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Análise anti-inflamatória do óleo essencial obtido de Piper amalago (Piperaceae)

Anti-inflammatory analysis of Essential Oils Obtained from Piper amalago (Piperaceae)

Aline Lima de Barros[1], Renan Donomae Iwamoto[1], Claudia Andrea Lima Cardoso[2], Giuliana Thomas Vitorino[2], Jonas da Silva Mota[2], Cayo Vinícius Fernandes[2], Ubirajara Lanza Junior[1], Arielle Cristina Arena[3], Márcia Midori Shinzato[1], Candida Aparecida Leite Kassuya[1*].

[1]Federal University of Grande Dourados, College of Health Science, Dourados, Mato Grosso do Sul, Brazil
[2]Centro de Pesquisa em Biodiversidade, Universidade Estadual de Mato Grosso do Sul, MS, Brazil
[3]Universidade Estadual Paulista Júlio de Mesquita Filho, Instituto de Biociências, Brazil

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*Autor correspondente: Candida Aparecida Leite Kassuya. Instituição – UFGD. E-mail: candida2005@gmail.com

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Key-words: Piper amalago; rats; anti-inflammatory; essential oil.

Resumo

Piper amalago é uma plantas utilizada na medicina popular brasileira como agente anti-inflamatório e analgésico. Como são escassos os dados que comprovem estes efeitos, objetivou-se no presente estudo avaliar a atividade anti-inflamatório e análise fitoquímica do óleo essencial extraído de *P. amalago*. O óleo essencial de folhas, flores, raízes e caules de *P. amalago* (EOPA) foi extraído hidrodestilação, e a análise fitoquímica foi realizada por GC/MS. Os principais compostos identificados no EOPA foram allo-ocimeno (19,29%), carotol (11,44%), (Z)-β-ocimeno (7,67%), trans-vertocitral C (7,19%), álcool trans-Arteannuic (4,82%), cadineno γ-(3,95%) e (E)-α-ionona (3,86%). A administração oral do EOPA (30-300mg/kg) e a injeção subcutânea de dexametasona inibiu significativamente o edema da pata induzido por carragenina (dose de 300mg/kg) e pleurisia (dose de 100 e 300mg/kg) induzida em ratos. Este trabalho descreve pela primeira vez os efeitos anti-inflamatórios do óleo essencial de *P. amalago* e sua composição e contribuiu, ao menos em parte, para validar os usos etnofarmacológicos de *P. amalago* como agente anti-inflamatório natural. Considerando que os medicamentos atualmente disponíveis para o tratamento de condições inflamatórias mostram efeitos colaterais indesejáveis, os presentes resultados podem ter relevância clínica e abrem novas possibilidades para o desenvolvimento de novas drogas anti-inflamatórias.

Abstract

Piper amalago is a plant used in Brazilian folk medicine as anti-inflammatory and analgesic agent. As there are few data showing the effect aimed in the present study was to evaluate the anti-inflammatory activity and phytochemical analysis of essential oil extracted from *P. amalago*. The essential oil from the leaves, flowers, roots and stems of *P. amalago* (EOPA) was extracted by hydrodistillation, and the phytochemical analysis was performed by GC/MS. The major compounds identified in the EOPA were allo-ocimene (19.29%), carotol (11.44%), (Z)-β-ocimene (7.67%), trans-vertocitral C (7.19%), trans-Arteannuic alcohol (4.82%), γ- cadinene (3.95%) and (E)-α-Ionone (3.86%). Oral administration of the EOPA (30-300mg/kg) and subcutaneous injection of dexamethasone significantly inhibited carrageenan-induced paw edema (dose of 300mg/kg) and pleurisy-induced (dose of 100 and 300mg/kg) in rats. This work describes for the first time the anti-inflammatory effects of the essential oil of *Piper amalago* and its composition and contributed, at leat in part, to validate the ethnopharmacological uses of *P. amalago* as anti-inflammatory natural agent. Considering that drugs currently available for the treatment of inflammatory conditions show undesirable side effects, the present results may have clinical relevance and open new possibilities for the development of novel anti-inflammatory drugs.

1. Introduction

The inflammatory process occurs in response to an injury and could be initiated by physical factors (burning), biological (microorganism) or chemical (caustic) (Maldini et al., 2009). During inflammation, homeostasis is affected, which in turns, stimulate the proinflammatory mediators activation and release such as tumor necrosis factor (TNF), interleukin-6 (IL-6), eicosanoids and nitric oxide (NO), (Haddad, 2003). There is a close relationship between activation of inflammatory mediators and intracellular redox homeostasis (Baldwin, 2001).

The inflammatory process could be classified in relation to time in acute or chronic. Acute inflammation is characterized by fever, pain, redness, edema and the inflammatory process is resolved and finished. Chronic inflammation process is characterized by cellular proliferation (Maroon et al., 2010) that is able to develop several inflammatory diseases such as rheumatoid arthritis, Alzheimer's disease and atherosclerosis. However, an excessive inflammatory response can provide adverse effects in healing process, since inflammatory cells can produce tissue damage (de Melo et al., 2012) and a stronger inflammatory response can lead to chronification (Bogdan, 2001).

The conventional treatment (using steroidal and non- steroidal anti-inflammatory drugs) against the inflammatory response has several adverse side-effects when were used for a long time or in people that have sensibility to adverse effects (Batlouni, 2010). The use of natural products is becoming an alternative and complementary treatment of inflammatory diseases.

In Brazil, especially in Mato Grosso do Sul State, there are several plants that are used in traditional medicine as anti-inflammatory such as *Piper amalago* (Achenbach et al., 1986, Parmar et al., 1997). *P. amalago* commonly known as "jaborandi" in Brazil, distributed from Mexico to Brazil, is used to alleviate chest pain and inflammation (Domínguez and Alcorn 1985; Achenbach et al., 1986; Parmar et al., 1997). The tea of leaves of *P. amalago* is also used against burns (Alves et al., 2008).

The essential oil of some *Piper* species has a fungicide, acaricide, molluscicide bactericide and larvicide properties (Fazolin et al., 2005). Scientific research showed that *P. amalago* specie act on the central nervous system, showing anxiety properties in rats (evaluated in the elevated plus-maze) and with locomotion and exploration effects in an open field, without induce genetic toxicity, in the comet assay or the micronucleus assay (Lopes et al., 2012). Phytochemical analysis showed that, from the genus Piper, has been isolated lignans, flavonoids, lactones, alkaloids (pyrrolidines and piperidines), butenolides, and cyclohexane epoxide (Calle-Alvarez, 1983; Sengupta e Ray, 1987).

Considering that drugs currently available for the treatment of inflammation conditions show undesirable side effects our study focused in evaluation the anti-inflammatory activities of essential oil obtained from *P. amalago* in rats.

2. Material and Methods

2.1. Plant material

P. amalago leaves were colected in Dourados – MS, in June 2012, and identified by Prof. Dra. Elsie Franklin Guimarães. The voucher specimens, *P. amalago* (DDMS 4410), was deposited in the herbarium of the Universidade Federal da Grande Dourados, MS, Brazil.

2.2. Preparation and analysis of essential oil of *P. amalago*

The oil was obtained (EOPA) from 1800 g of fresh parts (leaves, flowers, roots and stems) by separate hydrodistillation using a Clevenger-type apparatus for 4 h. The oil percentage was expressed as w/w respect to fresh weight of the initial material.

The analysis was performed by capillary GC-MS. The GC-MS analyses were performed on a gas chromatograph (GC-17A, Shimadzu, Kyoto, Japan) equipped with mass spectrometer detector (QP 5050a), using DB-5 (J & W, 5% de phenyl-dimethylpolysiloxane), fused-silica capillary column (30 in length x 0,25 mm i.d., 0.25µm film thickness), under the following conditions: carrier gas helium (99,999% and flow rate 1.0mL/min); 1µL injection volume, split ratio (1:20), with initial oven temperature of 50ºC and heating from 50º to 250ºC at 3ºC/min. The injector temperature and quadrupole detector temperature were 250ºC and line transfer 250ºC. The MS scan parameters included electron impact ionization voltage at 70 eV, a mass range of 40 to 500m/z and a scan interval of 0.5s.

Temperature-programmed retention indices (Isidorov et al., 1998, Zhao et al., 2005) were calculated using a mixture of normal paraffin (C8-C28) as the external references. The identifications were completed by comparing the spectra of the masses obtained with those of NIST 2.0, Saturn Libraries and literature data (Adams et al., 2005). The determination of the relative area by GC/FID analyses were performed using a gas chromatograph GC-17A (Shimadzu) equipped with the same DB-5 fused silica capillary column and under the same conditions as the GC/MS.

2.2.3. Material and reagents

Carrageenan was obtained from Sigma while dexamethasone (Decadronal®) was adquired from Prodome Laboratories (Campinas, Brazil).

2.3. Animals

The experiments were conducted using male and female Wistar rats provided by Universidade Federal da Grande Dourados. The animals were maintained under a 12h light-dark cycle, with controlled humidity (60-80%), and temperature (22 ± 1ºC). The animals were allowed free access to tap water and standard laboratory rat food. The care and handling of rats were in accordance with the internationally accepted standard guidelines for use of animals. The number of animals was the minimum necessary to show consistent effects of the drug treatments.

2.3.1. Carrageenan-induced paw-oedema in rats

Male rats were divided into 5 groups of 5 animals each. The groups were orally treated with the EOPA (30, 100 and 300 mg/kg) or vehicle (control group). Another group of rats was treated subcutaneously with the anti-inflammatory

dexamethasone (1 mg/kg). After 1 h, the animals received a 50 µl subcutaneous injection of carrageenan (Cg)(300 µg/paw) dissolved in sterile 0.9% saline into the right hindpaw. The contralateral paw received only saline and was used as the control. The thickness of the paw edema was measured using a digital micrometer 1 h before any treatment and at several time points (1, 2, and 4 h) after the injection of Cg. The results were expressed in µm, and the differences between basal and post-injection values were quantified as edema.

2.3.2. Pleural cell migration and protein exudation

Pleurisy was induced in animals by an intrapleural injection of carrageenan (1%) or a sterile saline solution (0.9% NaCl) into the right pleural space through the chest skin (final volume 0.1 ml). The female rats were pretreated by oral route (p.o.) with the essential oil of *P. amalago* at doses of (100 and 300mg/kg) or vehicle, 1 h prior to the induction of the edema or dexamethasone (1.0 mg/kg, subcutaneously, positive control), and naïve (0.9%-negative control) were administered orally by gavage, in different groups of rats. The carrageenan was diluted in saline buffered. Briefly, an adapted needle was inserted into the right side of the thoracic cavity of the animals to enable intrathoracic (i.t.) administration of carrageenan. Control rats received an equal volume (100 µL) of sterile, pyrogen free saline. The animals were sacrificed 4h after the carrageenan injection. Immediately, the thorax was opened, the pleural cavity was washed with 1ml of phosphate-buffered saline (PBS) and the exudate was collected. Instead of carrageenan, control animals received sterile saline solution (0.1ml) in the pleural cavity. The total leukocyte number in the pleural exudate was counted in a Neubauer chamber, after dilution in Turk solution (1:20). Slides of the cellular exudate were also prepared, dried, fixed, and stained with May-Grunwald-Giemsa. The number of mononuclear and polymorphonuclear leukocytes in the exudate was determined with the aid of a light microscope.

2.4 Statistical analysis

The results are expressed as mean ± S.E.M. of experiments. Statistical significance was determined through one-way analysis of variances (ANOVA), followed by either Newman–Keuls test or by Student's t-test. A P value less than 0.05 was considered statistically significant. Graphs were drawn and statistical analysis was carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

By hydrodistillation, the fresh products of P. amalago produced 0.20 % (w/w) of essential oil. The components of the studied essential oil are shown in Table 1. The different constituents were identified and quantified by GC-MS procedures, and thirty-eight compounds representing 92.85% of the oil were identified. The analysis of triplicates showed a coefficient of variation of less than 3% of retention times.

The main constituents found of the EOPA were allo-ocimene (19.29%), carotol (11.44%), (Z)-β-ocimene (7.67%), trans-vertocitral C (7.19%), trans-Arteannuic alcohol (4.82%), γ- cadinene (3.95%) and (E)-α-Ionone (3.86%).

Table 1 - Percentage composition of essential oils of P. amalago

Compounds	I_{Cal}	I_{lit}	Essential oil
β-citronellene	950	950	0,43
(Z)- β-ocimene	1036	1037	7,67
(E)-β-Ocimene	1050	1050	0,30
p-Mentha-3,8-diene	1072	1073	0,27
p-cresol	1076	1076	0,53
p-Mentha-2,4(8)-diene	1087	1088	2,20
trans-Vertocitral C	1106	1106	7,19
myrcenol	1123	1123	0,34
allo-Ocimene	1132	1132	19,29
neo-allo-)Ocimene	1145	1144	0,53
Pentyl-Benzene	1157	1157	0,11
iso-Menthol	1183	1183	0,48
(E)-α-Ionone	1430	1430	3,86
β-acoradiene	1470	1471	0,16
γ-Himachalene	1483	1483	1,25
γ- cadinene	1513	1514	3,95
Silphiperfol-5-en-3-ol B	1534	1535	0,32
Elemol	1550	1550	0,62
cis-Cadinene ether	1554	1554	0,27
Longicamphenylone	1564	1564	1,75
β- acoradiene	1570	1571	0,45
Silphiperfol-5-en-3-one A	1576	1575	1,52
thujopsan-2- β –ol	1586	1587	0,48
carotol	1594	1595	11,44
guaiol	1602	1601	1,71
β -Oplopenone	1608	1608	1,11
trans-Arteannuic alcohol	1614	1613	4,82
1,10-di-epi-cubenol	1619	1619	0,65
γ- eudesmol	1632	1632	0,23
Geranyl valerate	1658	1657	1,44
Bulnesol	1672	1672	1,79
Khusinol	1679	1680	0,57
Eudesma-4(15),7-dien-1-beta-ol(impure)	1688	1688	0,20
crysolide	1722	1723	0,57
(E)- Nuciferol	1726	1726	0,17
(Z)-Ligustilide	1737	1736	0,96
Khusimol	1739	1740	1,14
xanthorrhizol	1753	1753	3,04
Total			92%

[a]Compounds listed in order of elution from a DB-5 column; Ical = Programmed temperature retention indices determined on apolar DB-5 column (50-250 °C; 3 °C/min). Ilit=Retention indices literature (Admas, 2001).

Oral treatment with EOPA (30-300mg/kg) significantly inhibited the formation of paw edema induced by Cg. Figure 1 shows that EOPA at a dose of 300 mg/kg significantly reduced edema formation in 1, 2 and 4 h after injection of Cg and inhibitions at were 50 ± 10%, 60 ± 8%,

57 ± 10%. Group that received 100 mg/kg dose showed a significant reduction of edema only 2 h after injection of Cg, with inhibition of 55 ± 9%. However, there was no statistically significant inhibition of paw edema for group that received EOPA 30 mg/kg, compared to the control group. The positive control also induced significant reduction when compared to control group (not shown).

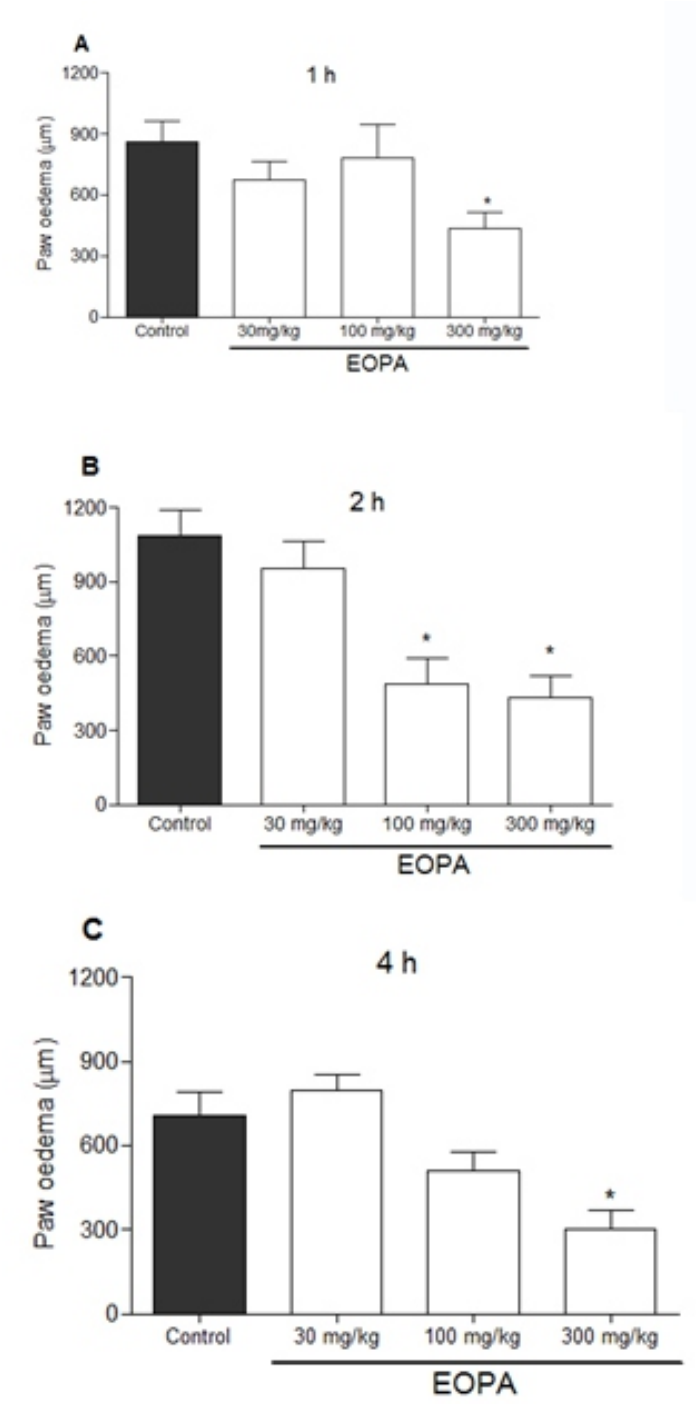

Figure 1 - Effect of essential oil of P. amalago (EOPA) in paw edema induced by carrageenan in rats. 1h after injection of intraplantar carrageenan, animals were treated orally EOPA (30, 100 and 300 mg/kg) and vehicle. In panel A, the paw edema was showed after 1 h of carrageenan treatment; In B, the paw edema was showed after 2 h of treatment; In C, the paw edema was showed after 4 h of treatment. Each bar or point represents mean ± S.E.M of 5 animals. * P <0.05, ** P <0.01 compared with vehicle-treated group.

As shown in Figure 2, EOPA significantly inhibited leukocyte migration into the pleural cavity in doses of 100 and 300mg/kg, as well as in group treated with dexamethasone. After 4 h injection of Cg, it was observed inhibition of 71 ± 2% at dose of 100 mg/kg and 79 ± 3% at dose of 300 mg/kg and 90 ± 1% for dexamethasone.

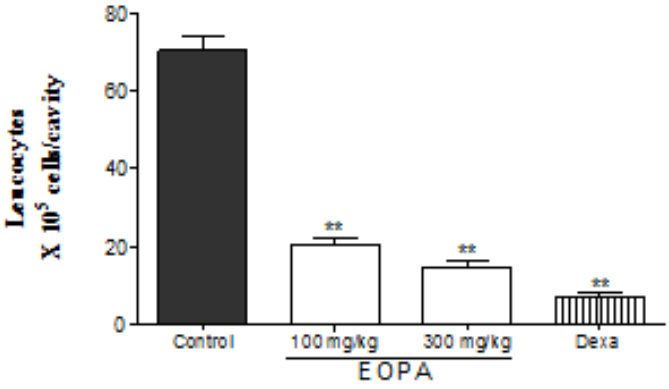

Figure 2 - Effect of essential oil of P. amalago (EOPA) on the carrageenan-induced pleurisy in rats. Animals received EOPA (100 and 300 mg / kg) and vehicle orally or dexamethasone 1 mg/kg (dexa). After 1h, carrageenan (Cg) (100 mL of a 1% solution / cavity) was injected into the pleural cavity of each animal. Animals were sacrificed 4 hours after Cg injection and the leukocyte migration was analysed. Each bar represents mean ± S.E.M. of 5 animals. Asterisks indicate significance levels: * P<0.05, ** P<0.01 compared with vehicle-treated group.

4. Discussion

In the study was evaluated the potential anti-inflammatory effects the EOPA and performed the identification of their chemical components.

Regarding secondary metabolites previous studies with leaves have shown that α-pinene (30.50%), camphene (8.90%), limonene (6.80%), borneol (5.70%), δ-cadinene (4.70%) and spathulenol (4.20%) were the major components. In essential oil of leaves P. amalago caryophyllene oxide (18.0%), E-caryophyllene (17.80%), bicyclogermacrene (16.40%), α-pinene (9.30%), and germacrene D (10.90%) were the major components. Biclyclogermacrene (27.91%), spathulenol (19.22%) germacrene D (9.94%), and γ-muurolene (7.27%) were the major components in oil of P. amalago and oil showed activity against Candida albicans, C. parapsilosis, C. krusei and Cryptococcus neoformans (Morandim-Giannetti, et al., 2010). Another study showed as major compounds β-copaen-4-α-ol (26.00%), 7-epi-α-eudesmol (21.84%), epi-α-cadinol (12.70%), and n-hexyl-benzoate (12.29%) and this oil showed antifungal activity against nine Candida strains.

In the present work, contrasting with those previous works, the essential oil of P. amalago collected in Mato Grosso do Sul State showed the allo-ocimene (19.29%), as major constituent. For the major component there are no reports in the literature of anti-inflammatory activity, the antifungal activity was reported to carotol (Jasicka-Misiak, et al., 2004).

The oral treatment with EOPA significantly reduced paw edema and leukocyte migration into the pleural cavity on rats, both induced by carrageenan injection. These two models are commonly used in experimental acute inflammation to evaluate the anti-inflammatory activity of drugs (Maldini et al., 2009). Carrageenan (Cg) is a sulfated polysaccharide that stimulate acute inflammation by releasing biochemical mediators involved in this stage of vascular inflammation (Di Rosa et al., 1971). The administration of Cg in the pleural cavity induces pleurisy that is an inflammation process characterized by immediately recruitment of polymorphonuclear cells (PMN) (Almeida et al., 1980, Higgs et al., 1980). The other experimental model used was Cg induced paw edema and it is believed that the formation of edema induced by this inflammatory agent has

two phases (Vinegar et al., 1969). First phase is mediated by the release of histamine and serotonin and the second phase is involved in neutrophils infiltration, eicosanoids releasing, free radicals production and others neutrophil derived mediators (Cuzzocrea et al., 1998).

The existing reports about the anti-inflammatory activity are just for other species of the genus Piper. The Peperomia pellucida showed dose dependent anti-inflammatory activity on paw edema induced by Cg. Edema was inhibited in first hour and during all phases of inflammation (de Fatima Arrigoni-Blank et al., 2004). Anti-inflammatory activity of P. ovatum Vahl (Piperaceae) was evaluated in rats through the ear edema induced by croton oil and pleurisy induced by Cg and it was found that the mixture of amides showed a good topical anti-inflammatory activity, but no activity was detected by pleurisy model. There are others species from Piperaceae family that showed anti-inflammatory activity like P. pellucida (de Fatima Arrigoni-Blank et al., 2004), Piper umbellatta (Perazzo et al., 2005) and P. lenticellosum (de las Heras et al., 1998).

The results of this study demonstrate that the EOPA at tested doses has anti-inflammatory properties. However, studies of anti-inflammatory activity shall be performed on compounds isolated in order to identify the component responsible for the anti-inflammatory action.

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