Antinociceptive effect of the *Orbignya speciosa* Mart. (Babassu) leaves: Evidence for the involvement of apigenin

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**Abstract**

**Aims:** Babassu is the common Brazilian name of *Orbignya speciosa* Mart. (Arecaceae). The fruits are used for several disorders. In the present study, the antinociceptive effects of the ethanol extract (EE) and dichloromethane fraction (DF) obtained from leaves were investigated, as well as apigenin using nociception models (acetic acid-induced abdominal writhing, formalin, and hot plate).

**Main methods:** Mice were treated with EE, DF (10, 30, and 100 mg/kg, p.o.), apigenin (1 mg/kg, p.o.), morphine (5 mg/kg, s.c.), aetylesalicylic acid (100 mg/kg, p.o.) or vehicle (0.1 ml, p.o.). The EE and DF reduced the contractions induced by acetic acid. Both also reduced the licking response in the formalin model. In the hot plate model, the antinociceptive effects were, at least, equal to that shown by morphine. To elucidate the antinociceptive mechanism of action of EE, DF, and apigenin the animals were pre-treated with atropine (nonselective muscarinic receptor antagonist, 1 mg/kg, s.c.), naloxone (opioid receptor antagonist, 1 mg/kg, s.c.), i-nitro arginine methyl ester (L-NAME, nitric oxide synthase inhibitor, 3 mg/kg, s.c.) or mecamylamine (nicotinic receptor antagonist, 2 mg/kg, s.c.) and evaluated in the hot plate model.

**Key findings:** The antinociception produced by DF was abolished by atropine, naloxone or mecamylamine. The effect of apigenin was significantly blocked by atropine or naloxone.

**Significance:** The results obtained indicated that EE and DF have antinociceptive activity that is mediated, at least, in part, by opioid and cholinergic systems. This effect can be attributed to the presence of apigenin, a flavonoid in the dichloromethane fraction.

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**Introduction**

Pain is one of the most prevalent conditions that limit the quality of life. Although there is a great number of analgesic opioid and non-opioid drugs, there is some concern regarding the safety and side effects of these drugs, such as ulcers, bleeding, and dependence, which limit their clinical use (Jage, 2005).

The use of medicinal plants is a traditional method of providing relief from illness and can be traced back over five millennia in several civilizations. Over the years, natural products have contributed enormously to the development of important therapeutic drugs used currently in modern medicine. The potential of higher plants as sources for new drugs is still largely unexplored (Calixto et al., 2000).

Babassu is the popular name of *Orbignya speciosa* Mart. Barbosa Rodrigues (Arecaceae, Palmae). This palm is native to the north, northeast and central regions of Brazil. It forms extensive forests in the states of Maranhão, Piauí, Tocantins, and Goiás. The babassu palm has been used by Apinaye and Guajajara Indians from north-eastern Brazil, yielding a variety of important products. The palms provide food, fuel, shelter, fibre, construction materials, and medicine for these people (Balick, 1998).

Ethnopharmacological studies have indicated the use of mesocarp from Babassu fruits for the treatment of chronic wounds, ulcers, dysmenorrhea, menstrual pain, constipation, obesity, rheumatism, leukaemia and inflammatory and venous diseases (Rego, 1995; Silva and Parente, 2001; Renno et al., 2008). The kernels of the fruits are also used for the treatment of urinary diseases. The ability of this extract to reduce urinary tract symptoms (LUTS) among aging men, usually caused by Benign Prostatic Hyperplasia (BPH), has been confirmed by our group (Reis de Souza et al., 2011).

The analgesic and anti-inflammatory properties of babassu mesocarp were confirmed experimentally using a chloroform extract of the dried fruits (Maia, 1987). In addition, a polysaccharide isolated from the mesocarp of fruits enhanced macrophage phagocytic activity (Silva and Parente, 2001). The chronic oral treatment with an aqueous extract of babassu mesocarp in C58B1/6 mice showed significant anti-thrombotic effect (Azevedo et al., 2007) and induced an increase...
in nitric oxide production by peritoneal macrophages (Nascimento et al., 2006). Traditionally, people use the fruits from Babassu to treat disorders mentioned above and discard the leaves. The fruit may be used when green in the smoking of rubber. When ripe, it is eaten as a nutritious food. A liquid contained in the fruit stalk, or peduncle, is fermented and drunk as an alcoholic beverage, much prized locally. Fiber is taken from the exocarp, or outer layer of the fruit, and used for various purposes. Stalks serve as timbers, and the leaves as coverings and partitions in dwellings. Leaves are also used domestically for making baskets and other plaited objects. Due to the great ethnobotanical importance of this plant, finding out another use for its different parts is an interesting approach. Therefore, in the present study, we aimed to investigate the possible antinociceptive activity of fractions obtained from O. speciosa leaves collected in a private farm in the northeast of Brazil.

Materials and methods

Plant material

O. speciosa leaves were collected at city of Teresina, Piauí state, Brazil, in December, 2002. The plant was identified by Dr. Roseli Faria Melo Barros. A voucher sample was deposited at the Herbarium Graziaela Barroso, Federal University of Piauí, and received the number 18,985.

Preparation of fractions

The leaves of O. speciosa (1,000 g) were dried under airflow in an oven between 40° and 50 °C and powdered in a mill of knives. The extraction was performed by static maceration with ethanol. After filtration, the ethanol extract (EE) was concentrated under reduced pressure yielding 29 g of brown syrup. EE was suspended in ethanol/water (1:4) and partitioned successively in hexane, dichloromethane, ethyl acetate and n-butanol, yielding 4.6, 4.9, 10.6, and 3.1 g, respectively, after solvent evaporation. This procedure is in accordance with previous publications from our group (Berrondo et al., 2003; Amado et al., 2009).

Isolation, purification, and identification of apigenin

Part of the dichloromethane fraction (1.5 g) was fractioned using column chromatography in silica gel (Vetec 70–230 mesh) and gradient mixtures of dichloromethane and methanol up to pure methanol. A total of 70 fractions were collected. Fractions 25 to 31, eluted with dichloromethane/methanol 1:1.5, have shown a yellow spot after thin layer chromatography. The combination of these fractions was re-chromatographed to yield 3 mg of the flavonoid apigenin, comprising 0.2% of the dichloromethane fraction. Also, one mg of apigenin standard has been mixed with one mg of the isolate and a TLC has been performed using different mobile phases, showing unequivocally that the isolate was in fact apigenin. The structure of apigenin (Fig. 1) was established by NMR analysis, using mono- and bi-dimensional experiments. It was also confirmed by comparison with literature data (Van Loo et al., 1986). Apigenin was also analysed by HPLC and found to be greater than 98% pure (Fig. 2). The analysis was performed on Waters HPLC system (Waters 600 controller with PDA detector and 717 plus autosampler) using a C18 column Nucleosil 120–5 C18 (4.6×250 mm). The optimum separation of HPLC was carried out with a mobile phase composed of methanol and 0.2% phosphoric acid aqueous solution (58:42, v/v) at a flow-rate of 1.0 ml/min. The volume of sample injected was 50 ml, the detective wavelength and the column temperature were set at 350 nm and 30 °C, respectively.

Other constituents in the extract could be suggested based on their TLC results. They are complex mixtures of terpenes, steroids and possibly other flavonoids. It is important to mention that this extract is a complex mixture of small quantities of compounds from where apigenin has been isolated and identified (Fig. 3).

Animals

All experiments were performed with male Swiss mice (18–25 g) obtained from our own animal facilities. Animals were maintained in a room with controlled temperature 22±2 °C for 12 hr light/dark cycle with free access to food and water. Twelve hours before each experiment animals received only water, in order to avoid food interference with substances absorption. Animal care and research protocols were in accordance with the principles and guidelines adopted by Brazilian College of Animal Experimentation (COBEA), approved by the Biomedical Science Institute/UFJF, Ethical Committee for Animal Research, and received the number DFBCICB-015.

Drugs and O. speciosa fractions administration

Acetylsalicylic acid (ASA), L-nitro arginine methyl ester (L-NAME), apigenin and atropine were purchased from Sigma (St. Louis, MO.). Acetic acid, formalin, and morphine hydrochloride were from Merck Inc., and naloxone was obtained from Cristália (São Paulo, Brazil). All drugs were dissolved in phosphate buffer saline (PBS) just before
use. The ethanol extract and dichloromethane fraction were dissolved in dimethylsulfoxide (DMSO) in order to prepare a stock solution at a concentration of 100 mg/ml. In all experiments, the final concentration of DMSO did not exceed 0.5% at which had no effect per se. The ethanol extract and the dichloromethane fraction were administered by oral gavage, at doses of 10 to 100 mg/kg; apigenin was administered at 1 mg/kg (p.o.). Morphine (5 mg/kg, s.c.) and ASA (100 mg/kg, p.o.) were used as reference drugs. The antagonists were used at doses indicated in each protocol. The control group was composed by vehicle (PBS with the same amount of DMSO used in the highest dose).

Peripheral antinociceptive effect

Acetic acid-induced abdominal writhing

The peripheral antinociceptive activity was evaluated in mice using the writhing test adapted by Pinheiro et al. (2010). Briefly, mice were pre-treated with vehicle, ASA, morphine, O. speciosa fractions, or apigenin 60 min before intraperitoneal administration of a 2% (v/v) acetic acid solution (AA). The total number of writhing was recorded over a period of 20 min, starting 5 min after AA injection.

Formalin-induced licking

The procedure was similar to the method described by Hunskaar and Hole (1987) with some modifications done by Gomes et al. (2007). Animals received the injection of 20 μl of formalin (2.5% v/v) into the dorsal surface of the left hind paw. Immediately, the time that the animal spent licking the injected paw was recorded. The nociceptive and inflammatory responses develop two phases: the first 5 min after formalin injection (first phase, neurogenic pain response) and 15–30 min after formalin injection (second phase, inflammatory pain response). The animals were pre-treated with oral doses of vehicle, ASA, morphine, O. speciosa fractions or apigenin, 60 min before administration of formalin.

Central antinociceptive effects

Hot-plate test

In the central antinociceptive test mice were tested according to the method described by Sahley and Berntson (1979) and adapted by Pinheiro et al. (2010). Animals were placed on a hot plate (Insight equipments, Brazil) set at 55 ± 1 °C. The reaction time for licking (fore- and hind- paws) and jumping was recorded at several intervals of 30 min after oral administration of vehicle, morphine, O. speciosa fractions or apigenin. Baseline was designated as the average of reaction time obtained at 60 and 30 min before administration of vehicle, morphine, O. speciosa fractions or apigenin and defined as normal reaction of animal to the temperature. Increase in baseline (%) was calculated by the formula: ([(reaction time×100)/baseline] − 100. The following formula based on the trapezoid rule (Matthews et al., 1990) was used to calculate the AUC: $AUC = 30 \times IB \left[ (\text{min}30) + (\text{min} 60) + \ldots + (\text{min} 180)/2 \right]$, where IB is the increase in baseline (in %).

Evaluation of the mechanism of action of the O. speciosa fractions and apigenin

To assess whether the opioid, nitric oxide or cholinergic systems are involved in the antinociceptive action of the dichloromethane fraction and apigenin from O. speciosa leaves, mice were pre-treated with atropine (1 mg/kg, s.c.), naloxone (1 mg/kg, s.c.), L-nitro arginine methyl ester (L-NAME, 3 mg/kg, s.c.) or mecamylamine (2 mg/kg, s.c.) 30 min before receiving ethanol, dichloromethane fraction (30 mg/kg, p.o.), or apigenin (1 mg/kg, p.o.). The doses of antagonists and NO synthase inhibitor were selected based on previous experiments from our laboratory (Pinheiro et al., 2010). Antinociception was quantified as either the increase in baseline (%) calculated by the formula (reaction time×100/baseline)−100, or the AUC of responses from 30 to 180 min after drug administration.

Acute toxicity

These parameters were determined as previously described by Lorke, 1983). A single oral dose of ethanol or dichloromethane fractions (500 mg/kg) was administered to a group of ten mice (five males and five females). Behaviour parameters including convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration, and food and water intake were observed over a period of 5 days. After this period animals were killed by cervical dislocation, stomachs were removed, an incision along the greater curvature was made, and the number of ulcers (single or multiple erosion, ulcer or perforation) and hyperaemia were counted.

Statistical analysis

All experimental groups were composed by 6–10 mice. The results are presented as mean ± S.D. The area under the curve (AUC) used was calculated by Prism Software 5.0 (GraphPad Software). Statistical significance between groups was performed by the application of analyses of variance ANOVA followed by Bonferroni’s test. $p$ values less than 0.05 ($p < 0.05$) were used as the significant level.

Results

Isolation and characterization of Apigenin

The phytochemical study was performed with the dichloromethane fraction from O. speciosa leaves. After several chromatographic procedures, a compound was isolated from the very complex and mixed crude dichloromethane fraction and identified by NMR analysis as the flavonoid 5,7,4-trihydroxyflavone, known as apigenin (Fig. 1). This was confirmed by means of TLC and HPLC analysis (Figs. 2 and 3).
Peripheral antinociceptive effect from ethanol and dichloromethane fractions

Injection of acetic acid into the peritoneal cavity of mice develops 51.5 ± 6.9 writhing in an interval of 20 min. The pre-treatment of animals with different doses of ethanol and dichloromethane fractions (10 to 100 mg/kg) resulted in a significant inhibition on writhing, indicating a peripheral antinociceptive effect (Fig. 4). The highest antinociceptive effect from both fractions was observed with the dose of 10 mg/kg (48.7 and 66.8% reduction; 51.5 ± 6.9 in control group versus 26.4 ± 2.2 and 16.6 ± 5.8 for ethanol and dichloromethane fractions, respectively). Despite this result, there was no significant difference between the three doses tested (Fig. 4A).

Fig. 4B shows the inhibitory effect of ethanol and dichloromethane fractions in the model of formalin-induced licking response. Intraplantar injection of formalin resulted in a two-phase nociceptive behaviour in mice. In the first phase (neurogenic phase), the response was immediate and lasted up to 5 min following formalin injection resulting in the licking time of the 47.8 ± 12.1 s whereas the second phase (inflammatory phase) began at 15 min and lasted up to 30 min and resulted in 136.1 ± 13.3 s. The oral administration of ethanol fraction before formalin stimulus decreased the licking behaviour in both phases. Dichloromethane fraction was also able to significantly reduce both phases. The dose of 100 mg/kg inhibited 78% (47.8 ± 12.1 s in control group versus 10.5 ± 4.1 in dichloromethane-treated group) and 62% (136.1 ± 13.2 s in control group versus 51.6 ± 8.1 s in dichloromethane-treated group) the licking response in the first and second phase, respectively (Fig. 4B). It is interesting to note that both fractions had an inhibitory effect similar to that obtained with acetylsalicylic acid (100 mg/kg).

Central antinociceptive effect from ethanol and dichloromethane fractions

The pre-treatment of animals with the ethanol extract and dichloromethane fraction also resulted in a central antinociceptive effect. Both fractions increased the time of response from animals in the hot plate model. All three doses significantly increased the baseline (when comparing with vehicle-treated group). It is also interesting to note that the doses of 30 and 100 mg/kg from both fractions demonstrated an anti-hyperalgesic effect higher than morphine-treated animals (Fig. 5).

Apigenin isolated from dichloromethane fraction develops a peripheral and central antinociceptive effect

The apigenin isolated from the dichloromethane fraction was evaluated for its antinociceptive potential. Thus, a dose 1 mg/kg was used based on the amount in the dichloromethane fraction. The pre-treatment of mice with apigenin resulted in a significant reduction in the writhing induced by i.p. injection of acetic acid. This effect was similar to that observed with ASA or morphine-treated animals (Fig. 6A). Apigenin also reduced the licking response in the first and second phase of formalin model. In the 1st phase the effect was similar to morphine and in the 2nd phase the effect was similar to that of ASA.

Evaluation of the mechanism of action of dichloromethane fraction and apigenin

The anti-hyperalgesic effect observed in the hot plate model is indicative that fractions and apigenin has central antinociceptive activity. The next step was then to evaluate the antinociceptive
mechanism of action. In this regard, mice were divided in several groups and treated with different antagonists 30 min before oral administration of dichloromethane fraction (30 mg/kg) or apigenin (1 mg/kg). The results demonstrate that the anti-hyperalgesic effect of dichloromethane fraction observed in the hot plate model was reversed by the pre-treatment of mice with atropine (1 mg/kg, s.c.), naloxone (1 mg/kg, s.c.), mecamylamine (2 mg/kg, s.c.) or L-NAME (3 mg/kg). The same antagonists were used to evaluate the mechanism of action from apigenin. Our results demonstrate that only atropine and naloxone significantly reduced the antinociceptive effect from apigenin (Fig. 7).

Acute toxicity

To evaluate possible toxic effects on mice, 500 mg/kg of ethanol extract, dichloromethane fraction, or apigenin were orally administered. After 5 days of a single administration, treated mice did not present behavioural changes and no lesions or bleedings in stomachs were observed. Any signals of intoxication such as convulsion, death, gastric ulcer was present during the days of observation. These results indicate that the treatment with ethanol extract, dichloromethane fraction or apigenin was nontoxic in animals up to the oral dose of 500 mg/kg.

Discussion

In this study, we have demonstrated that ethanol extract and dichloromethane fraction from O. speciosa leaves produce antinociceptive effect when tested on peripheral and central models. The intraperitoneal injection of acetic acid induced a pain-related behaviour of writhing. This algic response involves the intraperitoneal liberation of several mediators, such as neurotransmitters and neuromodulators, kinins, histamine, acetylcholine, substance P, and prostaglandins (Deraedt et al., 1980; Cervero and Laird, 1999). These mediators increase vascular permeability, reduce the threshold of the nociception and stimulate the nervous terminal of nociceptive fibers. The effect obtained with ethanol extract and dichloromethane fraction may be related with the reduction on the liberation of those inflammatory mediators into the peritoneal cavity or by direct blockage of its receptors resulting in an antinociceptive effect. Also their activity could be due to an enhancement on the nociceptive threshold or by the interruption of the stimulus propagation in the pain nervous fibre.

The injection of formalin induced a pain-related licking response of the injected paw in two distinct phases. The first phase is characterized by neurogenic pain caused by direct chemical stimulation of nociceptors. The second phase is characterized by inflammatory pain triggered by a combination of stimuli — inflammation of the peripheral tissues and mechanisms of central sensitization. In this latter phase, different mediators are involved, as excitatory amino acids, neuropeptides, nitric oxide, PGE2, and kinins (Tjolen et al., 1992). Various studies have reported that both phases are sensitive to central action drugs, as opioids. However, the second phase is also sensitive to nonsteroidal anti-inflammatory analgesic and corticosteroids (Hunskaar and Hole, 1987). Our results showed that ethanol and dichloromethane fractions decreased both phases of the licking response induced by formalin. These results indicate that these fractions could be acting via inhibition of any inflammatory mediator liberated in mice paw. They could inhibit the formation and/or liberation of those mediators or directly blocking it receptor. These results corroborate with the inhibitory effect of O. speciosa leaves on the acetic acid-induced writhing response. Although we observed an inhibitory effect in both models, we did not get a dose response curve. This is probably due to synergism/antagonism between several substances presented in the ethanol extract or in the dichloromethane fraction. Due to the observation that the fractions were able to inhibit the first phase of the formalin response (neurogenic phase), we decided to test the fractions in a model of central antinociceptive activity,
the hot plate test. In this model ethanol extract and dichloromethane fraction developed an antinociceptive effect greater than morphine (at doses of 30 and 100 mg/kg).

To test the hypothesis that the antinociceptive effect of dichloromethane fraction could be due to the presence of apigenin, the flavonoid isolated in the fraction, we used the same models mentioned above. We observed that the apigenin treatment with the doses of 1 or 3 mg/kg induced an inhibitory effect in acetic acid-induced contractions and formalin-induced licking (1st and 2nd phases), indicating a peripheral antinociceptive activity. It was interesting to note that apigenin effect was similar to or greater than the drugs used as positive control groups, morphine and ASA. Apigenin also demonstrated a central antinociceptive effect, increasing the area under the curve in the hot plate model. Some studies have reported the anti-cancer, anti-oxidant, anti-inflammatory, and neuroprotective properties of the flavonoid apigenin (Kowalski et al., 2005; Lee et al., 2007; Nicholas et al., 2007; Ha Keun et al., 2008; Zhong et al., 2010), but to the best of our knowledge this is the first study that demonstrated the antinociceptive activity of apigenin.

In order to determine the mechanism involved in the central antinociception caused by dichloromethane fraction and apigenin classical antagonists of cholinergic and opioid receptors (atropine, mecamylamine, and naloxone, respectively) and an inhibitor of nitric oxide pathway (L-NAME), was used. The results indicate that there is an involvement of cholinergic receptors since atropine inhibited the antinociceptive effect of dichloromethane fraction and apigenin. A major site of action for cholinomimetics in analgesia is the spinal cord. Painful stimuli are known to increase acetylcholine in the spinal cord. The activation of muscarinic receptors in the spinal cord results in an increased release of inhibitory transmitters along with a decrease in the release of excitatory transmitters, and this in part mediates their antinociceptive effects (Jones and Dunlop, 2007). Based on these evidences, it is possible that some substances in dichloromethane fraction as well as apigenin activate cholinergic receptors resulting in an antinociceptive effect. The dichloromethane fraction contains apigenin and other compounds. This explains why the treatment with all four antagonists has reversed the antinociceptive effect from dichloromethane fraction and only two of them have inhibited the antinociceptive effect from apigenin.

Our results also demonstrated that dichloromethane fraction and apigenin activities seem to involve the participation of opioid system. Both dichloromethane fraction and apigenin effects were reversed by naloxone, similarly to the compounds morphine-like. Together, these data are consistent with the interpretation that systemically administered morphine exerts its analgesic effects by interacting with both central and peripheral opioid receptors (Perrot et al., 2001; Shannon and Lutz, 2002).
Conclusion

The present study demonstrated that the fractionation of the *O. speciosa* Mart. leaves resulted in the isolation and identification of the flavonoid apigenin in the dichloromethane fraction from leaves. The evidence supports our hypothesis suggesting that apigenin may contribute to the antinociceptive effect observed and that the opioid and cholinergic systems are involved in this effect.

Conflict of interest statement

I declare any competing interests.

Acknowledgements

We are in debt to Mr. Mark English for the English revision of the manuscript. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant support and fellowship to PDF), Fundação Carlos Chagas Filho de Apoio à Pesquisa do Estado do Rio de Janeiro (FAPERJ, grant support to PDF), and Conselho de Administração de Pessoal de Ensino Superior (CAPES, fellowship to MMGP).

References


