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Anti-inflammatory and antinociceptive activities of the hexane extract of *Lacistema pubescens* mart. leaves

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Abstract

Traditionally, leaves of *Lacistema* genus are used to combat rheumatism, vomiting, dysentery, body aches and as antipyretic. Due to the ethnopharmacological importance of this genus, the aims of this study were to investigate the *in vivo* anti-inflammatory and antinociceptive effects of the hexane extract from the leaves of *L. pubescens* (PHEX). Also, the total amount of flavonoids was determined and the chemical composition of the extract was analyzed. The antinociceptive effects were carried out using acetic acid-induced abdominal constriction, formalin and tail flick tests and the anti-inflammatory effect was investigated employing the croton oil induced ear edema in mice. The total amount of flavonoids was determined by $AlCl_3$ reagent method. The composition of the extract was analyzed using TLC and GC/MS. PHEX significantly decreased the number of acetic acid-induced abdominal contortions being as potent than indomethacin used as reference drug. Also PHEX decreased the paw licking time in early (0 - 5 min) and late (15 - 30 min) phases of formalin-induced nociception, similar to morphine. No effect was observed using tail flick test. PHEX showed a topical anti-inflammatory effect on croton oil induced ear edema similar to the anti-inflammatory drug, dexamethasone, but when administered orally, no effect was observed. Besides the presence of triterpenes and sterols, phytochemical screening also showed the presence of flavonoids, which constituted 32.7 mg/g of PHEX. In conclusion, the findings support some of *L. pubescens* traditional uses involving anti-inflammatory and antinociceptive effects, and also represent the first report on the chemical profile and biological activities of this specie.

Key words: Lacistema pubescens; anti-inflammatory; antinociceptive

Introduction

The Lacistemataceae family is represented by the genera *Lacistema* and *Lozania* at about 11 and 4 species, respectively [1]. Traditionally, *Lacistema* sp is extensively used by Brazilian indigenous people of Amazon to combat rheumatism, vomiting, dysentery, body aches and as antipyretic [2-4]. Roumy et al. (2007), listing the Peruvian Amazonian plants used for medicinal purposes by the local indigenous population, mentioned *Lacistema* sp as antipyretic and against rheumatism. There are also records on the use of the stalk as a fuel [5].

Experimental studies have demonstrated potential pharmacological properties of some species belonging to *Lacistema*. Roumy et al. (2007) found a significant antiplasmodial activity and low cytotoxicity of the dichloromethane extract prepared from the bark of *L. agregatum* and Wall et al. (1988) demonstrated its antimutagenic properties. In addition, bioautography tests showed that the crude ethanolic extract of the leaves of *L. lucidum* presented antifungal activity against *Cladosporium cladosporioides* [7] and *L. hasslerianum* exhibited potent antiviral activity [8]. Likewise, *Lozania pittieri* was able to selectively inhibit cyclo-oxygenase activity and the reverse transcriptase of HIV-1 [9].

Originally from Brazil, *Lacistema pubescens* Mart. is widely distributed in other countries such as Bolivia, Guyana and Venezuela. In Brazil, it takes different popular names as "espeto vermelho", "canela vermelha" [10, 11], "sabãozinho" [5] and "cafezinho" [12].

Despite several records on popular use of species of *Lacistema*, there are no phytochemical records on chemical composition and/or pharmacological properties of *L. pubescens*. Therefore, the aims of this study were to identify the major constituents of the hexane extract of the leaves of *L. pubescens* by TLC and GC-MS and to evaluate its *in vivo* antinociceptive and anti-inflammatory properties.

Materials and Methods

Plant material: *L. pubescens* leaves were collected in Juiz de Fora, State of Minas Gerais, Brazil, in December 2008. A voucher specimen (CESJ 49751) has been deposited at the Leopoldo Krieger Herbarium of the Federal University of Juiz de Fora.

Preparation of extract: The dried leaves (375 g) were powdered and macerated with methanol (5 x 300 mL) for five days at room temperature. The crude extract (65 g), after removal of solvent, was dissolved in MeOH-H_2O (8:2) and partitioned with hexane. The hexane extract was then concentrated using a rotary evaporator under reduced pressure (yield 16 g), and kept in tightly stoppered bottle under refrigeration until used for the pharmacological testing, phytochemical screening and GC-MS analysis.

Gas chromatography and mass spectrometry: Capillary gas chromatography was performed using a Hewlett-Packard 6890 gas chromatograph coupled with a Hewlett-Packard 5972 mass spectrometer and equipped with a DB-5MS 30 m × 0.25 mm, 0.25 ìm capillary column; helium as carrier gas with a flow rate 2.0 mL/min; and temperature programming from 70 °C to 290 °C (2 °C/min); injector temperature 270 °C and detector temperature 300 °C. The MS operating parameters were: 70 eV, ion source 250 °C equipped with EI. Compounds were identified by computer comparison of the mass spectra with those in the Wiley and NIST libraries and by mass fragmentation [13].

Phytochemical screening: A portion of PHEX that was subjected to biological screening was used for the identification of the major secondary metabolites employing thin layer chromatography (TLC) as described by Wagner (1996).

Amount of flavonoids: The amount of flavonoids was determined by $AlCl_3$ reagent as previously described by Miliauskas et al. (2004) with slight modifications. Rutin was used as the standard for the calibration curve. Total amount of flavonoids was expressed in mg/g extract, in rutin equivalents (RE).

Animals: Male Swiss mice (weighing 25–35 g), obtained from the Reproduction Biology Center of the Federal University of Juiz de Fora, were housed in a room kept under controlled conditions with temperature maintained at 23 °C ± 2 °C, on a 12h light: 12h dark cycle and free access to water and complete commercial chow (Nuvital™, Colombo, PR, BR). For experimentation, six animals were included in each group and before each experiment they were fasted for a period of 12 h with free access to water. Throughout the experiments, animals were processed according to the ethical guidelines for the care of laboratory animals. The study was approved by the Brazilian College of Animal Experimentation (COBEA).

Acetic acid-induced writhing test: Writhing activity was determined by the method of Koster et al. (1959). The mice were divided into 3 groups: group I, treated with vehicle (saline + DMSO 2%), group II, treated with indomethacin (10 mg/kg) and group III, treated with PHEX (300 mg/kg). The antinociceptive activity was evaluated by the writhing induced by acetic acid. Treatments were made orally and after one hour a solution of acetic acid 0.6% (v/v) in saline was administered intraperitoneally. The total number of writhing was observed for 30 minutes to quantify the intensity of nociception and the results were expressed as mean ± SEM of the number of contortions.

Formalin test: The formalin test was carried out as described by Hunskaar et al. (1985). Mice were divided into 4 groups: group I, treated with vehicle (saline + DMSO 2%), group II, treated with indomethacin (10 mg/kg), group III, treated with morphine (7.5 mg/kg) and group IV, treated with PHEX (300 mg/kg). They were injected with 20 µL of 2% formalin (in 0.9% NaCl) into the subplantar space of the right hind paw, and the duration of paw licking was determined 0-5 minutes (early phase) and 15-30 minutes (late phase) after formalin. PHEX, vehicle and indomethacin were given orally 60 minutes prior to formalin injection, while morphine was administered intraperitoneally 45 min prior. The results were expressed as mean ± SEM of the licking-time.

Tail-flick: Hot tail-flick method was performed by the method described by D`Amour and Smith (1941). The mice were divided into 3 groups: group I, treated with vehicle (saline + DMSO 2%), group II, treated with morphine (7.5 mg/kg) and group III, treated with PHEX (300 mg/kg). The lower two-thirds of the tail were immersed in water bath at 55 °C. The mice were exposed to hot water for no longer than 15 seconds to avoid tissue damage. The latent period of the tail-flick response was taken as the index of antinociception and was determined before and at 20, 40, 60, 80, 100 and 120 minutes after the administration of PHEX, vehicle and morphine. The results were expressed as mean ± SEM of the time taken by the mice to deflect their tails.

Croton oil-induced ear edema: The croton oil ear test was performed as previously described [19]. For oral activity, the mice were divided into 3 groups: group I, treated with vehicle (saline + DMSO 2%), group II, treated with indomethacin (10 mg/kg) and group III, treated with PHEX (300 mg/kg). For topical activity, the mice were divided into 3 groups: group I, treated with vehicle (acetone + DMSO 4%), group II, treated with dexamethasone (0.1 mg/20 µL acetone) and group III, treated with PHEX (1 mg/20 µL), diluted in DMSO 4%, ethanol 20% and acetone 76%. For all groups, a total of 20 µL of an acetone solution of croton oil (2.5%) was applied to the inner surface of the right ear of each mouse. The left ear remained untreated. The animals were euthanized 4 hours later, and a plug (6 mm in diameter) was removed from both the treated and untreated ear. The difference in weight between the two plugs was taken as a measure of edematous response.

Statistical analysis: The data for anti-inflammatory and antinociceptive activities were expressed as mean ± SEM and the statistical significance was determined by analysis of variance (ANOVA) followed by Newman-Keuls or Bonferroni test using the Prism 5.0 (GraphPad Software Inc.) statistic program.

Results

PHEX was obtained from the dried leaves of *L. pubescens* and yielded 25% (w/w). The analysis of volatile compounds by gas chromatography/mass spectrometry (GC-MS) resulted in the identification of fatty acids such as palmitic acid (4.21%), stearic (1.19%) and linoleic acid (2.15%). Also á and â tocopherol (2.50%), the diterpene phytol (5.98%), the sterol gamma sitosterol (9.14%) and triterpenes (41.05%) were identified. Although triterpenes were presented in great amount in PHEX, their complete identification was not possible from the mass spectra obtained in the total extractives, but they were classified as such due the typical fragments presented in mass spectra. Besides the presence of triterpenes and sterols, phytochemical screening also showed the presence of flavonoids, which constituted 32.7 ± 2.8 mg/g of PHEX.

In the acetic acid-induced writhing (Figure 1), the PHEX presented 70% inhibition of the nociceptive response, superior to that observed to the positive control indomethacin at 10 mg/kg (60% inhibition). This assay is used for detecting both central and peripheral analgesia [20].

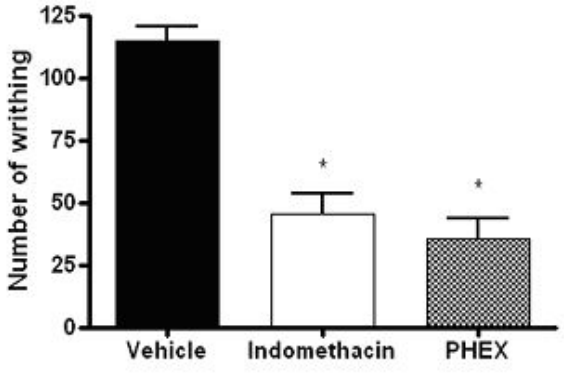


Fig.1. Effect PHEX administered orally against acetic acid induced writhing movements in mice. Animals were pretreated orally with vehicle, extract (300 mg/kg) or indomethacin (10 mg/kg) one hour prior to acetic acid application (0.6%, i.p.). The values in each column represent the mean ± SEM for number of writhing in each group. *P<0.001 compared with the control group (one-way analysis of variance followed by Newman-Keuls test).

In the formalin test, PHEX reduced pain-related behavior in both the early and the late phases, although it was less pronounced in the early phase (Figure 2a) with inhibition of 47%, while in the late phase (Figure 2b) the inhibition was 68%, which suggests that its activity may be the result of its peripheral action associated with inflammatory pain reaction. As expected, indomethacin acted only in the second phase of formalin test.

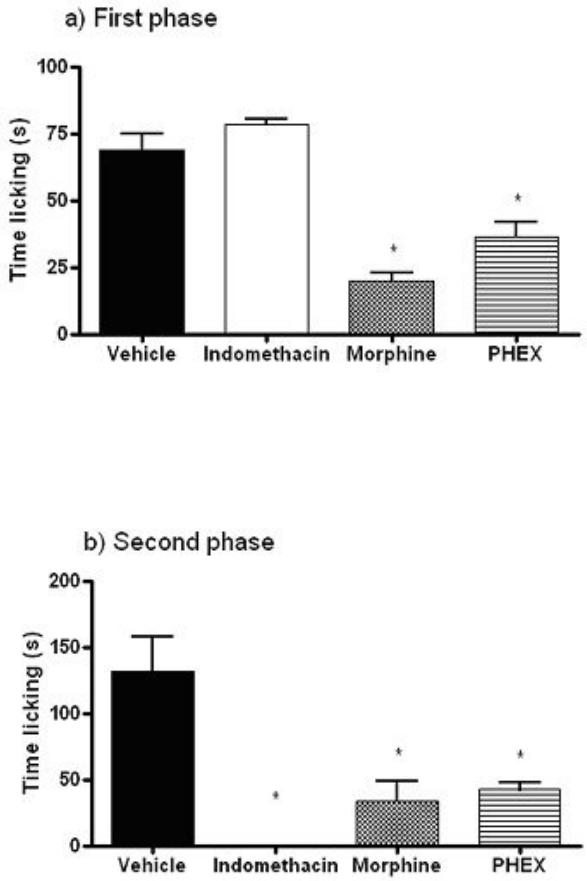


Fig.2. Effect of the PHEX administered orally on the time of licking induced by formalin in mice. Animals were pretreated orally with vehicle, extract (300 mg/kg) and indomethacin (10 mg/kg) for one hour prior to formalin application and morphine (5 mg/kg) for 30 minutes prior to formalin application. The total time spent licking the hindpaw was measured in the (a) first and (b) second phases after intraplantar injection of formalin. The values in each column represent the mean ± SEM for time of licking in each group. *P<0.001 compared with the control group (one-way analysis of variance followed by Newman-Keuls test).

In the tail-flick (Figure 3), morphine (7.5 mg/kg, i.p.) significantly increased the latency time. Unlike morphine, PHEX did not manifest a significant effect.

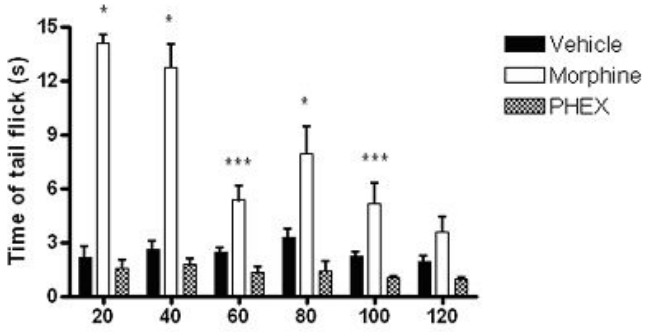
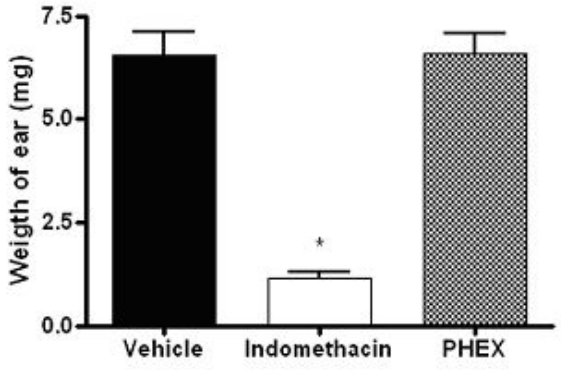


Fig.3. Effect of the PHEX administered orally on the reaction time in tail flick test in mice. Animals were pretreated orally with vehicle, extract (300 mg/kg) and morphine (5 mg/kg) 30 or 60 minutes prior to the thermal stimulus to the tail of the animal. The values in each column represent the mean ± SEM of the time of tail flick in seconds in each group. *P<0.001; ***P<0.05 compared with the control group (two-way analysis of variance followed by Bonferroni test).

As a model of inflammation, the croton oil-induced mouse ear edema model was used. The previous oral administration (60 minutes) of the PHEX did not show an edema reduction (Figure 4a). However, when applied topically, PHEX reduced significantly the edema (88%), similar to the positive control dexamethasone (93%) (Figure 4b). Therefore, topical administration of PHEX on the site of inflammation was more effective than oral administration. It is known that the route of administration determines the activity observed. This was probably due to the fact that higher concentrations of drugs can be achieved topically [21].



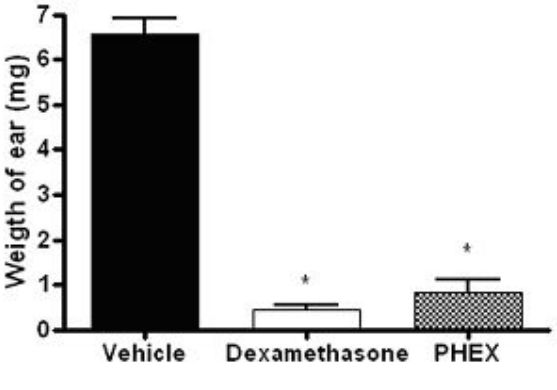


Fig.4. Effect of PHEX administered orally on the ear edema induced by croton oil in mice. Animals were pretreated orally with vehicle, PHEX (300 mg/kg) and indomethacin (10 mg/kg) one hour prior application of croton oil (a) and effect of PHEX given topically on the ear edema induced by croton oil in mice. Animals were pretreated topically with dexamethasone (0.1 mg/20 µL) and PHEX (1 mg/20 µL) immediately after the application of the oil (b). The values in each column represent the mean ± SEM of the weight of the ears (mg). *P<0.001 compared with the control group (one-way analysis of variance followed by Newman-Keuls test).

Discussion

The antinociceptive effect produced by the PHEX was assessed using three models of nociception, as the writhing and the formalin which involve chemical stimuli and the tail flick which involves thermal stimuli.

The acetic acid-induced writhing in mice is an inflammatory visceral pain model and it is very useful to detect painful complaints due to inflammatory disorders of internal organs such as the stomach or intestines [22]. It has been suggested that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons. In this model, the processor releases arachidonic acid via cyclo-oxygenase (COX); notably, prostaglandins biosynthesis plays an important role in the nociceptive mechanism [23]. This method is sensitive to non-steroidal anti-inflammatory drugs (NSAIDs), to narcotics and other centrally acting drugs [24], but shows poor specificity because abdominal constrictions could be suppressed by smooth muscle relaxants so results could be misunderstood [25].

Injection of formalin produced distinct biphasic responses being the first phase associated to the irritant effect of formalin in the C type sensorial fibers, which characterize the neurogenic pain. Otherwise, the second phase is associated to the inflammatory pain. Analgesics for central action, such as morphine, inhibit the both phases in contrast to drugs of peripheral action such as anti-inflammatories and corticoids, which inhibit only the second phase [17, 26]. The formalin test is believed to represent a more valid model for clinical pain, and is a very useful method, not only for assessing antinociceptive drugs, but also helping in the elucidation of the action mechanism. The neurogenic phase is probably a direct result of stimulation in the paw and reflects centrally mediated pain with release of substance P while the late phase is due to the release of histamine, serotonin, bradykinin and prostaglandins. Drugs that act primarily on the central nervous system, such as narcotics, inhibit both phases equally while peripherally acting drugs such as anti- inflammatory non-steroidal (NSAID) and anti-inflammatory steroidal only inhibit the late phase [27].

The tail-flick is only predictive for substances that are morphinomimetic in the strictest sense [28], therefore, it seems that the mechanism of the antinociceptive and anti-inflammatory actions of extract are not related to the opioid system.

The croton oil-induced mouse ear edema model is an *in vivo* model useful to screen of the topical anti-inflammatory activity of synthetic and natural compounds, often available in limited amounts during the bioassay-oriented fractionation procedure due to the small amount of substances necessary to carry out the assay [29]. In this edema the most important mediators involved are prostaglandins, histamine and serotonin, whereas the lypoxygenase pathway has no important role [30].

Compounds identified in PHEX like -tocopherol [31], gamma sitosterol [32], phytol [33], triterpenes [34] and lipophilic flavonoids [35], could be involved in the biological/pharmacological activities presented by PHEX. Many studies have indicated the increasing importance of lipophilic flavonoid as biologically active natural products [36]. Lipophilic flavonoids presented in plants are usually flavones or flavonol methyl ethers but flavanones and other classes of flavonoids may also be present [37]. Some of those are known to inhibit the major pathways of arachidonate metabolism in leukocytes [38,39] and possibly act as cyclo-oxygenase or/and lipoxygenase inhibitors, therefore serving as anti-inflammatory and non-narcotic analgesic agents [40]. Naturally occurring terpenoids, among other actions, also present anti-inflammatory and antinociceptive properties, inhibit platelet aggregation, and interfere at the intracellular level with several steps of signal transduction mechanisms [41].

Conclusion

The results support some of the traditional use of *L. pubescens* involving anti-inflammatory and antinociceptive effects. This study also represents the first report on the chemical profile and biological activities not only for *L. pubescens* but also for the *Lacistema* genus.

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