitory action; p.o. = per oral; WSDP = water-soluble derivate of propolis; it = intratumorally; GC = gas chromatography; GC-MS = gas chromatography-mass spectrometry; TLC = thin layer chromatography; HPLC = high performance liquid chromatography.

3. Conclusions

Propolis' chemical composition as well as the identification of its vegetal sources enables us to carry out the assays with chemically characterized samples. The fact that no seasonal effect was seen on Brazilian propolis composition and variations were predominantly quantitative suggests to use samples collected in the same place all over the year, although in some regions, such as the temperate zone of the Northern Hemisphere, bees collect propolis mainly in summer. Biochemical, microbiological and immunological assays with Brazilian samples reveal no seasonal effect on its activities, what is in agreement with results on propolis composition.

Propolis is safe and shows no side effect after administration. Propolis shows antimicrobial activities, and its effects may occur through a direct action on microorganisms, as well indirectly, via stimulation of the immune system and further microorganism killing. Propolis may also show synergistic effects with antimicrobial drugs. These data are promising but lack investigation, in order to associate it or not to commercially disposable drugs, even to new products in the pharmaceutical industry.

The knowledge of propolis mechanisms of action on the immune system has advanced in the last years. *In vitro* and *in vivo* assays demonstrated that propolis may activate macrophages, increasing their microbicidal activity. Propolis enhances the lytic activity of natural killer cells against tumor cells. It also stimulates higher antibody production, suggesting its use in vaccines, as an adjuvant. Propolis inhibitory effects on lymphoproliferation may be associated to its anti-inflammatory property. Ethanol (propolis solvent) did not influence its activities in immunological assays. The best results were observed when propolis was administered over a short-term to animals, nevertheless further assays should be carried out with humans to establish

dose levels and intake period. Although related articles provide new information to postulate some hypothesis and explanations, propolis' mechanisms of action is not fully elucidated, and further investigation will help to a better understanding of its effects on the immune system.

Propolis shows antitumor properties and its anticarcinogenic and antimutagenic potential is promising, but the mechanisms involved in the chemoprevention by propolis are still obscure.

In vitro assays were very useful in order to understand some mechanisms of action, and propolis-treated animals revealed some of its effects *in vivo*. Although the published evidence to date supports propolis safety and effectiveness, its importance to human health is not known with sufficient detail, what opens a new perspective for further studies Since humans have been using propolis for a long time, scientific-based information bring an important contribution, evidencing the necessity of basic researches in this field and opening perspectives for new works.

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