Anti-inflammatory and analgesic evaluation of hydroalcoholic extract and fractions from seeds of *Piper cubeba* L. (Piperaceae)

F.F. Perazzo a,b,c, I.V. Rodrigues d, E.L. Maistro e, S.M. Souza d, N.P.D. Nanaykkara c, J.K. Bastos a, J.C.T. Carvalho b, G.H.B. de Souza a,d,*

a Faculdade de Ciências Farmacêuticas de Ribeirão Preto, USP, Ribeirão Preto, SP, CEP 14040-903, Brazil
b Departamento de Ciências Exatas e da Terra, UNIFESP, Diadema, SP, CEP 09972-270, Brazil
c National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, MS 38677, USA
d Faculdade de Farmácia, UFOP, Ouro Preto, MG, CEP 35400-000, Brazil

* Corresponding author. Lab. de Farmacognosia, Escola de Farmácia, UFOP, Ouro Preto, MG, CEP 35400-000, Brazil.
E-mail address: guhbs@yahoo.com.br (G.H.B. de Souza).

A B S T R A C T

Some of the *Piper* species have been applied for the treatment of several diseases (anti-oxidant, anti-microbial, anti-inflammatory, and analgesic), considering multiple applications used in traditional medicine of different countries. About these, the present study evaluated some biological activities of *Piper cubeba*, as writhing test induced by acetic acid, ear edema induced by croton oil and paw edema induced by carrageenan were used to evaluated the analgesic and anti-inflammatory activities of crude hydroalcoholic extract (PCE) and its fractions of different polarities of *P. cubeba* seeds. The lethal dose (LD₅₀) and the effective dose (ED₅₀) were evaluated too. Both the PCE and dichloromethane fraction showed decrease values of edema and abdominal constrictions. The results obtained in this study confirm the low toxicity and analgesic and anti-inflammatory activities of PCE from *P. cubeba* seeds, justifying its use in folk medicine.

1. Introduction

*Piper* species are widely distributed around the world, especially in tropical and subtropical regions. Some of the *Piper* species have been applied for the treatment of several diseases, considering multiple applications used in traditional medicine of different countries. Species of genus *Piper* are also used as flavor food additive by many communities. Some biological activities attributed to this genus involving anti-oxidant, antimicrobial, anti-inflammatory and analgesic effects have been reported. Chinese *Piper* species also have been used in traditional medicine for rheumatic diseases, as well as ailments of the respiratory tract. In recent years, *Piper* species have been widely studied by various research groups, leading to the identification of more than 600 different organic compounds.

*Piper cubeba* L. is native species from India that has been used in its traditional medicine to treat ailments associated to inflammatory and pain processes. The seeds of *P. cubeba* contain cubebin, a dibenzylbutirolactone lignan, which has been previously described as anti-inflammatory and analgesic with no clastogenicity associated.

The aim of the present work was to study the anti-inflammatory effect of *P. cubeba* L. seed extract (PCE) and its fractions through *in vivo* assays. The effects on rat paw carrageenan-induced edema, the median effective dose (ED₅₀) and on ear edema induced by croton oil were investigated. The median lethal dose (LD₅₀) was also established to guarantee low intoxication risk of the animals used in the experiments.

2. Material and methods

2.1. *P. cubeba* seeds

Seeds of *P. cubeba* L. were imported from India. The seeds were air dried (60 °C/3 days) and pulverized to a coarse powder (500.0 g). This powdered material was submitted to maceration with water—ethanol (9:1) (6000 ml) by three days. The macerate was filtered and the extraction procedure repeated. The concentrate of combined extracts obtained under reduced pressure furnished 63.65 g (12.73% yield) of crude water—ethanol extract. Part of this *Piper* seed concentrated extract (PCE) (10.0 g) was dissolved in 3% Tween 80 to give 100 ml (PCE 1%).
80 and 0.9% NaCl solution and the resultant solution was aseptically filtered and administered to the animals.

In parallel, another part of the crude water—ethanol extract (53.0 g) was dissolved in MeOH:H2O (9:1) and sequentially partitioned with hexane, methylene chloride and ethyl acetate. After solvent removal in a rotatory evaporator, the hexane [7.81 g (14.73%)], methylene chloride [77.24 g (8.18%)] and ethyl acetate [0.62 g (1.16%)] dried fractions were obtained.

2.2. Animals

Male albinos Wistar rats (Rattus norvegicus) (150–200 g) and male albinos Swiss mice (Mus musculus) (20–25 g), specific pathogen free, were obtained from the Central Biotery of Universidade de São Paulo, S.P., Brazil. Groups of animals were kept at controlled conditions of humidity (53%) and temperature (23 ± 2 °C), and with food and water ad libitum. During 12 h before experimental assays, the animals were kept only with water ad libitum. The experiments were carried out according to the “Guide for the care and use of laboratory animals” (The National Academic Press, USA, 2011).

2.3. Determination of median lethal dose (LD50)

A single dose of PCE (500, 750, 1000, 1250, 1500 and 2000 mg/kg) was administered orally (p.o.) to groups of mice (n = 8). The animals of each group were observed during 72 h. The number of death animals was expressed as a percentile, and the LD50 was determined by probit test using death percentage versus dose’s log.

2.4. Anti-inflammatory activity

Rat paw edema was induced by Kappa carrageenan type III (Iota-Fluka-Biochemica Co.). The inflammatory agent (100.0 µg/paw) was injected in the right hind paw planter surface of the rat (n = 8). Left paw was used as control of edema and sterile saline solution (0.9% NaCl, 0.1 ml) was used as vehicle. The foot volume of all animals was determined by plestimographic method described by Ferreira (1979). The foot volume measurements were taken before and then at hourly intervals during the first 4 h after the injection of the inflammatory stimulus.

Determination of ED50 — groups of rats (n = 8) were treated orally with P. cubeba L seeds crude extract (PCE) (50, 100, 200 and 300 mg/kg) 30 min before the carrageenan injection. The inhibition of inflammatory process was calculated by measuring the volume difference between the right and left paws of PCE treated animals in comparison to the control group at the third hour of experimentation (edema peak). ED50 was determined through the data obtained from the curve of carrageenan-induced edema percent inhibition versus dose. This method was used to evaluate the effectiveness of P. cubeba hydroalcoholic extract and its fractions in relation to the inhibition of inflammatory process by comparison with the control and indomethacin (MSD Co.), a non steroidal anti-inflammatory drug (NSAID) used as positive control. To establish the ED50, different doses of PCE, indomethacin (5.0 mg/kg, p.o.) and 0.9% NaCl (0.5 ml, p.o.) were administered orally 30 min before the injection of carrageenan and the inflammatory process inhibition was determined.

Similarly, the anti-inflammatory effect of hexane, methylene chloride and ethyl acetate fractions (50.0 mg/kg, p.o.) were compared with the results of indomethacin and 0.9% NaCl.

2.5. Writhing test

Writhing test was carried out as described by Koster et al (1959), modified by Broadbear et al (1994). Groups of mice (n = 8) were treated orally with different doses of PCE, indomethacin (10 mg/kg) or 0.9% NaCl (0.5 ml) 30 min before the stimulus. The muscular contraction was induced by intraperitoneal injection of 0.6% acetic acid solution (Reagan Co.) (0.25 ml/animal). The number of muscular contractions was counted by a period of 20 min, starting 5 min after the injection, and the results expressed as the average of total writhes observed.

2.6. Ear edema induced by croton oil

The method used was described by Tubaro et al (1985). The cutaneous inflammation was induced by croton oil solution in acetone (10.0 mg/ml) on the right ear surface (0.1 ml, 1.0 mg/ear). The same volume of acetone was applied on the left ear. Thirty minutes after the stimulus, crescent doses of PCE, dexamethasone (MSD Co.) (0.2 mg/kg) and 0.9% NaCl (0.5 ml) were administered orally to different groups of mice (n = 8). After 6 h, the animals were sacrificed and a biopsy (8 mm diameter) of each ear was obtained. The weight difference (mg) between the stimulated ear (right) and the control ear (left) represented the inflammatory reference.

2.7. Statistical analysis

The statistical analyses were done using Analysis of Variance (ANOVA) followed by Tukey–Kramer multiple comparison test. Results with P < 0.05 were considered to be significant. Data are expressed as mean ± standard deviation (S.D.).

3. Results

3.1. The effectiveness of P. cubeba and lethal median dose (ED50 and LD50)

PCE has decreased the carrageenan-induced edema in a dose-dependence relationship (correlation coefficient r = 0.9407 and linear regression y = 0.2061x + 4.4297). ED50 for PCE was determined as 150.0 mg/kg (Fig. 1). During 72 h of accurate toxicity assay, no deaths have occurred in function of the doses administrated. The animals showed no stereotypical symptoms associated with toxicity. The calculated value of lethal dose (LD50) was determined to be higher than the highest dose tested (2000.0 mg/kg).

![Fig. 1. Effect of PCE (p.o.) on the carrageenan-induced rat paw edema (100.0 µg/paw). The straight line represents the equation Y = 0.2061x + 4.4297 of the administered doses with R = 0.9407.](image-url)
3.2. Carrageenan-induced rat paw edema

The group treated with PCE in the carrageenan-induced rat paw edema had a significant decrease when compared to the group treated with indomethacin ($P < 0.05$) (Fig. 2). The groups treated with the fractions of PCE (hexane, methylene chloride and ethyl acetate) also have shown statistically significant decrease of the edema ($P < 0.05$).

The treatment with PCE inhibited the formation of the edema by 22.50% in the third hour of experimentation (peak of edema). This result is similar to those observed for the group treated with indomethacin (26.2% of inhibition). Both results are statistically significant when compared to the control ($P < 0.05$), but not between them ($P > 0.05$). In the second and fourth hours of experimentation, the treatment with PCE decreased the edema formation by 28.1% and 30.9%, respectively. The group treated with indomethacin had the edema formation decreased by 51.9% and 48.2%, respectively, after the same period following treatment. Both treatments were significantly different to control group ($P < 0.05$), but not between them ($P > 0.05$).

The organic solvent soluble fractions obtained from PCE (hexane, methylene chloride and ethyl acetate) also produced inhibitory effect at 50.0 mg/kg dose on the carrageenan-induced edema at the third hour. The methylene chloride fraction inhibited the inflammatory process by 28.7% when compared to control ($P < 0.05$). This was comparable to the effect observed for indomethacin, which inhibited the edema by 26.2%. The hexane soluble fraction inhibited the inflammation by 11.2% and the ethyl acetate fraction inhibited it by 20.0% compared to control group (Fig. 3).

3.3. Writhing test

PCE (30.4 ± 5.7 writhes) and indomethacin (29.2 ± 4.3 writhes) were effective in inhibiting the writhing in mice when compared to the control group (41.1 ± 5.4 writhes) ($P < 0.05$). Treated groups did not show significant difference between them. These results are shown in Fig. 4.

3.4. Ear edema induced by croton oil

Compared to the control group, dexamethasone has inhibited the ear edema by 68.4% ($P < 0.01$), and PCE has decreased the edema formation by 20.8% ($P < 0.05$).

4. Discussion

Different *P. cubeba* L. preparations are commonly used in Indian traditional medicine for the treatment of inflammatory processes and diseases. In this study, the efficacy of a water-ethanolic extract prepared from seeds of *P. cubeba* L. was evaluated. ED$_{50}$ of PCE was established as 150 mg/kg by carrageenan-induced paw edema for anti-inflammatory evaluation. Using acute toxicity assay, the median lethal dose (LD$_{50}$) was determined to be higher than 2000.0 mg/kg. In this test, neither deaths nor symptoms associated with toxicity such as convulsion, ataxy, diarrhea or increased diuresis were noticed during the 72 h observation period. These results indicate the efficacy and relative safety of PCE for the treatment of conditions associated with inflammatory processes.

Edema formation in rat paws is the result of a synergism among several inflammatory mediators, promoting the increase of vascular permeability and/or mediators that increase blood flow.

Carrageenan-induced edema is a well-known experimental animal model applied to the studies of acute inflammation. It has been known that at the third hour after carrageenan inoculation the edema reaches its highest volume. The presence of prostaglandins and other mediators are responsible to slow reactions during the inflammatory processes. Carrageenan injection induces the liberation of bradykinin, and also the biosynthesis of PGI$_2$ and other autacoids, responsible for the inflammatory exudate. The early phase (1–2 h) of the carrageenan effect is mediated by histamine and serotonin and has been associated to the enhancement of prostaglandins synthesis around damaged tissue. The last phase is characterized by prostaglandin release and mediated by...
bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages.22

The inhibitory activity produced by P. cubeba seed extract (150.0 mg/kg) over a period of 4 h in carrageenan-induced rat paw inflammation was very similar to those exhibited by indomethacin treated group, the standard NSAID used as positive control. Results indicate that PCE acts during the third phase, probably involving arachidonic acid metabolites, which produce an edema dependent on neutrophils mobilization.23 Thus, it is suggested that the mechanism of action of this extract and fractions can be related to prostaglandin synthesis inhibition, as described for indomethacin.24

The intraperitoneal administration of irritant agents to serous membranes provokes stereotypic behavior in mice and rats characterized by abdominal contractions, anomalous body movements noted in the hind paws, twisting of dorsiabdominal muscles, and reduction of motor activity and coordination.25 Although, this model is widely used for analgesic drug screening and involves local receptors (cholinergic and histamine receptor) and ace- tycholine and histamine mediators.26

Quantification of prostaglandins by radioimmunoassay in the peritoneal exudates of rats obtained after intraperitoneal injection of acetic acid demonstrated that high levels of prostaglandins PGE2x and PGF2α are presented during the first 30 min after stimulus, suggesting that anti-inflammatory substances can be involved in the peripheral analgesic activity.27 The genesis of carrageenan-induced edema may cause prostaglandins and kinins releases, among other substances.28 The writhing test has shown results similar to those obtained through edematogenic assay using carrageenan, even probable due to because both assays likely induce the same inflammation mechanisms based in prostaglandin’s biosynthesis.

All fractions of PCE obtained with organic solvents showed different degrees of inhibition on the carrageenan-induced edema. Moreover, the methylene chloride fraction showed the best activity, indicating that the active compounds are concentrated in this fraction. These compounds, probably cubebin and other dibenzylbutyrolactones lignans as hinokinin, have been previously described as anti-inflammatory agents.29 Methylene chloride fraction (50.0 mg/kg) has decreased the inflammatory process by 28.75% in comparison to the negative control group. This effect was similar to those observed for the group treated with indomethacin (5.0 mg/kg), that showed 26.25% of edema inhibition (P < 0.05).

Elfahmi et al (2007) have isolated several compounds from the extract of P. cubeba. The main constituents are phenolic compounds such as lignans as cubebin, yatein and hinokinin, which have been described as anti-inflammatory.29,30 The presence of lignans in the P. cubeba extract, such as other compounds as alkaloids and terpenoids (essential oil) are related to the anti-inflammatory and analgesic activities, confirming the eth- nopharmacological use of this medicinal plant as food and medicine.

5. Conclusion

The results obtained in this study demonstrated that the seeds extract of P. cubeba L. have an anti-inflammatory activity supporting its traditional use for the treatment of inflammatory disorders.

Conflicts of interest

All authors have none to declare.

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