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Antiulcerogenic Effect of Brazilian Propolis Formulation in Mice

Niraldo Paulino[1*], Lucas Amorim Coutinho[1], José Reinaldo Coutinho[1], Giselle Cunha Vilela[1], Veroneide Pio da Silva Leandro[1], Amarilis Scremin Paulino[2]

[1]Universidade Anhanguera de São Paulo, São Paulo, Brasil
[2]Medical Lex Gestão de Informações & Cursos Ltda, Florianópolis, Brasil
Email: [*]niraldop@yahoo.com.br

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Abstract

Propolis is a natural resin used around the world to treatment of several diseases. We evaluated the effect of Brazilian propolis (P1) in guinea pig isolated trachea *in vitro* contracted with histamine, in paw oedema induced by histamine in mice and anti-ulcerogenic effect of propolis formulation in mice. We used a P1 extract from South of Brazil to produce a formulation containing sodium bicarbonate and propolis dry extract (2%). In the guinea pig trachea Brazilian propolis extract (10 μg/mL) produced a non-significant inhibition in the cumulative response curve for histamine. However, the dose of 100 μg/mL shown had a significant inhibitory effect on the contraction induced by histamine (51% ± 5%). In the mouse paw oedema induced by intraplantar injection of histamine (50 μg/paw), the results showed that the treatment of the mice with P1 (10 or 100 mg/Kg (p.o) or 1 or 10 mg/Kg (i.p)), 0.5 h beforehand, significantly inhibited the paw oedema with maximal inhibition (MI) of 25% or 42% by p.o. route or 22% or 37% by i.p. route, respectively. Finally we have pre-treated mice with P1 formulation (20 mg/Kg or 40 mg/Kg, p.o, one time) or vehicle (powder) in animals submitted to diclofenac-induced ulcer, after 8 h P1 formulation inhibited of 43% or 83% of ulcers scores, respectively. Results showed that P1 formulation inhibited the ulcerogenic effect induced by diclofenac. Results suggested also, at least in part, the antiulcerogenic effect of formulation containing standardized extract of propolis can be due to the antagonistic action on the histaminergic system.

Keywords

Propolis, Formulation, Ulcer, Stomach, Mice, Palavras Chaves: Própolis, Formulação Oral, Úlcera, Estômago, Camundongo

[*]Corresponding author.

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1. Introduction

Propolis is a natural resin produced by bees, and has been widely used for the treatment of several diseases, such as viral [1], bacterial [2] and fungal infections [3], as an antitumor [4], anti-hyperalgesic [5], and anti-inflammatory preclinical [6]-[8] and clinical [9] and also behaves as a free radical scavenger [10] [11].

Chemical studies carried out with propolis have revealed that it contains a complex mixture of many compounds, namely flavonoids, terpenes, cinnamic acid, phenolic acids, caffeic acid and several esters, which exhibit a broad spectrum of activities [12]-[15].

The biological activity of propolis is associated mainly with phenolic compounds such as flavonoids and derivatives of hydroxycinnamic acids. Three p-coumaric acid derivatives isolated from a Brazilian sample presented *in vitro* a relaxant effect on smooth muscle using preparations of guinea pig isolated trachea [16].

Brazilian propolis has recently been tested in the guinea pig isolated trachea, *in vitro*, and shows a potent relaxant effect. It has been demonstrated that this effect is related with nitric oxide-, VIP-, and potassium channels-pathways [17]. It can, at least in part, be related with the phenolic compounds isolated from these samples [16].

The objectives of the present study were to evaluate the effect of standardized Brazilian propolis extract (P1) on response to histamine cumulative-concentration contraction on guinea pig isolated trachea *in vitro*, on the oedema induced by histamine in mice, and to evaluate the effect of P1 on ulceration in mice's stomach treated with sodium diclofenac.

2. Materials and Methods

2.1. Propolis Extract Preparations

The propolis was collected from the beehive on March (1999) near Araranguá city, in the Santa Catarina state, Brazil (following a sample frozen stocked in our laboratory). Propolis was triturated and mixed with an extractive solution containing 96˚GL alcohol. The mixture was left for 10 days, with a single mixing of 10 minutes once a day. After this period, the mixture was concentrated in soxlhet, an extractor of compounds from plant and natural products, 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 96˚GL alcohol.

2.2. High Performance Liquid Chromatographic (HPLC) Assay

The ethanolic extracts of propolis were analyzed by HPLC (Merck-Hitachi, Germany), equipped with a pump (model L-6200, Merck-Hitachi, Germany) and a diode array detector (L-3000, Merck-Hitachi, Germany). Separation was achieved on a Lichrochart 125-4 column (Merck, Darmstadt, Germany) (RP-18, 12.5 × 0.4 cm, 5 µm 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 using standards as references [16]. For data analysis, the Merck-Hitachi D-6000 (Chromatography Data Station-DAD Manager) was used.

2.3. Pharmacological Assays *ex vivo*

For pharmacological *ex vivo* experiments, we used adult guinea pig of both sexes weighing 250 - 400 g. All animals were anesthetized, sacrificed and had the medial segment of trachea removed and quickly cleaned from the internal epithelial layer. Each segment containing at least 3 transverse rings (3 - 4 mm wide) as described previously [18]-[20]. Briefly, rings were opened and suspended in individual pots containing 10 mL Krebs-Henseleit solution maintained at 37˚C, pH 7.2, gassed with a mixture of 95% O_2 and 5% CO_2. The tissues remain in equilibrium for 60 minutes prior to the start of the experiments and were kept under the resting tension of 1 g coupled to a isometric transducer TRI-201 connected to polygraph (Letica Scientific Instruments, Barcelona, Spain). The preparations were subjected to cumulative concentration response curve to histamine (10 nM - 10 µM) in the absence or presence of the standardized extract of propolis 10 or 100 µg/mL. All animals were used in accordance with current ethical guidelines for laboratory animal care and under approval by the Ethics Committee of the University of Southern Santa Catarina.

2.4. Pharmacological Assays *in vivo*

2.4.1. Animals

Non-fasted adult Swiss mice males (18 - 30 g) were used in the experiments. The animals were maintained in an environment under controlled temperature (21˚C ± 2˚C), illuminated by daylight supplemented with electric light from 6:00 a.m. to 6:00 p.m., with free access to food and water. Throughout the experiments, the animals were managed using the principles and guidelines for the care of laboratory animals according to [21].

2.4.2. Measurement of Paw Oedema

The experiments were conducted according to [22], with minor modifications. Under slight anesthesia with 2,2,2 tribromoethanol (0.12 g/Kg), male Swiss mice received a 50 μl i.d. injection in one hind paw of phosphate buffered saline (PBS, composition mmol/L: NaCl 137, KCl 2.7 and phosphate buffer 10) containing histamine (50 μg/paw). The contralateral paw received 50 μL of PBS and was used as control. Oedema was measured by use of a plethysmometer (Ugo Basile, Italy) at several time points after injection of histamine. Oedema is expressed in μL as the difference between the test and control paws. In most experiments, animals were treated with ethanolic extract of Brazilian propolis (P1) (1 or 10 mg/Kg, i.p) or indomethacin (10 mg/Kg, i.p), 30 min before, or 10 or 100 mg/Kg, p.o) or indomethacin (100 mg/Kg, p.o) inflammatory agents. Control groups received the same volume of the vehicle (PBS solution).

2.4.3. Anti-Ulcerogenic Effect of Propolis Extract Formulation in Mice

The mice were treated with sodium diclofenac (150 mg/Kg, during 2 consecutive days 8 - 8 h), after this time, the mice received a single dose of propolis formulation containing sodium bicarbonate and propolis (P1) dry extract (2 g·%). After 8 h, animals were killed by lethal injection and its stomachs were removed to analysis of the ulcer scores (number of red points on the stomach wall analyzed on optical microscope). The results were expressed by mean ± S.E.M. of the 8 animals.

2.5. Drugs

Ethanolic extract of Brazilian propolis (P1) was obtained from commercial preparations available in southern Brazil (Prodapys, Produtos Naturais Ltda., Ararangu, SC, Brazil). Histamine and PBS solution (PBS-pH 7.6, composition mM: NaCl 137.0, KCl 2.0 and phosphate buffered salts 10.0) were acquired from Sigma Chemical Co., St Louis, MO, USA. The other chemical products and salts were supplied by MERCK (Darmstadt, Germany) and were of a high degree of analytic purity. The stock solutions of these drugs were prepared and stored at –20˚C. The bath concentration of ethanol did not exceed 0.03%, which was shown to have no effect *per se* on the basal tonus of the preparations or on the agonist-mediated contraction or relaxation.

2.6. Statistical Analysis

Data is reported as mean 6 ± SEM. Statistical differences in all experimental groups were determined by the use of one-way ANOVA test, followed by Dunnett's post-test or Student's unpaired "t" test, when indicated. Statistically significant differences were reported at P < 0.05 or less (Graph Pad Instat, Prism). When possible, the ID_{50} values were determined by use of the least-square method and reported as the geometric means accompanied by their 95% confidence limits.

3. Results

The samples used in these experiments present the following phenolic compound composition: 3-prenyl-4-hydroxycinnamic acid (1.72 mg/mL of standardized extract), 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopirane (38.04 mg/mL of standardized extract) and 3,5-diprenyl-4-hydroxycinnamic acid (26.21 mg/mL of standardized extract) [20].

We analyzed the role of the Brazilian propolis P1 in airway smooth muscle from guinea pig trachea, *in vitro*. In this case, the pre-incubation of the Brazilian propolis P1, 10 or 100 μg/mL induced a significant inhibition in the contraction promoted by histamine. Histamine produced a cumulative contraction response curve (100 nM - 10 μM) in the guinea pig isolated trachea without epithelium, with an EC_{50} mean of 52 (22 - 78) μM. The preincubation in the guinea pig isolated trachea, in vitro, with Brazilian propolis P1 10 μg/mL, produced a non-

significant inhibition in the cumulative response curve for histamine. However, the dose 100 μg/mL shown had a significant inhibitory effect on the contraction induced by histamine: 51% ± 5% (**Figure 1**).

Our results shown that the treatment of the mice with ethanolic extract of Brazilian propolis P1 10 or 100 mg/Kg (p.o) or 1 or 10 mg/Kg (i.p), 0.5 h beforehand, significantly inhibited the paw oedema induced by histamine (50 μg/paw) with maximal inhibition (MI) of 25 or 42% by oral route or 22% or 37% by intraperitoneal route, respectively (**Figure 2**).

Finally we pre-treated mice with P1 formulation (20 mg/Kg or 40 mg/Kg, p.o, one time) or vehicle (powder) in animals submitted to sodium diclofenac-induced ulcer, after 8 h P1 formulation inhibited of 43% ± 3% or 83% ± 5% the ulcers scores, respectively (**Figure 3**).

4. Discussion

Histamine is a biogenic amine found in mast cells, circulating basophiles, epithelial cells, gastric mucosal and in central nervous system neurons [23]. Its preferential occurrence is in the skin [24], lung and upper airway [25] and gastrointestinal system in special on the enterocromafins cells [26] [27], but is involved in the several deleterious stimuli such antigen activation, in response to venoms or toxins [28].

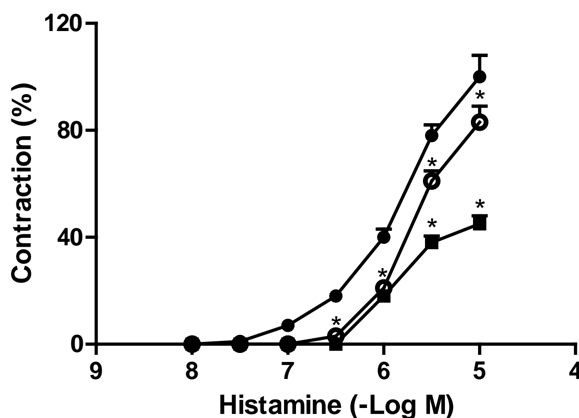

Figure 1. Mean contraction concentration-response curves for histamine in the guinea pig trachea without epithelium in the absence (●) or presence of Brazilian propolis extract P1 (○, 10 μg·mL^{-1} or ■, 100 μg·mL^{-1}). Values are mean ± S.E.M. of 6 experiments. *P < 0.05 for the difference between points.

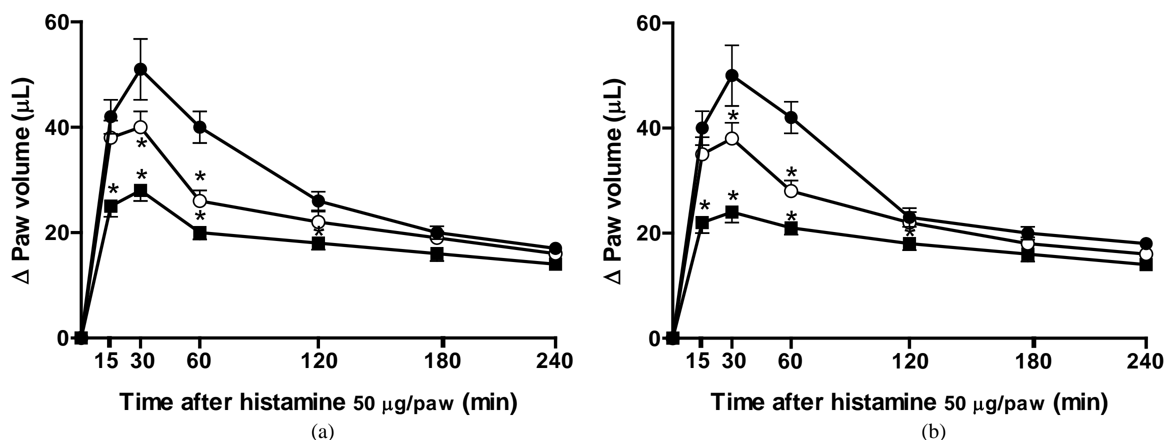

Figure 2. Effect of treatment with saline (●), propolis 1 (○) or 10 (■) mg/kg, i.p.) (a) or saline (●), propolis 10 (○) or 100 (■) mg/kg, p.o.) (b) on the mouse paw oedema induced by intraplantar injection of histamine (His, 50 μg/paw). Each point represents the mean of 6 animals and the vertical lines the SEM. Asterisks indicate the statistically-significant differences. *P < 0.05 when compared with respective control values.

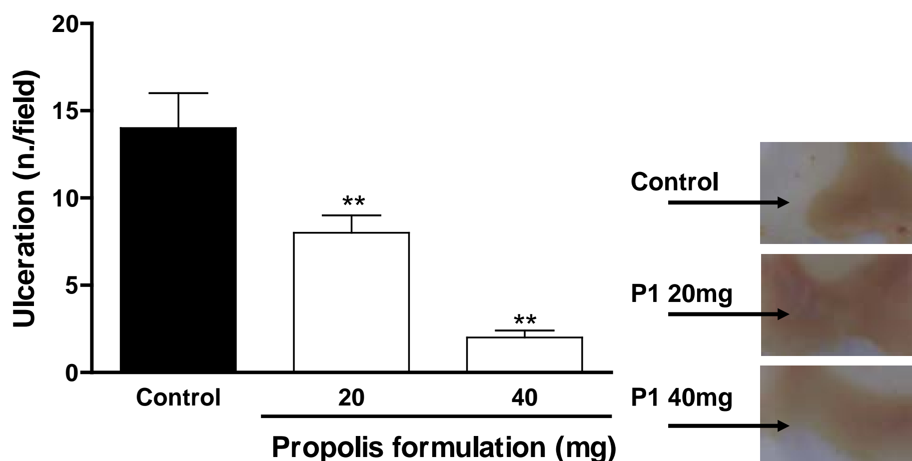

Figure 3. Antiulcerogenic effect of the treatment with base (bold column) or formulation containing propolis P1 (20 or 40 mg/Kg) plus sodium bicarbonate (blank column) on mice treated with toxical doses of sodium diclofenac. Each point represents the mean of 8 animals and the vertical lines the SEM. Asterisks indicate the statistically-significant differences. $^{*}P < 0.05$ when compared with respective control values.

Physiologically, histamine active three different receptors named, H_1, H_2 and H_3. The H_1 receptors are coupled to G protein and induce the activation of the phospholipase C and promote the increase of the IP3 and DAG, releasing intracellular calcium from sarcoplasmatic reticulum and activating C protein. In airway smooth muscle cells, the increase of citoplasmatic calcium mediated by IP_3 receptors in the sarcoplasmatic reticulum or by voltage-gated L type calcium channels produces a fast and consistent contraction [29] [30].

Pre-incubation of the guinea pig trachea with propolis P1 produce strong inhibition of the contractile effect of histamine, suggesting, at least in part, a direct interaction with the histamine receptors or indirectly with its transduction signal system. Thereby, we have shown that propolis P1 induce a relaxant effect on guinea pig isolated trachea by mean of modulation of the high conductance calcium-activated potassium channels (BKCa). This effect can antagonize electrophysiological response mediated by voltage-gated calcium channels coupled to histamine receptors on the airway smooth muscle cells [20].

When the histamine activates H_1 receptors on the endothelial vascular cells induce increase of endothelial nitric oxide synthase and a consequent elevation of the nitric oxide synthesis. Nitric oxide across the smooth muscle cell membrane and activate the cGMP-kinase protein (PKG), and to finish, re-uptake the citoplasmatic calcium cell and/or active the small conductance calcium-activated potassium channels (SKCa). These effects are related to vasorelaxation and increase of the vascular permeability induced by histamine in the oedematogenic and inflammation models [31].

We have shown here, that propolis P1 administrated by oral or intraperitoneal route, decrease the oedema produced during the inflammatory stimulation on mice paw's. This result is in accord with the literature that shown some informations about the anti-inflammatory, analgesic and immunomodulation effects or propolis [32]-[35]. This effect seems to be related with the inflammatory mediator such as bradykinin, prostaglandins, histamine, and modulated by NFκB pathway [8] [34] [36]-[40].

H_2 receptors are coupled to G protein and can interact with adenyl cyclase increasing the cAMP, activating protein kinase cAMP-dependent (PKA). In the enterocromafin cells this cellular signaling activates the H^+/K^+ ATPase and increases the acid secretion on the stomach. The histaminergic activity is a deleterious factor to produce gastric lesion and are involved in the patophysiology of ulcer [41]-[43].

Our results showed that the formulation containing propolis P1 and calcium bicarbonate reduce significantly the ulceration induced by toxically administration of diclofenac in the stomach.

Ulcer, in the gastrointestinal system, are linked to decrease on produce the endogenous mucus, and sometimes are related to conical use of non-steroidal anti-inflammatory like diclofenac, or can be associated to *Helicobacter pylori* (*H. pylori*). Recently, have demonstrated in the literature positive correlation between the use of non-steroidal anti-inflammatory is related to *H. pillory* presence in the stomach [44] [45].

Massignani [46] shown that *Baccharis dracunculifolia* oil produced a reduction of gastric juice volume and total acidity was observed, as well as an increase in the gastric pH. No sign of toxicity was observed in the acute

toxicity study. It has been shown also that propolis from *Baccharis dracunculifolia*, inhibits the grown of *H. pylori in vitro* [47] [48] reducing the incidence of ulceration in patients and It can be use in associated therapy to anti-ulcerogenic conventional treatment [49].

The principal factor involved in the ulcerogenic response is the inflammatory process instigated during the ulcer formation. Inflammation produces local pain and gastric discomfort when in the clinical evaluation. The gastric inflammation produces several oxygen free radicals involved in the attack to mucosal protection in the stomach [50].

We have demonstrated that propolis P1 shown potent anti-inflammatory effect related to presence of its major constituent, Artepillin C. This compound and another phenolic compounds present in Brazilian propolis are scavengers of free radicals and reduce the oxidative process associated to inflammation [51] [52].

5. Conclusions

Based in our results and in accord to published [53], we suggest that the formulation containing propolis P1 can be used to treat the gastric ulceration by mean of anti-histaminergic effect, anti-acid properties, anti-inflammatory antioxidants and anti-*H. pylori* effects.

The constituents responsible for the inhibition of histamine-contraction in the guinea pig trachea, oedema induced by histamine or anti-ulcerogenic effect in mice are currently not completely known. However, this effect is likely to be related to the presence of phenolic compounds and flavonoids [21]. Chemical and pharmacological studies are now in progress to isolate and chemically characterize the constituents responsible for such effects.

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