Antiedematogenic and antinociceptive effects of leaves extracts from Protium spruceanum Bentham (Engler)


Abstract

Ethnopharmacological relevance: In traditional medicine, gums and oil-resins from Protium species have been used for many diseases, however, there are no reports of studies of Protium spruceanum leaves. Materials and methods: The antiedematogenic and antinociceptive effects of crude ethanol extract (EEB) from leaves of P. spruceanum and its fractions were evaluated in biological models. The fractions were obtained with hexane (FHEX) and methanol (FMEOH). Rat paw edema induced by carrageenan, writhing test, formalin test, hot plate test and the toxicity of EEB were performed. Results: Phytochemical analysis has shown the presence of α and β-amyrins as major constituents of FHEX. Promising results of anti-inflammatory and antinociceptive activity were found for EEB, FHEX and FMEOH. Also were observed the EEB at 6000 mg/kg showed no toxicity. Conclusions: One might suggest that the activities of FHEX are due to the presence of a and β-amyrins and contributes to the biological activities of EEB.

1. Introduction

The Burseraceae family comprises 21 genera with 700 species divided in three major tribes: Proteae (three genera), Canarieae (eight genera) and Burseraceae (seven genera). These species are widely spread throughout tropical and subtropical regions, mainly in the Amazon Rain Forest. It can be found all over Brazil, where the genus Protium makes up 80% of the Burseraceae family. This family has been a well-known source of exudates and oil-resins rich in aromatic substances that are used in perfumery industries, and appliances by native tribes in the regions where they are found, as anti-inflammatory and insect repellent.

The Protium genus (Tribe Proteae) has been known as the main family member with 150 species, and it is the most representative. Species of Protium are widely found in all countries of South America, especially in the Rain Forest. The oil-resins and gums produced by many species of this genus are commonly known as “breus”.

In traditional medicine, gums and oil-resins from Protium species have been used for many diseases, e.g. as tonic and stimulant, expectorant, as analgesic and anti-inflammatory agent, healing of ulcers and as insect repellent.

Phytochemical studies performed on gums and oil resin, obtained from Protium heptaphyllum revealed the presence of binary mixtures of triterpenes, especially α- and β-amyrins. In the essential oil of P. heptaphyllum was detected p-cymene (39.93%) and α-tetradecane (13.38%) as the main constituents. The pharmacological assays using substances isolated from species of Protium reveal proprieties as gastroprotective and anti-inflammatory, antiallergic, hepatoprotective. These biological activities have been attributed to the pentacyclic triterpenes mixture α- and β-amyrin, major component of the resin and also due to the presence of compounds with hydroxyl groups. The mixture of these two triterpenes is frequently found in medicinal plants mainly in species of the genera Maytenus. The triterpenes α- and β-amyrin are considered the most important constituents of non-polar fractions and resins obtained from species of the genus Protium.

P. spruceanum (Fig. 1) grows in abundance in the regions of Amazon, the rain forest, “Mata Atlântica” and in “Cerrado”, the most extensive woodland-savanna in South America, characterized by a pronounced dry season. This species is popularly known as...
“almécega-de-casca-lisa” or “breu”. Reports on its floristic relationships\(^{16,17}\) and its differences in chemical composition of essential oils obtained from leaves, branches and resin associated to edaphical conditions\(^{18}\) have been described.

Based on the traditional medicine uses related to this genus, the analgesic activity was evaluated using the hot plate method in mice and acetic acid-induced writhing test. The anti-inflammatory action was studied by rat paw edema induced by carrageenan. This manuscript reports the pharmacological properties of *Protium spruceanum* Benth. (Engler) for the first time.

### 2. Materials and methods

#### 2.1. Plant material and extract EEB preparation

Leaves of *P. spruceanum* were collected around the Lavras City, Minas Gerais, Brazil (between coordinates 21°17’33”, 6°56’35′′E e 44°59’15”, 1°25’11”, 9°57’42′′E e 44°59’18”, 8°50’49′′W). A voucher specimen was deposited at the Herbário da Universidade Federal de Lavras (No 16399 HESAL). The leaves were dyed at room temperature and fragmented in a mill. The powder (200.0 g) was subjected to exhaustive percolation with ethanol. The ethanol was recovered using a rotary evaporator, yielding the ethanolic extract [EEB (52.39 g, 26.2%)]. The crude extract EEB (35.05 g) was resuspended using a rotary evaporator, yielding the ethanolic extract [EEB (35.05 g)] and methanolic fraction [FMEOH (27.05 g, 77.16%)].

#### 2.2. Isolation of \(\alpha\)- and \(\beta\)-amyrin

Fraction FHEX (2.0 g) was subject to column chromatography on silica gel 60 (70–230 mesh), glass column (2.0 cm internal diameter), using hexane, dichloromethane, ethyl acetate and methanol on a gradient polarity elution technique, producing 166 fractions of 10 mL each. The fractions were analyzed by thin layer chromatography (TLC) using vanillin-perchloric acid solution as the revealing reagent. Fractions with similar TLC patterns based on retention factor (RF) were combined and selected for subsequent bioassays.

The group of fractions fr97–135 (3000.0 mg), eluted with hexane:dichloromethane (70:30 to 50:50), was submitted to silica gel column chromatography using a glass column (diameter 0.5 cm) at the similar conditions mentioned above. Thirty three fractions of 10 mL were collected and the group of fractions fr7–9, after recrystallization in dichloromethane, had presented white solid crystals (melting point: 176.5–177.5 °C). By TLC analysis, only a single constituent was observed both under UV light (254 nm) and after treatment with vanillin-perchloric acid, it produced a pink to purple spot. The solid material of fraction fr7–9 (16.95 mg) was analyzed through \(^1\)H and \(^{13}\)C NMR spectrometry.

#### 2.3. Animals

For the animal assays, health male Swiss mice (*Mus musculus*), weighing 25–30 g, were supplied by the Centro de Ciência Animal of Universidade Federal de Ouro Preto. They were randomly divided into groups (\(N = 6\) per cage). All experimental animals were kept in controlled conditions of temperature (25 ± 2 °C) and relative humidity (~80%), at 12 h light–dark cycles. All mice were fed with standard pellet diet *ad libitum* and allowed free access to drinking water. The animals were solid food-deprived for 12 h, and housed acclimatized to laboratory conditions 30 min prior the assays. All experiments involving animals were performed with strict adherence to ethical guidelines (protocol N° 2010/07) accepted by institutional Animal Care and Use Committee.

#### 2.4. Preparation of samples

The extract and its fractions were dissolved in saline solution of Tween 80 (3%). Volume solutions of 0.2 mL corresponding to doses of 200.0, 300.0 or 400.0 mg/kg were orally administered in each experimental animal (\(N = 6\)). The control group was treated only with Tween 80 saline solution.

#### 2.5. Acute toxicity

The LD\(_{50}\) (50% lethal dose) was evaluated for the acute toxicity. Doses ranging from 200.0 to 6000.0 mg/kg of EEB were orally administered to the experimental animals (\(N = 6\), per group). According to the original protocol suggested by Litchfield et al., \(^{19}\) all animals were observed during 48 h to detect toxicity signs such as convulsions, diarrhea, alertness, piloerection, sedation, ptosis, urination, spontaneous motor activity and death.

#### 2.6. Paw edema induced by carrageenan

Anti-edema effect was evaluated through induced carrageenan paw edema method according to previously described by Winter et al.\(^{20}\) Thirty minutes after the administration of EEB, its fractions or the standard indomethacin (10.0 mg/kg, orally, 0.2 mL/animal) each animal was treated by giving an intraplantar injection of 0.1% carrageenan (Sigma, St Louis) in a 20.0 μL volume into the right hind paw using a 26 gauge needle. The opposite paw was treated with 20.0 μL volume of saline solution. The volumes of the carrageenan injected paws edemas of mice (mm) were measured 1 h prior to the injection of carrageenan and at hourly interval for 7 h after the injection using a Starrett digital caliper (300 mm 12” × 0.005” 0.01 mm resolution).\(^{21}\)

The anti-edema effect was estimated based on the difference of right and left paw volume. Relevant difference between treated or non-treated paws volumes, compared with control group (vehicle) was considered as positive anti-edema response.

#### 2.7. Antinociceptive activity

Three models, acetic acid-induced abdominal writhing response,\(^{22}\) hot plate method\(^{23}\) and formalin induced hind paw licking\(^{24}\) were employed to study the antinociceptive effect of the extract EEB and its fractions.
2.8. Acetic acid-induced abdominal writhing response

Acetic acid (0.2 mL of 0.6% v/v solution) was administered intraperitoneally to all groups at the dose of 10 ml/kg body weight 30 min after the application of extract EEB, its fractions, and indomethacin (10.0 mg/kg, orally, 0.2 mL/animal) used as standard drug administration. An abdominal constriction is indicated as the full extension of hind limb. The number of abdominal constrictions (writhing) and stretching with a shudder of the hind limb was counted for 25 min after administering acetic acid. Percent protection against writhing movement was taken as index of antinociception. Antinociceptive activity was expressed as the percentage inhibition of abdominal constrictions among control animals and mice pre-treated (N = 6) with the EEB, its fractions using the formula (Control mean – treated mean) / Control mean. A statistical reduction in the contortions number compared with control group was considered positive antinociceptive response.

2.9. Analgesic response using hot plate method

Mice were divided into three groups consisting of six animals in each group. After 30 min of oral administration of extract EEB and its fractions, and morphine (7.5 mg/kg, 0.2 mL/animal) used as standard drug. Each animal was placed on the Eddy’s hot plate (56 ± 2 °C). Then, the latency time (LT) was recorded as the nociceptive effect in the animals, expressed through its reflex of lick, jump, tap dance or stand on its hind paws. The reaction time in control and treated animals was recorded at 0, 30, 60, 90 and 120 min after the treatment. The maximum animal stay on hot plate was 30 s to prevent tissue damage.

2.10. Formalin induced hind paw licking in mice

The nociception action was induced injecting 30 μL of dilute formalin (1.5% in saline solution) under the skin of the dorsal surface of the hind paw of the mice. Each animal was challenged with formalin 30 min after being pretreated with standard indomethacin (10.0 mg/kg, 0.2 mL/animal); morphine (7.5 mg/kg, 0.2 mL/animal) or dipyrone (200.0 mg/kg, 0.2 mL/animal) and the test extract EEB and its fractions. The animals were placed into a transparent glass container. The licking response was monitored 0–5 min (phase I or neurogenic phase) and 20–25 min (phase II or inflammatory phase) starting immediately after the injection of formalin. The amount of time spent licking the injected paw was considered as indicative of pain. Antinociception was defined as a statistical reduction in the time spent in licking the injected paw in comparison with the control group during the phases I and/or II.

2.11. Statistical analysis

The statistical analysis of the results was carried out using one-way analysis of variance (ANOVA) followed by multiple comparison test of Bonferroni. The results obtained in the study were compared with the control group. P values < 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Chemical analysis of α- and β-amyris

The signals observed in the 13C NMR spectra of α- and β-amyrins were in accordance with those reported by Mahato & Kundu for α and β-amyrins (Fig. 2). The signal at δC 79.31 and 79.34 were respectively attributed to C-3 of α and β-amyrin. The signals at δC 124.71 and 122.01 (C-12), and at δC 139.87 and 145.47 (C-13) were associated to double bond present in these two pentacyclic triterpenes. Characteristic signals also were observed at δC 40.08 and 31.36 attributed to C-20 of α and β-amynir, respectively. The signal at δC 17.74 and 33.23, associated to C-29, as well as the signal at δC 21.86...
and 23.97 attributed to C-30 are other chemical shift assignments used to establish the structures of \( \alpha \)- and \( \beta \)-amyrin.

Reports describing phytochemical studies of Protium species have evidenced the presence of \( \alpha \)- and \( \beta \)-amyrins mainly in non-polar extracts.\(^2,8,26\) The mixture of these triterpenes was considered as being responsible for the anti-inflammatory, analgesic, anti-pruritic, anti-ulcer and hepatoprotective properties found in species of the genera Protium.\(^27\) One possible explanation for pharmacological activity of this mixture is related to the presence of the perhydroaromatic ring, which is closely similar to the molecular structure of steroidal drugs.\(^12\)

3.2. Acute toxicity

The extract from leaves of P. spruceanum was administered orally to mice (\( N = 6 \)) in order to evaluate the acute toxicity. The extract administration up to a dose of 6000.0 mg/kg had shown no apparent toxicity and did not cause animal death during the assay period. This result suggests a low toxicity for the extract. The data is in accordance to some researchers that attribute the absence of toxicity for Protium species.\(^5,27\) Siani and coworkers\(^5\) report the toxicity absence of essential oil from leaves and resin of Protium species. By research conducted by Oliveira\(^27\) it was not possible to estimate the LD\(_{50}\) of the resin (\(<5000.0 \text{ mg/kg}\)) and a mixture of \( \alpha- \) and \( \beta \)-amyrins (\(<2000.0 \text{ mg/kg}\)), obtained from Protium sp in rats through oral administration, and also suggested the low toxicity of these substances.\(^27\)

3.3. Paw edema induced by carrageenan

The volume difference of the carrageenan-induced paw edema in animals treated with EEB and indomethacin in relation to negative control group is presented in Fig. 3.

The maximum inflammatory response induced by subplantar administration of carrageenan was observed at the third hour in the control group (0.8 ± 0.06 mm). It was observed that the extract and indomethacin (10.0 mg/kg) administration caused edema inhibition at the third hour. However, only EEB at dosage 300.0 mg/kg (0.3 ± 0.18 mm) was able to inhibit the edema, when compared with the control group (0.8 ± 0.06 mm). Indomethacin was able to inhibit the edema induced by carrageenan (\( -0.3 \pm 0.28 \text{ mm}\)) at the third hour, when compared with control group (1.0 ± 0.08 mm) (Fig. 4).

The extract FHEX, rich in \( \alpha \)- and \( \beta \)-amyrin, induced an important anti-edematogenic activity at all doses tested, at the third hour (\(-0.01 \pm 0.12 \text{ mm}, -0.2 \pm 0.08 \text{ mm} \) and \(-0.2 \pm 0.18 \text{ mm}\) for 200.0, 300.0 and 400.0 mg/kg, respectively) in comparison with control group (1.0 ± 0.08 mm) (Fig. 4B). The anti-edematogenic activity of FHEX was attributed to the presence of \( \alpha \)- and \( \beta \)-amyrins, isolated from this fraction. This activity has already been reported and
attributed to this mixture also found in other species of *Protium* genus.²⁸,²⁹

For the first time, the anti-edematogenic activity is reported for FMEOH. The maximum edema inhibition was observed at the third hour (Fig. 4) induced by all doses administered (1/C₇ 0.01 mm; 1/C₆ 0.12 mm and 1/C₅ 0.04 mm for 200.0, 300.0 and 400.0 mg/kg, respectively) in comparison with control group (1.0 mm).

### 3.4. Antinociceptive activity

#### 3.4.1. Acetic acid-induced abdominal writhing response

The abdominal constrictions observed after oral treatment with EEB, FHEX and FMEOH, at doses of 200.0, 300.0 and 400.0 mg/kg and indomethacin (10.0 mg/kg) compared with the control group is respectively showed in Figs. 5 and 6.

The EEB (Fig. 4) decreased the total number of acetic acid-abdominal constrictions in the mice at doses of 200.0, 300.0 and 400.0 mg/kg (24.1 ± 3.78; 22.3 ± 3.26 and 20.8 ± 3.82, respectively). Indomethacin, used as gold standard, also reduced the abdominal constriction number (27.0 ± 2.83). Reduction of the abdominal constriction number was also observed for fractions FHEX and FMEOH (Fig. 5). FMEOH (200.0 and 300.0 mg/kg) decrease the constriction number (18.0 ± 4.55 and 14.4 ± 3.29, respectively) in comparison with control group (62.0 ± 4.93).

Fraction FHEX presented an expressive antinociceptive activity at doses of 200.0 and 400.0 mg/kg (22.1 ± 9.79 and 19.8 ± 2.73, respectively). The antinociceptive activity of this fraction was also attributed to the presence of α- and β-amyrians. Although no antinociceptive activity was considered for FMEOH, the dose of 300.0 mg/kg induced a decrease of animals constritions (38.3 ± 6.13) when compared with the control group (62.0 ± 4.93).

#### 3.4.2. Hot plate method

The results of hot plate test observed after oral administration of extract and fractions (EEB, FMEOH and FHEX, doses 200.0, 300.0 and 400.0 mg/kg), morphine (7.5 mg/kg) and vehicle are respectively showed in Figs. 7 and 8.

Morphine induces antinociceptive activity at 90 min showing a remarkable enhancement of the latency time (LT) (20.6 ± 1.72 s) when compared to initial LT (3.1 ± 0.89 s). Antinociceptive property was observed for EEB at dose of 400.0 mg/kg (Fig. 7). The initial LT (4.1 ± 0.85 s) was increased in 60 min (12.8 ± 1.24 s). No antinociceptive effect was observed for the other dosages of EEB.

The fraction FHEX (400.0 mg/kg) induced a relevant increase in LT (16.0 ± 3.45 s, Fig. 8B) 90 min after the administration, in comparison with the initial LT (3.5 ± 1.00 s). Morphine (7.5 mg/kg) used as standard drugproduced similar increase: initial LT of 3.1 ± 0.89 s and after 90 min LT of 20.6 ± 1.72 s (Fig. 8B). It was not observed significative reduction in LT induced by the MEOH fraction (Fig. 8A).

#### 3.5. Formalin method

The results of antinociceptive assays determined through the formalin method are showed in accordance with phase I (Fig. 9A) and phase II (Fig. 9B) of inflammatory process. The latency time (LT) observed for EEB and fractions were compared with the results of
During the first phase, receptors involved in antinociceptive response are constituents of opioid receptor system. At second phase, the inhibition of cyclooxygenase enzyme is responsible to decrease the nociceptive effect. Some compounds that act as analgesics of central action can inhibit both phases. However, compounds with peripheral action, inhibit only the second phase. Holanda Pinto and co-workers using the capsaicin test and formalin test found that pre-treatment with α- and β-arymins produced pronounced antinociceptive effect in the second phase. This analgesic effect may be associated to the release of some neuropeptides and prostaglandins. Indomethacin, dipyrone, morphine, EEB, FHEX and FMEOH were administered 30 min before subplantar paw injection of 1.5% formalin solution. The latency time was measured during the first 5 min [phase I (A)] and from 20 to 25 min [phase II (B)] after injection of the nociceptive agent.

Fig. 9. Effect of oral administration of EEB and fractions (150 mg/kg, v.o.) from leaves of P. spruceanum on latency time on formalin induced hind paw nociception. Negative control, indomethacin, dipyrone, morphine, EEB, FHEX and FMEOH were administered 30 min before subplantar paw injection of 1.5% formalin solution. The latency time was measured during the first 5 min [phase I (A)] and from 20 to 25 min [phase II (B)] after injection of the nociceptive agent.

4. Conclusion

The phytochemical study of EEB from leaves of P. spruceanum allowed to establish that the mixture of α- and β-arymins represent the major constituents of FHEX. It was observed that EEB has low acute toxicity. Promising results of anti-inflammatory activity were observed for the EEB and its fractions. The activity observed for FHEX must be attributed to α- and β-arymins which was further isolated by this fraction. Through the analysis of the results obtained through the methods used to evaluate the analgesic activity, it was possible to suggest that FHEX has relevant central and peripheral antinociceptive activity that can also be attributed to the mixture of α- and β-arymins. The results of FMEOH by writhing test suggested that this fraction has only peripheral analgesic properties. Then, it is possible to attribute to the mixture of pentacyclic triterpenes α- and β-arymins is sponsor for anti-inflammatory and analgesic activities of FHEX and contributes to the biological activities of EEB, both extracts isolated from leaves of P. spruceanum.

Conflicts of interest

All authors have none to declare.

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References


