INTRODUCTION

Propolis or bee glue, is a natural resin produced by bees, which is produced by mixing the exudates collected from various plants by honeybees [1, 2]. Propolis is a traditional remedy, in folk medicine, and is widely used around the world for the treatment of numerous diseases, such as inflammatory airway affections, cutaneo-mucosal infections, viral infections, etc [3].

It has been reported that water extract of propolis showed hepatoprotective activity in both chemical and immunological liver injury models [4], antiviral activity, inhibition of platelet aggregation [5], and anti-inflammatory activity [6]. However, there are few studies of propolis extracts from South of Brazil on reactive oxygen species (ROS) in and against autoxidation and free radicals, such as superoxide anion, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals and their relation with the hepatoprotective effect of propolis.

The Brazilian propolis G1, classified as BRG [11] from Shoutheast of Brazil, induces a potent anti-nociceptive effect in the chemical models of nociception in mice [8]. In addition to the analgesic effect, we have demonstrated anti-edematogenic properties induced by Brazilian propolis G1 [9]. Recently, we have demonstrated, that Brazilian propolis G1 induce relaxation in the guinea pig isolated trachea in vitro by mean modulation of the several cellular signaling pathways, such as potassium channels, vasoactive intestinal polypeptide and nitric oxide (NO) [10].

Chemical analysis of propolis show more than 150 polyphenolic compounds including flavonoids, cinnamic acid derivatives and prenylated compounds [10, 11]. It has been reported that propolis and/or its active constituents exert potent biological actions such as free radical scavenging and antioxidant properties [12-14], anti-carcinogenic action [15-16], antiviral [3] and antibacterial [17] effects, anti-protozoan action against Tripanossoma cruzi [18], immunomodulatory and anti-inflammatory properties [19], which are related or not to propolis antioxidant properties.

Recently, there has been growing interest in the involvement of ROS in several pathological situations, such as cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases involved in the genesis of cancer, cardiovascular disease and liver injury [20, 21].

In the current study we therefore investigated the ability of standardized Brazilian propolis extract (G1), to act as scavenger of ROS, superoxide and hydroxyl...
We investigated also, the hepatoprotective effect of G1 on hepatic damage induced by toxic concentration of carbon tetrachloride (CCl4) in rats.

MATERIALS AND METHODS

Standards and reagents
Nitroblue tetrazolium (NBT) chloride, 2-thiobarbituric acid (TBA), TBA deoxyribose, 2-deoxy-d-ribose, xanthine, xanthine oxidase from butter milk (XOD; 0.34 U/mg powder), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, ferric chloride anhydrous (FeCl3), β-nicotinamide adenine dinucleotide (NADH), ethylenediaminetetraacetic acid (EDTA) disodium salt, ascorbic acid, CCl4, and acetaminophen were obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. Ultra pure milli Q water was used through-out. Propolis extract were supplied by Pharmaneck Ltda (Belo Horizonte, MG, Brazil).

Propolis extract preparations
Standardized ethanolic extract of propolis, G1, obtained from commercial preparations available in Brazil, supplied by Pharmaneck Ltda, was prepared as follows: the alcohol of this preparation was evaporated and the dry resin was diluted in stock solution at a concentration of 10% (w/v). The propolis was collected from the beehive on March near Caetá city, in the Minas Gerais state, Brazil (following a sample frozen stocked in our laboratory). Propolis was triturated and mixed with an extractive solution containing 96GL alcohol. The mixture was left for 10 days, with a single mixing of 10 min once a day. After this period, the mixture was concentrated in Soxhlet extractor and the alcohol was removed from the solution to make a dry residue. The product of this extraction was diluted in a concentration of 10% (w/v) in 96GL alcohol.

High performance liquid chromatography
The ethanolic extracts of propolis were analyzed by means of an HPLC (Merck-Hitachi; Darmstadt, Germany), equipped with a pump (Merck-Hitachi: model L-6200) and a diode array detector (Merck-Hitachi: model L-3000). Separation was achieved on a Lichrocart 125-4 column (Merck, Darmstadt, Germany; RP-18, 12.5 x 0.4 cm, 5 mm particle size) using water, formic acid (95:5, v/v) (solvent A) and methanol (solvent B). The elution was carried out with a linear gradient and a flow rate of 1 ml/min. The detection was monitored at 280 nm and the compounds were identified and quantified by a method described previously [11]. For data analysis, the Merck-Hitachi D-6000 Chromatography Data Station-DAD Manager was used. The classification of propolis type was measured using commercial software. The exact concentration of major compounds of propolis was calculated by means of comparison with authentic standards previously isolated from Brazilian green propolis.

Effect of propolis G1 on DPPH free radicals
The effect of propolis G1 (1-100 µg/ml) on DPPH radical was evaluated by the following assay: the mixture contained 0.3 ml of 1 mM DPPH radical solution, 2.4 ml of 99% ethanol, and 0.3 ml of sample propolis G1 solution. The solution was rapidly mixed and scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. Ascorbic acid (1 µM) was used as positive control.

Effect of propolis G1 on superoxide anion radical
The effect of propolis G1 (0.1-10 µg/ml) on superoxide anion radical production was evaluated by the method described by Nagai et al [22]. This system contained 0.48 ml of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 ml of 3 mM xanthine, 0.02 ml of 3 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 0.02 ml of 0.15% bovine serum albumin, 0.02 ml of 0.75 mM NBT and 0.02 ml of sample solution. The reaction was started by adding 6 µM XOD and carried out at 25°C for 20 min. After this time, the reaction was stopped by adding 0.02 ml of 6 mM CuCl2. The absorbance of the reaction mixture was measured at 560 nm and the inhibition rate was calculated by measuring the amount of the formazan that was reduced from NBT by superoxide. Ascorbic acid (1 µM) was used as positive control.

Effect of propolis G1 on hydroxyl radical
The effect of propolis G1 on hydroxyl radical production was assayed by using the deoxyribose method. The reaction mixture contained 0.45 ml of 0.2 M sodium phosphate buffer (pH 7), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM FeSO4 EDTA, 0.15 ml of 10 mM H2O2, 0.525 ml of H2O, and 0.075 ml of sample propolis G1 solution in an eppendorf tube. The reaction was started by the addition of H2O2. After incubation at 37°C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% trichloroacetic acid and 0.75 ml of 1% of TBA in 50 mM NaOH; the solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at 520 nm using a Hitachi u2010 spectrophotometer. Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical [23]. Ascorbic acid (1 µM) was used as positive control.

Inhibition of superoxide-induced contraction of isolated guinea pig trachea
Tissue preparations: guinea pigs (250-400 g) of both sexes were anesthetized and killed by cervical
The trachea was rapidly removed, and after being freed from connective tissue, each trachea was cut into six transverse rings (3.4 mm wide), each containing 3 cartilages as described previously [25]. The rings were opened (usually 5 strips of 8-10 mm in length were obtained from the same animal) and were suspended in individual 10 ml jacketed organ baths containing Krebs-Henseleit solution maintained at 37ºC, pH 7.8, and gassed with a mixture of 95% O₂ and 5% CO₂. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.4, MgSO₄ 1.1, CaCl₂ 2.5, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11. Tissues were allowed to equilibrate for at least 60 min before drug addition, during which time the fresh buffer solution was renewed every 15 min, under a resting tension of 1 g. Isometric responses were measured by means of TRI-201 force displacement transducers (Panlab apparatus; Barcelona, Spain) and were recorded on a polygraph (Letica Scientific Instruments; Barcelona, Spain). In most experiments, the epithelial layer of the trachea was gently removed with a cotton-tipped applicator. The integrity of the epithelium was assessed by the ability of bradykinin to induce relaxation [24]. The animals were used in accordance with current ethical guidelines for the care of laboratory animals.

**Experimental procedure;** after the equilibration period of at least 60 min, the preparations without epithelium were contracted with histamine (1 nM, approx. the E₅₀) to evaluate the tonic responsivity of the smooth muscle. After 60 min and when tonic baseline became stable (usually after 5 min) they were exposed to the superoxide radicals producing system, in absence or in presence of G1 (0.1, 1 or 10 μg/ml) or superoxide dismutase. Superoxide was generated in the organ bath by the xanthine/xanthine oxidase system following the reaction mixtures: xanthine (44 mM), xanthine oxidase (0.29 U/ml) in a final volume of 2.5 ml. Xanthine was dissolved in NaOH (1 mM) and after in phosphate buffered saline plus EDTA 0.1 mM, in pH 7.8; xanthine oxidase in EDTA 0.1 mM. Usually, two to three complete cumulative concentration-response curves were obtained in each preparation at 60 min intervals between curves.

**Hepatoprotective effect of propolis G1 on hepatic damage induced by carbon tetrachloride and acetaminophen in rats**

Male rats (180-250 g), from UNISUL facilities, were housed at 22 ± 2°C under a 12 h light-dark cycle. Food and water were offered *ad libitum*. The animals were acclimatized to the laboratory for at least 1 day before testing and the experiments were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experimental conscious animals [26] and the current ethical guideline to use of animals approved by Committee at UNISUL 021/2006. The animals were treated orally (p.o.) with propolis G1 (1, 3 or 10 mg/kg) during 7 consecutive days, once a day. After treatment the animals received carbon tetrachloride (1.5 ml/Kg, p.o.) twice during 48 h. During the experiments, toxic effect and compartmental reactions were monitored.

The animals were treated orally with propolis G1 (1, 3 or 10 mg/kg) during 7 consecutive days, once a day. After treatment the animals received carbon tetrachloride (1.5 ml/Kg, p.o.) twice during 48 h. During the experiments, toxic effect and compartmental reactions were monitored.

After the treatment, the rats were anesthetized and killed by cervical dislocation, and the liver and kidney were isolated by histological analysis. Slices of liver and kidney (near microvascular place) were cored by means of hematoxylin-eosin method and analyzed by mean optical microscopy. We evaluate the neutrophil migration and Kupffer cells on micro-vascular liver- and kidney-cored slices, respectively. In this time the blood (3 ml) was collected by cardiac puncture using sterile disposable syringes. Serum was separated by centrifugation (3000 rpm) and aspartate transaminase (AST), alanine transaminase (ALT) and gamma glutamyl transferase (γ-GT) were estimated on the same day using Merck Diagnostic kits on UNISUL Biochemical Laboratory.

**Statistical analysis**

Responses were expressed as means ± SD. Statistical analysis of the results was carried out by means of the unpaired Student’s *t*-test (Graph Pad InStat software), by comparison of individual points of the treated groups with the control groups, during the experiments. *P* < 0.05 was considered as indicative of significance. The IC₅₀ values were determined from individual experiments for the complete dose-response by graphical interpretation test. The IC₅₀ values are reported as geometric means accompanied by their respective 95% confidence limits.

**RESULTS**

**Phenolic composition of Brazilian propolis G1**

The chemical composition of green propolis was evaluated by HPLC analysis (Fig.1), showing high levels of phenolic compounds. The total content of phenolic compounds is 151.69 mg/g of dried extract.

**Antioxidant effect of Brazilian propolis G1**

**DPPH radical scavenging assay:** DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various chemicals and natural products around the world. Our results showed Brazilian propolis G1 produced a significant and dose-dependent reduction of the relative activity of DPPH with mean IC₅₀ of 0.96 ± 0.4 μg/ml. In these experiments, ascorbic acid (0.1 or 1 mM) was used as positive control (Fig.2).
Figure 1. The chemical composition of G1 has been determined by high performance liquid chromatography: The amount of phenolic compounds was estimated as follows (mg/g): (1) coumaric acid 3.81, (2) rutin 9.87, (3) pinobanksin 3.48, (4) quercetin 2.15, (5) kaempferol 0.78, (6) apigenin 1.86, (7) pinocembrin 22.55, (8) pinobanksin-3-acetate 4.1, (9) chrysin 2.49, (10) galangin 4.14, (11) kaempferide 5.59, (12) tectochrysin 2.90, (13) artepillin C 87.97.

Figure 2. Effect of propolis (1, 10 or 100 µg/ml) or ascorbic acid (AA, 1 µM) on DPPH relative activity in vitro. The results represent the mean of three experiments. *P < 0.05 for control vs treated group.

Figure 3. Effect of propolis (1, 10 or 100 µg/ml) or ascorbic acid (AA, 1 µM) on superoxide production in vitro. The results represent the mean of three experiments. *P < 0.05 for control vs treated group.

Superoxide-scavenging activity of propolis
Superoxide-scavenging activity of Brazilian propolis extract G1 was measured using the xanthine–xanthine oxidase system and these results are indicated as the superoxide productivity. Our results showed that propolis G1 (0.1-10 µg/ml) exhibited high superoxide-scavenging activity by dose dependent manner with mean IC_{50} of 0.28 ± 0.09 µg/ml and the pre-incubation of 10 µg/ml completely inhibited the production of superoxide in this system. The activities of propolis G1 were higher than that of 1 mM ascorbic acid (Fig.3).

Hydroxyl radical scavenging activity of propolis
We used the Fenton reaction to determine the scavenging effect of propolis G1 (1-100 µg/ml) against hydroxyl radical. We have shown here that propolis G1 present a potent hydroxyl radical scavenging activity and its activity was increased with concentration of the sample and completely abolished the hydroxyl radical when 100 µg/ml was add on solution. The mean IC_{50} of this effect was 15.7 ± 2.5µg/ml (Fig.3).

Effect of propolis on superoxide radical-induced contraction in the guinea pig isolated trachea
Cumulative addition of the standardized propolis extract (G1) (0.1, 1 or 10 µg/ml) inhibited the superoxide radical-induced contraction in the guinea pig isolated trachea, with significant inhibition rate of 56.6 ± 4.2% or 97.3 ± 2.2% to 1 or 10 mg/ml, respectively, and with IC_{50} mean of 0.79 ± 0.2 µg/ml (Fig.3).
Effect of propolis on \( \text{CCl}_4 \)-induced neutrophil margination on liver

\( \text{CCl}_4 \)-induced hepatic injuries are commonly used models for hepatoprotective drug screening. \( \text{CCl}_4 \) can be converted into halogenated free radicals that spread propagation of the alkylation as well as peroxidation, causing damage to macromolecules in membrane, focal neutrophil margination and inflammation. In in vivo experiments, propolis extract G1 (1, 3 or 10 mg/kg, p.o., during 7 consecutive days), inhibited the hepatic neutrophil margination induced by \( \text{CCl}_4 \) with IC\(_{50}\) mean of 5.78 ± 0.9 mg/kg. Our results showed that propolis G1 can be a potent lipoperoxide free radical scavenger and this effect can be related with hepatoprotective action on liver damage induced by \( \text{CCl}_4 \).

Effect of propolis G1 on liver biochemical function during \( \text{CCl}_4 \)-induced hepatotoxicity

The transaminases and \( \gamma \)-GT levels were determined in rat’s serum before induction of. The initial values of serum AST, ALT and \( \gamma \)-GT in control (saline + vehicle) group (169 ± 25, 70 ± 3 and 0.25 ± 0.02 U/l, respectively) were found to be increased (755 ± 72, 475 ± 32 and 3.55 ± 0.25 U/l, respectively) after administration of the toxic dose of \( \text{CCl}_4 \) (1.5 mg/kg, p.o.). Treatment with propolis G1 (1, 10 and 100 mg/kg, p.o., during 7 consecutive days) reduced the AST (578 ± 43, 354 ± 30 and 184 ± 12 U/l, respectively), ALT (325 ± 29, 170 ± 18 and 77 ± 5 U/l, respectively) and \( \gamma \)-GT (2.8 ± 0.2, 1.8 ± 0.2 and 1.2 ± 0.1 U/l, respectively) levels significantly (Fig.7).

DISCUSSION

Propolis is a natural product produced by bees and used in the folk medicine to treat many pathologies, including pain, inflammatory diseases, cancer, etc. We have recently shown that Brazilian propolis can induce a potent relaxant effect on guinea pig isolated trachea by means of potassium channel, NO and VIP receptor modulations [9]. In a recent review Marcucci and Bankova [7] described that Brazilian propolis have a complex chemical composition with a majority of compounds linked to phenolic and prenylated compounds family.

Phenolic compounds are substances of low molecular weight and are present in several plants and other natural products. It was previously shown that phenolic compounds isolated from Brazilian propolis, such as 3-prenyl-4-hydroxycinnamic acid, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran, 3,5-diprenyl-4-hydroxycinnamic acid and 2,2-dimethyl-6-carboethenyl-8-prenyl-2H-1-benzopyran, have potent anti-protozoan, antibacterial and relaxant effect on guinea pig isolated...
trachea [27]. Kimoto et al. [28, 29] showed that 3,5-diprenyl-4-hydroxycinnamic acid has anti-tumoral, anti-leukemic and anti-carcinogenic effects in isolated cell line. In addition, flavonoids and phenolic compounds possess anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, anti-thrombotic, antiviral and anti-carcinogenic activities [30].

In this work we showed that Brazilian propolis, named G1, present a chemical constitution based on phenolic and prenylated compounds. This propolis sample show antioxidant effect, scavenging ability on free radicals and inhibitory effect on the superoxide radical mediated contractile activity of guinea pig isolated trachea.

It has been shown that free radicals can induce several pathologies including aging, atherosclerosis, neuro-degenerative diseases [31], hepatic damage and inflammatory response [32]. Some flavonoids and phenolic compounds act by antioxidant mechanisms including the inhibition of enzymes involved in the formation of ROS (xantine oxidase, protein kinase C, lipooxygenase, cyclooxygenase, NADH oxidase, etc) or the chelation of trace elements (free iron or copper) which are potential enhancers of free radical generation or stabilizing free radicals involved in oxidative processes by complexing with them [33, 34].

The pharmacological effect of propolis G1 on free radical system, i.e. inhibiting DPPH, superoxide and hydroxyl radicals can establish its relationship with anti-inflammatory and hepatoprotective properties, shown here on hepatic toxicity experiments. We have demonstrated that the treatment with propolis G1, orally, can induce a potent reduction of the CCl4-induced inflammatory response and hepatotoxicity, clearly mediated by lipoperoxide free radical reaction. On the other hand, when the hepatotoxicity were paracetamol-mediated, the treatment with propolis G1 was poorly effective to prevent the neutrophil migration on liver or on the enzymatic hepatic function.

The increase in serum levels of ASP, ALT and γ-GT has been attributed to the damaged structural integrity of the liver induced by CCl4 because these are cytoplasmic in location and are released into circulation after cellular damage. The treatment of the animals with propolis G1 seems to preserve the structural integrity of the hepatocellular membrane producing a significant reduction in the paracetamol and CCl4-induced increase in serum enzymes of rats. The results of this study indicate that propolis G1, a common folk remedy in several countries, exhibits hepatoprotective activity, related with antioxidant effect, and the presence of phenolic and prenylated compounds in this propolis extract confirmed, at least in part, the folkloric use of propolis in hepatic damage or to treat other pathologies with free radical-mediated inflammation.

In fact, our research group has studied several samples of propolis and recently published a report on the anti-inflammatory and analgesic effects of green propolis from Brazil. We have shown that green propolis produces potent anti-edematogenic, anti-inflammatory and analgesic effects using several animal models and molecular biology methods. Propolis G1 inhibits prostaglandin E2 production during the acute inflammation induced by carrageenan, the NO production in the murine macrophage cell line RAW 264.7 and the nuclear factor kappa B (NF-κB) over-expression in human embryonic kidney (HEK) cells. This inhibitory effect reduced the transcription and expression of the inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2). These results are also reproducible for artepillin C [35]. Therefore, the incubation of green propolis on vascular endothelial cells did not affect the activity or expression of endothelial nitric oxide synthase (eNOS), but it did have a dual effect on the protein kinase B (PKB)/Akt activity in smooth muscle cells from rat aorta in the presence of angiotensin II: in low concentration it induced phosphorylation and the activation of this system, and in high concentration it decrease the phosphorylated form and the activity. This pharmacological effect may indicate that green propolis from Baccharis dracunculifolia induces the analgesic and anti-inflammatory effect, at least in part, by means of NF-κB modulation.

We also have shown that artepillin C, the main compound identified in G1, reduced NF-κB expression suggesting anti-inflammatory activity, particularly during acute inflammation. Lastly, artepillin C was absorbed after an oral dose (10 mg/kg) with maximal peaks found at 1 h [36].

In addition, Fonseca et al. [37] suggest the potential applicability of propolis extracts for preventing UV-induced skin damages. Green propolis extracts exhibited considerable antioxidant activity and inhibited UV irradiation-induced GSH depletion. In agreement of this effect demonstrated that B.dracunculifolia exhibit potent antioxidant activity protecting liver mitochondria against oxidative damage and such action probably contribute to the antioxidant and hepatoprotective effects of green propolis [38].

The antioxidant effect is directly linked to the anti-inflammatory action, as demonstrated by Szliszka et al. [39] showed that propolis exerted strong antioxidant activity and significantly inhibited the production of ROS, reactive nitrogen species (RNS), NO, cytokines IL-1α, IL-1β, IL-4, IL-6, IL-12p40, IL-13, TNF-α, G-CSF, GM-CSF, monocyte chemotactic protein-(MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES in stimulated J774A.1 macrophages.
Collectively, our results showed for the first time that propolis G1 can protect the liver from oxidative stress and that its effect can be modulated by antioxidant and anti-inflammatory action mediated, at least in part, by prostaglandin E2 and NO inhibition through NF-κB modulation. This effect was produced by phenolic compounds that exhibited bioavailability after oral administration, such as artepillin C. Taken together, our results suggest a strong evidence to use propolis G1 like an antioxidant and anti-inflammatory natural remedy.

REFERENCES

ACKNOWLEDGEMENTS
The authors are grateful to MEDLEX Gestao de Informacees & Cursos Ltda and to the Pharmanectar Ltda for providing propolis sample.

COMPETING INTERESTS
The authors declare that they have no conflict of interest.

http://www.oamsjournal.com
Paulino et al: Antioxidant and hepatoprotective effect of green propolis


This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided that the work is properly cited.

DOI 10.5455/oams.150214.or.058