Investigation of the Anti-inflammatory and Analgesic Activities of a Sample of Brazilian Propolis

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SUMMARY. Propolis is extensively used in Brazilian folk medicine for inflammatory diseases. This work studied the chemical composition, anti-inflammatory and antinociceptive activities of a hydro alcoholic extract of propolis (HEP) from Atibaia, São Paulo, Brazil. The results of the analyses reveal a predominance of 3,5-diprenyl-4-hydroxynamic acid, and derivatives, as well as p-coumaric acid, caffeic acid its derivatives. The anti-inflammatory activity of HEP, per os was evaluated in the carrageenin-induced rat paw oedema model and mouse ear oedema induced by croton oil. At doses of 250 mg.Kg−1, HEP caused a significant reduction in paw oedema two hours (51.36%) after the subplantar injection of carrageenin in rats, persisting until the third hour (29.68%). Doses of 500 and 1000 mg.Kg−1, promoted the reduction of edema from the first hour until the fourth; after the first hour 52.1% and 60.3%; after two hours 65.6% and 59.7%; after three hours 56.1% and 52.8% and after four hours 41.4% and 36.9%, respectively. The effects of HEP administered on croton oil-induced ear oedema in mice at doses of 250, 500 and 1000 mg.Kg−1, showed reduction of oedema of 47%, 50.5% and 48.1%, respectively. To evaluate the antinociceptive activity of HEP by oral administration, two experimental models were used (writhing test in mice and tail-flick in rats). The results obtained in the writhing test showed that HEP at a dose of 1000 mg.Kg−1 significantly inhibited the acetic acid-induced writhing in mice, decreasing the contortions in 52.8%. In the tail-flick assay, the results obtained with the oral administration of HEP did not show evidence of analgesic activities at the doses used, but an ip administration of 1000 mg.Kg−1, demonstrated significative antinociceptive activity increasing the latency of tail retreat in rats, comparable to the control drug, morphine. The ip administration of naloxone associated with HEP or morphine, decreased the latency of tail retreat in rats, such results suggest that HEP contain antinociceptive substances which appear to be unrelated to the activation of opioid receptors.

RESUMEN. “Investigación de las Actividades Anti-inflamatoria y Analgésica de una Muestra de Propóleo brasileño”. El Propóleo es ampliamente usado en Brasil en la medicina popular en las enfermedades inflamatorias. Este trabajo estudió la composición química y las actividades antiinflamatoria y antinociceptiva del extracto hidroalcohólico de propóleo (EHP) de Atibaia, São Paulo, Brazil. Los resultados de los análisis químicos revelaron el predominio del ácido 3,5-diprenil-4-hidroxinámico y sus derivados y de los ácidos p-cumárico, caffeico y derivados. La actividad antiinflamatoria del EHP per os fue evaluada en el edema de pata inducido por la carragenina en ratas y edema de oreja de ratón inducido por aceite de croton. En dosis de 250 mg.Kg−1 causó una reducción significativa en el edema de la pata en dos horas (51,36%) después de inyección subplantar de carragenina en las ratas, persistiendo el efecto hasta la tercera hora (29,68%). Las dosis de 500 y 1000 mg.Kg−1 promovieron la reducción del edema del 52,1% y 60,3% en la primera hora, del 65,6% y 59,7% a la segunda hora, del 56,1% y 52,8% a la tercera hora y del 41,4% y 36,9% en la cuarta hora, respectivamente. Los efectos del EHP administrados en el edema de la oreja inducido por aceite de croton en los ratones en las dosis de 250, 500 y 1000 mg.Kg−1 mostraron reducción del edema en 47%, 50,5% y 48,1%, respectivamente. Para evaluar la actividad antinociceptiva del EHP, administrado oralmente, fueron usados los dos modelos experimentales (“writhing test” en los ratones y “tail-flick” en las ratas). Los resultados obtenidos en el “writhing test” con EHP solamente en la dosis de 1000 mg.Kg−1 significativamente inhibieron las contorsiones en 52,8%. En el análisis “tail-flick”, los resultados obtenidos con la administración por vía oral de EHP no evidenciaron actividades analgésicas en ninguna de las dosis utilizadas, pero en administración de 1000 mg.Kg−1, ip, demostraron significativa actividad antinociceptiva, incrementando la latencia de retirada de la cola de las ratas, valor comparable a la droga control morfina. La administración de naloxone asociada al EHP o morfina, ip, disminuyó la latencia de la retirada de las colas de las ratas, respectivamente, en los tiempos 9,67 y 4,9 s; estos resultados sugieren que el EHP contiene sustancias antinociceptivas que parecen estar relacionadas con la activación de los receptores opioides.

KEY WORDS: Anti-oxidant, Inflammation, Pain, Phenolic compounds, Propolis.
PALABRAS CLAVE: Anti-oxidante, Dolor, Inflamación, Compuestos fenólicos, Propolis.

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INTRODUCTION

Propolis is a resinous hive product colleted by honeybees from parts of plants, buds, and exudates and has been used as a folk medicine since around 300 BC. Many medical properties, including antiseptic, bacteriostatic, antmycotic, antiprotozoan, antiviral, spasmolytic, astringent and immunostimulatory activities, have been ascribed to propolis. Recent research also supports its anti-inflammatory and analgesic properties, being that these effects are mainly attributed to the presence of certain compounds in its composition, such as phenolic acids and flavonoids. Propolis is extensively used in Brazilian folk medicine, and found in the form of suspensions, extracts, tablets, powder, creams for the treatment of wounds and as a beauty product against wrinkles, soap, shampoo, talcum powder, after-shave lotion and throat lozenges. One of the biggest problems in the therapeutic use of this, and other, apicultural products (honey, bees-wax and royal jelly), is the variation of its composition depending on the flora, seasonal changes in a given geographic region, the time of collection and the presence of contaminants. Therefore, one of the challenges faced for the therapeutic use of propolis is to define which type of propolis is more adequate for the treatment of specific pathologies, based on its chemical and pharmacological characterization. The aim of the present paper was to study the anti-inflammatory and antinociceptive effect of a sample of propolis from the city of Atibaia, São Paulo (Brazil), in experimental models of oedema and nociception in mice and rats, as well as its toxicological and chemical profiles.

MATERIAL AND METHODS

Origin of propolis sample

The sample of propolis used in this experiment was collected in April 2000 from Apis mellifera bee hives in the proximity of the town of Atibaia, São Paulo, Brazil. Local vegetation was composed of native plants, Pinus elliotti and Eucalyptus tereticornis. The crude sample had a characteristic odor, was dark brown in color and weighed approximately 72 g.

Preparation of propolis extract

The sample was kept desiccated with silica, then ground in a food processor to a particle size of 2 mm previous to extraction in order to guarantee the homogeneity of the sample as well as a large surface area in contact with the solvent, absolute ethanol. The sample was then put in a paper cartridge inside the Soxhlet extractor and refluxed for 24 h with maximum temperature of 60 °C. The liquid extract thus obtained (HEP) was evaporated to dryness in a water bath. The dry extract was subsequently dissolved in absolute ethanol for the chemical analyses and in a solution of olive oil for the pharmacological and toxicological tests.

Chemical analysis of phenolic acids

The propolis extract was analyzed by High Performance Liquid Chromatography (HPLC) on a Merck-Hitachi HPLC equipment model D-7000 (Darmstadt, Germany), equipped with a pump and a diode array detector (L-3000, Merck-Hitachi). Separation was achieved on a Lichrochart 100 RP-18 column (Merck, Darmstadt, Germany) (12.5 x 0.4 cm, 5 µm particle size) using water, formic acid (95:5, v/v) as solvent A and methanol as solvent B. The elution was carried out with a linear gradient and a flow rate of 1 mL.min⁻¹, with maximum time of 60 minutes and detection monitored at 280 and 340 nm.

Animals

Albino Wistar rats (150-180 g) and albino Swiss mice (20-25 g) were used in this study. The animals were housed in plastic cages (groups of six rats or mice/cage) in a room with controlled temperature (22±2 °C) under a 12 h light/dark cycle with access to standard certified rodent diet and water ad libitum. They were fasted 24 and 6 h prior use, respectively.

Acute toxicity

Acute toxicity was analyzed according the method described previously by Campbell & Richter. Increasing doses of HEP (250-5000 g.Kg⁻¹) and the vehicle (olive oil), were administered per os (1mL100 g⁻¹) to mice (n = 10) and the mortality rate was observed for 48 h. The animals were monitored as to their body weight, water and ration consumption and excreta over a period of 14 days, then killed and examined macroscopically.

Anti inflammatory activity

Carrageenin-induced rat paw edema

The carrageenin-induced oedema assay was carried out according to Winter et al. Wistar rats were divided into groups of 7 animals. Edema was induced on the left hind foot of the rats by subplantar injection of 0.1 mL of a solution of 1% (w/v) lambda carrageenin in a 0.9% NaCl
(w/v) solution. Swelling of the carrageenin and contra lateral 0.9% NaCl injected feet was measured before injection the carrageenin (time 0) and at 30, 60, 120, 180, 240 e 300 min after the injection of carrageenin. Reference groups were treated with indomethacin (10 mg.Kg⁻¹, per os). HEP was administered to other groups in doses 250, 500 and 1000 mg.Kg⁻¹, per os 1 h before carrageenin administration. The control group received vehicle only (olive oil). The degree of pedal edema was determined by measuring the left hind paw volume by water plethysmography (Ugo Basile, Italy). Percentile edema inhibition was calculated according to the following formula: percentile inhibition = 1 - Vt/Vc x 100, where Vt and Vc represent the mean difference in paw measurement between the treated and control groups.

Croton oil induced ear edema in mice

The mouse ear oedema was obtained by topical application of croton oil (CO) and carried out according to the method described previously by Tubaro et al. CO was dissolved in a 5% acetone solution (v/v) and 10 µL were applied with an automatic pipette to both anterior and posterior surfaces of the right ear. The left ear (control) received the vehicle (acetone 80, 10 mL.Kg⁻¹, v/v). Groups of mice (n = 10) received orally the graded doses of HEP (250, 500 and 1000 mg.Kg⁻¹), vehicle (olive oil) or indomethacin (10 mg.Kg⁻¹), 1 h before CO application. Inflammation was allowed to develop for 5 h, after which the animals were killed by cervical dislocation, and a section (6 mm diameter) of the central portion of both ears was obtained and weighed. The swelling induced by CO was assessed in terms of the increase in the weight of the right ear punch biopsy over that of the left ear. Inhibition percentages were calculated by comparison with the control group that only received the CO application but none of the treatments.

Analgesic activity
Writhing test

In the acetic acid test, groups of mice (n = 10 per group) received orally the graded doses of HEP (250, 500 and 1000 mg.Kg⁻¹, per os), vehicle (olive oil) or dipyrone (200 mg.Kg⁻¹) 30 min prior to an i.p. injection of 0.6% acetic acid, v/v (10 mL.Kg⁻¹). The number of writhing movements of each mouse was counted for 15 min, commencing 5 min after the injection of acetic acid. The results permitted the percentage of protection to be expressed according to the following ratio: percent inhibition = 1 - Wt/Wc x 100, where Wt and Wc represent the mean difference in writhing movements between the treated and control groups.

Tail-flick test

The Wistar rats (n = 7) were secured on the apparatus so that their tail rested in a groove and shielded the aperture of a photocell, which could sense the light emitted by an 80-W projector bulb. Activation of the ‘start’ switch by the experimenter simultaneously turned on the light and started a digital timer on the Ugo Basile tail-flick apparatus. The animals flicked their tail due to increasing heat, allowing the light from the bulb to activate the photocell and stop the timer. The latency was recorded to give an estimation of analgesia. The apparatus had been calibrated to produce tail-flick latencies (TFL) of approximately 0.5-1.5 s for all animals used in this experiment prior to treatment. The minimum and maximum TFL after treatment were of 0 and 15 s, respectively for the control animals (olive oil) as well as animals treated with HEP (250, 500 and 1000 mg.Kg⁻¹) and standard pure chemicals (morphine sulfate and naloxone hydrochloride). Test latencies were assessed 30 min after the administration of drugs to rats for all test substances and control groups. Naloxone was applied 15 min prior to administration of drugs and test substances (0.4 mg.Kg⁻¹, i.p.). Morphine sulfate was used as a standard opioid agonist (1 mg.Kg⁻¹, ip). Analgesia was expressed as the percent of the maximal possible effect (%MPE):

\[
\text{%MPE} = \left(\frac{\text{postdrug latency} - \text{predrug latency}}{\text{cutoff-time-predrug latency}}\right) \times 100
\]

Statistical analysis

The results are expressed as mean ± S.E.M. The statistical analysis involving two groups was performed by means of the Student’s t-test, whereas analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test were used in order to compare more than two groups. P values of 0.05 or less were considered as indicative of significance.

RESULTS

Chemical analysis

The results of the chemical analysis of the sample of HEP by High Performance Liquid Chromatography (HPLC) are shown on Table 1, and reveal the predominance of 3,5-diprenyl-4-
hydroxycinnamic acid and its derivatives, totaling 64.52 mg.g\(^{-1}\) of the sample. P-coumaric and caffeic acids as well as their derivatives, were found in lesser quantities, totaling 18.15 and 5.62 mg.g\(^{-1}\), respectively.

**Toxicological analysis**

Propolis extract had no lethal or noxious effects on the central nervous system, autonomous nervous system and motor activity in mice at the doses tested (250-5000 g.Kg\(^{-1}\), administered per os). No alteration of body weight, water and ration consumption and excreta production, over a period of 14 days were observed.

**Anti-inflammatory activity**

Table 2 shows the results of the anti-edematous effect of orally administered HEP on carrageenin paw oedema in rats. At doses of 250 mg.Kg\(^{-1}\), HEP caused a significant reduction in paw edema (51.7%) two hours after the subplantar injection of carrageenin, the effect persisting until the third hour (29.68%). Doses of 500 and 1000 mg.Kg\(^{-1}\) promoted the reduction of oedema from the first hour until the fourth; after the first hour 52.1% and 60.3%; after two hours 65.6% and 59.7%; after three hours 56.1% and 52.8% and after four hours 41.4% and 36.9%, respectively. In this model, the standard drug, indomethacin (10 mg.Kg\(^{-1}\), per os), produced a greater anti-edematous effect at all times, inhibiting the development of edema from the first to the fourth hour in percentages of 59.4%, 48.5 %, 59.7% and 66.8% respectively.

The effects of orally administered HEP and dexamethasone on croton-oil-induced ear oedema in mice are shown in Table 3. In the test, HEP at doses of 250, 500 and 1000 mg.Kg\(^{-1}\), showed reduction of oedema by croton-oil ear in mice at 47%, 50.5% and 48.1%, respectively. In this assay, the standard drug, dexamethasone (3 mg.Kg\(^{-1}\), per os), produced a greater anti edematous effect in mice (74% inhibition).

**Analgesic activity**

To evaluate the antinociceptive activity of HEP, administered orally and intra peritoneally, two experimental models were used. The results obtained in the writhing test with oral administration the HEP showed that only a dose of 1000 mg.Kg\(^{-1}\) significantly inhibited of acetic acid-in-
duced writhing in mice; decreasing the contor-
tions in 52.8% (Table 4). In this assay, the stan-
dard drug dipyrone (200 mg.Kg\(^{-1}\), per os), pro-
duced a significant antinociceptive effect. The
incidence of abdominal constriction responses
(writhing movements) induced by i.p. of acetic
(Table 4) was found to be significantly less for
dipyrone than for all the test doses of HEP; de-
creasing the writhing movements in 72.1% com-
pared to the control group.

In the tail-flick assay (Table 5), the results
obtained with the administration of HEP by oral
route did not give evidence of analgesic activi-
ties at any of the doses utilized, but an ip ad-
ministration of 1000 mg.Kg\(^{-1}\), demonstrated
significative results in the antinociceptive activity
increasing the latency of tail retreat in rats to 15
s. The ip administration of naloxone associated
with HEP or morphine decreased the latency of
tail retreat in rats, to 9.67 s and 4.9 s respective-
ly.

**DISCUSSION**

Propolis proved to be non-toxic, per os, in
mice in the single dose acute toxicity tests, not
presenting any deleterious effects involving the
central nervous system, autonomous nervous
system or motor activity. The value of the LD\(_{50}\)
was superior to 5000 mg.Kg\(^{-1}\) of body weight,
therefore the doses used in the present experi-
ment were deemed safe 19. According to Diet-
rich 20, substances with LD\(_{50}\) above 5000
mg.Kg\(^{-1}\) are considered to be of low toxicity
and the LD\(_{50}\) results obtained with doses above
these values are considered to be imprecise. In
the present study, two animal models for inves-
tigation of the anti-inflammatory and anti-noci-
ceptive effects of hydro alcoholic propolis ex-
tract were used. Carrageenin-induced paw oede-
ma in rats and croton oil-induced ear swelling
in mice were selected to represent models of
acute (exudative phase) inflammation. Car-
rageenin is a sulfated polysaccharide derived
from certain species of algae. Its subplantar in-
jection is followed by an immediate increase in
capillary permeability, leading to the exudation
of plasmatic liquid and proteins, with the pre-
dominant leukocytic migration of neutrophils 21.
During this event, there is the sequential libera-
tion of several mediators of inflammation, such
as histamine, 5-hydroxitriptamine, bradykinin
and finally prostaglandins 22. The migration of

<table>
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<th>Treatment</th>
<th>Mean±SEM and % inhibition</th>
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<td>Dose (mg.Kg(^{-1}))</td>
<td>Time (h)</td>
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<td>-</td>
</tr>
<tr>
<td>Indomethacin</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>500</td>
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<td>1000</td>
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</table>

**Table 2.** Effect of indomethacin and HEP on carrageenin-induced paw edema in rats. The drugs were administered orally 1 h before injection of 1% carrageenin (n = 07). The results represent the increase in paw volume (mL) (mean±SEM) and the percent inhibition of anti-inflammatory activity. * p < 0.05 ; b p < 0.01; c p < 0.001; d p < 0.0001 compared with control (ANOVA followed by Dunnett’s test).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (m.Kg(^{-1}) p.o.)</th>
<th>n</th>
<th>Weight (mg)</th>
<th>Inhibition ear edema (%)</th>
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<td>Dexamethasone</td>
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<tr>
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<td>0.0092±0.0002(^{a})</td>
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<tr>
<td></td>
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<td>10</td>
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<tr>
<td></td>
<td>1000</td>
<td>10</td>
<td>0.0091±0.0003(^{*})</td>
<td>48.1</td>
</tr>
</tbody>
</table>

**Table 3.** Inhibitory effects of HEP administered orally on croton oil-induced ear edema in mice. Each value is the mean±SEM of the results. * p < 0.001 significant compared with control value (ANOVA followed by Dunnett’s test).
neutrophils to the affected area constitutes an important pro-inflammatory factor, as they liberate toxic oxygen radicals in the extracellular medium (such as O$_2^-$, H$_2$O$_2$ and OH$^-$) that react with nitrous oxide forming reactive radicals (such as ONOO$^-$, NO$_2^-$ and NO$_3^-$) which promote the intensification and amplification of the inflammatory process. The present study demonstrated that HPE was effective in an animal model of acute inflammation. Oral administration of HPE was capable of reducing the evolution of the oedema produced by the subplanar injection of carrageenin between the second and third hours for a dose of 250 mg.Kg$^{-1}$ and from the first to fourth hour for doses of 500 mg.Kg$^{-1}$ e 1000 mg.Kg$^{-1}$. These results are in agreement with those of Calixto and Park. Scavenging of free radicals generated by neutrophils in inflammatory processes may be an important mechanism of the anti-inflammatory effect of propolis. Acceleration of regenerative processes have been observed in clinical trials after treatment with ethanolic propolis extract as a result of the phenolic compounds in its chemical composition. In the present work, several phenolic compounds were identified and quantified (Table 1). Since these substances are involved in the anti-inflammatory and antioxidant properties of propolis, one can presume, in the present investigation, that one anti-oedematous effect of HPE is the neutralization of free radicals generated by neutrophils at the site of the carrageenin induced inflammation. A second experimental model of exudative inflammation was conducted in mice. Croton oil is a highly irritant agent which, applied topically, provokes an intense dermatitis characterized by vasodilatation, edema and leukocytic migration, as well as the local liberation of inflammation mediators such as histamine e 5-hydroxitriptamine, bradykinin and prostaglandins. The results show that HEP, in all the doses tested, was capable of inhibiting the development of ear oedema in the treated animals, which is in agreement with results found in literature. Since the anti inflammatory and analgesic activities are mostly associated with a particular compound, the acetic acid induced writhing in mice and tail-flick for thermal stimulation in rats were selected to investigate the peripheral and central analgesic effects of different doses of HPE. These tests are frequently used to evaluate the anti-inflammatory and analgesic activity of new compounds and are based on the reflexes of nociception evoked by thermal or chemical stimuli. The writhing test is a classical model for the evaluation of analgesic compounds, while the tail-flick test is used to assess nociceptive behaviors. The results obtained in this study showed that HPE had a significant analgesic effect in the writhing test, with a dose-dependent inhibition of writhing movements. In the tail-flick test, HPE also showed a significant analgesic effect, with a dose-dependent latency increase. The results of these tests are presented in Table 4 and Table 5, respectively. The data were analyzed using ANOVA followed by Dunnett’s test.
anti-nociceptive effects of HPE respectively. The writhing test method is based on the ip administration of a 0.6% solution of acetic acid, an agent that induces endogenous pain mediators, such as prostaglandins, histamine and bradykinin, which stimulate the free nerve ends, causing a sensation of pain locally. The administration of HEP (1000 mg, per os) to the animals produced a significant inhibition of acetic acid-induced abdominal constriction response in mice (Table 2), reducing by 52.8% the number of constrictions, suggesting an antinociceptive effect. For doses of 250 and 500 mg Kg\(^{-1}\), per os, the analgesic effect was not significant. Antinociceptive activity was also evaluated in the tail-flick test. This model measures the period of latency for the tail retreat reflex in rats subjected to the incidence of light (55 °C) directed at the median portion of the tail. This test is adequate for the detection of analgesic substances that act centrally such as opioids. The present results show that HPE induced analgesic protective effect against thermal stimuli (tail flick) only at the dose of 1000 mg, ip. administration, showing a significant analgesic effect when compared to morphine, a narcotic drug (Table 5). The oral administration of HEP, at the three doses administered, did not show a significant effect on the latency of tail retreat in rats subjected to thermal stimulus, when compared to the control group, indicating that its active principle(s) seem to be poorly absorbed by the gastrointestinal tract. Naloxone, an opioid antagonist of membrane-bound receptors δ (OP\(_{1}\)), κ (OP\(_{2}\)) and μ (OP\(_{3}\))\(^{18}\), was also employed in an attempt to provide some insight into the mechanism involved in the antinociceptive effects of propolis. In Table 5 one can observe that the association of naloxone with HPE (1000 mg, ip), reverted the analgesic effect of propolis, measured by the increase in the period of latency of tail retreat in rats, measured in seconds (9.67±3.37). Such results suggest that HEP contains antinociceptive substances that appear to be unrelated to activation of opioid receptors.

REFERENCES