Further Antinociceptive Properties of Extracts and Phenolic Compounds from *Plinia glomerata* (Myrtaceae) Leaves

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This study describes the antinociceptive activity of extracts (methanolic (ME) and acetonic (AE)) and two phenolic compounds, 3,4,3'-trimethoxyflavellagic acid (1) and 3,4,3'-trimethoxy flavellagic acid 4'-O-glucoside (2), from *Plinia glomerata* leaves, against different experimental models of pain in mice. When evaluated against writhing test, by i.p. route, ME and AE presented calculated ID₅₀ values (and respective confidence interval) of 3.28 (1.63—6.61) and 24.79 (16.57—37.09) mg/kg, respectively. Given by the oral route at 500 mg/kg, AE and ME extracts inhibited the abdominal constrictions by 60.5% and 35.3%, respectively. In the formalin test (10 mg/kg, i.p.), AE inhibited both phases of pain (45.6% in the first phase; 99.8% in the second phase) whereas ME inhibited 47.8% the first phase, and 92.6% the second phase. In the capsaicin test both extracts showed activity, with calculated ID₅₀ values of 6.56 (5.69—7.56) and 7.68 (4.94—11.93) mg/kg for AE and ME, respectively. When evaluated against the hot-plate test, both extracts demonstrated activity, but only in high doses. Compound 2, when evaluated against the formalin test (10 mg/kg, i.p.), inhibited both phases of pain (77.6%, first phase; 62%, second phase) whereas 1 inhibited only the first phase, with inhibition of 70%. When tested in the capsaicin and glutamate tests, at 10 mg/kg, i.p., 1 and 2 caused inhibitions of 41.5% and 37.9%, and 37.7% and 54.5%, respectively. These results confirm previous studies carried out by our research group regarding the antinociceptive properties of *P. glomerata*, stimulating other studies on mechanism of action as well as the determination of additional active principles in this plant.

Key words *Plinia glomerata*; antinociception; mouse; phenolic compound

*Plinia glomerata*, popularly known as “cabeluinha” or “yellow jaboticaba,” is used as an ornamental tree, and its edible fruits are also appreciated for their flavor. This species has some synonymous names, such as *Myrciaria glomerata* Bergr. *Eugenia cabelluda* Klaersk and *Eugenia tomentosa* Berg.

Several experimental studies have suggested different pharmacological properties for various species belonging to the family Myrtaceae, particularly their analgesic action.1,2) Recently, we have demonstrated in preliminary assays, that some extracts, fractions and flavellagic acid derivatives from *P. glomerata* leaves, against different experimental models of pain in mice. When evaluated against writhing test, by i.p. route, ME and AE presented calculated ID₅₀ values (and respective confidence interval) of 3.28 (1.63—6.61) and 24.79 (16.57—37.09) mg/kg, respectively. Given by the oral route at 500 mg/kg, AE and ME extracts inhibited the abdominal constrictions by 60.5% and 35.3%, respectively. In the formalin test (10 mg/kg, i.p.), AE inhibited both phases of pain (45.6% in the first phase; 99.8% in the second phase) whereas ME inhibited 47.8% the first phase, and 92.6% the second phase. In the capsaicin test both extracts showed activity, with calculated ID₅₀ values of 6.56 (5.69—7.56) and 7.68 (4.94—11.93) mg/kg for AE and ME, respectively. When evaluated against the hot-plate test, both extracts demonstrated activity, but only in high doses. Compound 2, when evaluated against the formalin test (10 mg/kg, i.p.), inhibited both phases of pain (77.6%, first phase; 62%, second phase) whereas 1 inhibited only the first phase, with inhibition of 70%. When tested in the capsaicin and glutamate tests, at 10 mg/kg, i.p., 1 and 2 caused inhibitions of 41.5% and 37.9%, and 37.7% and 54.5%, respectively. These results confirm previous studies carried out by our research group regarding the antinociceptive properties of *P. glomerata*, stimulating other studies on mechanism of action as well as the determination of additional active principles in this plant.

**MATERIAL AND METHODS**

**Plant Material** Extracts were obtained from the aerial parts of *P. glomerata*, cultivated at EPA GRI (Itajaí, Brazil). The plant was identified by Prof. Oscar B. Iza (UNIVALI). A voucher sample was deposited in the Barbosa Rodrigues Herbarium (Itajaí, Brazil) under number VC Filho 052.

**Preparation of Extracts and Isolation of Constituents** Dried aerial parts of *P. glomerata* (1.7 kg) were cut into small pieces and macerated in acetone for 10 d and then in methanol for 3 d. The solvent was concentrated under reduced pressure to give the respective extracts of acetone (110 g) and methanol (60 g). Part of the acetonic extract (30 g) was chromatographed using a silica gel column, furnishing the compounds 3,4,3'-trimethoxyflavellagic acid (1) (150 mg) and 3,4,3'-trimethoxy flavellagic acid 4'-O-glucoside (2) (139 mg), according to the method previously described.3) The molecular structure for both compounds are indicated in Fig. 1.

**Pharmacological Assays. Animals** Male swiss male mice (25—35 g) were obtained from Central Bioterio of the University of Vale do Itajaí (Itajaí, Brazil). They were kept in a temperature-controlled environment (23±2 °C) with a 12 h light–dark cycle. Food and water were freely available. The experiments were performed after gaining approval of the protocol by the Institutional Ethics Committee, 116/06 CEP/UNIVALI, and in accordance with the current guide-
lines for the care of laboratory animals, and the ethical guidelines for investigations of experimental pain in conscious animals. For each experiment, one group of animals was used. The numbers of animals (8—10 for group of treatment) and the intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. After the experiments, the animals were sacrificed by exposure to CO₂.

**Abdominal Constriction Response Caused by Injection of Acetic Acid** The abdominal constriction induced by intraperitoneal injection of acetic acid (0.6%), which consisted of contractions of the abdominal muscle, together with stretching of hind limbs, was carried out according to previously described procedures. The animals were pretreated with the extracts and compounds from *P. glomerata* or standard drugs, administered intraperitoneally or orally, at different concentrations, based on previous studies, 30 and 60 min prior to acetic acid injection respectively. Extract ME was analyzed intraperitoneally at 3, 6, 10 mg/kg and orally at 100, 150, 300, 500 mg/kg. Extract AE was evaluated intraperitoneally at 10, 20, 30 mg/kg and orally at 100, 150, 300 mg/kg. The control animals received a similar volume of 0.9% NaCl solution (10 ml/kg). After the challenge, pairs of mice were placed in separate boxes, and the number of abdominal constrictions was cumulatively counted over a period of 20 min. Antinociception was expressed as the reduction of the number of abdominal constrictions, comparing the control animals and the mice pretreated with extracts or standard drugs.

**Formalin-Induced Pain** The procedure was similar to that described previously. The mice were observed for 30 min, following 20 μl of formalin (2.5% formaldehyde in saline solution) which was injected under the paw surface of the right hind paw. The amount of time spent licking the injected paw was considered as indicative of pain. The initial nociceptive scores normally peaked 5 min after formalin injection (early phase) and 15—30 min after formalin injection (late phase), representing the tonic and inflammatory pain responses, respectively. The animals were treated with ME and AE intraperitoneally at 5, 10, 20 mg/kg, and with compounds 1 and 2 intraperitoneally at 10 mg/kg, 30 min prior to formalin injection. Following intraplantar injection of formalin, the animals were immediately placed into a glass cylinder of 20 cm in diameter, and the time spent licking the injected paw was recorded.

**Capsaicin Test** In an attempt to provide more direct evidence concerning its possible antinociceptive effect on neurogenic nociception, the extracts and compounds 1 and 2 from *P. glomerata* were investigated in capsaicin-induced licking in the mouse paw. The experiments were performed essentially according to the method previously described. After the adaptation period, 20 μl of capsaicin (1.6 μg/paw) was injected intraplantarly into the right hindpaw. The animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. The animals were treated with ME, AE and compound 1, intraperitoneally, at 3, 6, 10 mg/kg and compound 2, intraperitoneally, at 10 mg/kg, 30 min before capsaicin injection, respectively. The control animals received a similar volume of vehicle.

**Glutamate Test** The animals were treated with ME, AE and compounds 1 and 2, intraperitoneally (i.p.), at 10 mg/kg, 30 min before glutamate injection. A volume of 20 μl of glutamate solution (30 μmol/paw), made up in phosphate buffered saline (PBS), was injected intraplantarly under the surface of the right hindpaw as described previously. After injection with glutamate, the animals were individually placed into glass cylinders 20 cm in diameter and observed from 0 to 15 min. The time spent licking and biting the injected paw was timed with a chronometer and considered as indicative of pain.

**Hot-Plate Test** The hot-plate test was used to estimate the latency of responses of thermal stimuli according to the method described by Eddy and Leimback with minor modifications. The temperature of the hot-plate was maintained at 56±1°C. The animals (n=10) were placed in glass funnels, on the heated surface and the time between placing the animals and the beginning of licking paws or jumping were recorded as latency of response in the non-treated animals (saline 10 ml/kg, i.p.), and those treated with extract ME and AE (10, 30, 60 mg/kg, i.p.) or morphine (5 mg/kg, s.c.). Mice with baseline latencies of more than 20 s were eliminated from the study, in a pre-test, and the cutoff time for the hot-plate latencies was set at 30 s.

**Statistical Analysis** The results are presented as mean±S.E. mean, except the mean ID₅₀ values (i.e., the dose of drugs or fractions reducing the algesic responses by 50% relative to control value) which are reported as geometric means accompanied by their respective 95% confidence limits. Data were analyzed by analysis of variance (ANOVA) or post-hoc test and complemented by Dunnett’s or Newman–Keul’s *post-hoc* test. *p*-values of less than 0.05 were considered as indicative of significance. Where possible, the ID₅₀ value was determined using at least three dosages of extract or compounds by linear regression from the individual experiments, using linear regression software (GraphPad software, San Diego, CA, U.S.A.). Maximal inhibition values were calculated at the most effective dose used.

**RESULTS AND DISCUSSION**

The first test used for pharmacological evaluation was the writhing test, administered by the intraperitoneal and oral routes. Past studies have postulated that the acetic acid acts indirectly by inducing the release of endogenous mediators which stimulate the nociceptive neurons sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. Although abdominal writhing induced by acetic acid represents a peripheral nociception model, and is normally used for screening synthetic and natural compounds, this is not a specific model, since several compounds, including opioid analogues, tricyclic antidepressants and anti-histaminic compounds inhibit acetic acid induced writhing. In this model, both the methanolic (ME) and aceticone (AE) extracts, showed a significant reduction in writhing test.

The results indicate that AE and ME produced dose-dependent inhibition of the acetic acid-induced abdominal constrictions in mice, with a calculated ID₅₀ value (and their respective 95% confidence limits) of 24.79 (16.57—37.09) and 3.28 (1.63—6.61) mg/kg and the maximal inhibitions observed were 64.5% and 70.3% for the doses of 30 and
10 mg/kg, respectively. When given by oral route, AE and ME also produced a significant inhibition (60.5% and 35.3%, respectively). The mean ID$_{50}$ value for AE was 208.71 (186.26—233.91) mg/kg. These important results suggest that both AE and ME extracts, but particularly AE, contain ingredients which may be absorbed by gastrointestinal tract.

When evaluated against the formalin test, both extracts were significantly active at 10 mg/kg, i.p. AE showed inhibition of 45.6±6.0% in the early phase and 99.8±0.3% in the late phase (Fig. 2), whereas ME caused inhibition of 47.8±2.5% in the early phase (0—5 min) and 92.6±6.6% in the later phase (15—30 min) (Fig. 3). The results show that AE and ME are effective in both phases of the test, but are more effective against the later phase of formalin test.

This test, which causes local tissue injury to the paw, has been used as a model for tonic pain and localized inflammatory pain.\textsuperscript{16} It has been demonstrated that intraplantar injection of formalin in rodents produces significant increases in spinal levels of different mediators, such as excitatory amino acids, PGE$_2$, nitric oxide and tachykinin, kinins among other peptides.\textsuperscript{17,18} Furthermore, systemic spinal and supraspinal administration of tachkinin receptor antagonists, nitric oxide synthase (NOS) inhibitors, N-methyl-D-aspartate (NMDA) receptor antagonists, opioids, $\alpha_2$ adrenoceptor agonist, and NSAIDS, were all found to be effective in antagonizing formalin-induced nociception.\textsuperscript{17,19,20} Intraplantary injection of formalin produces distinct biphasic pain, called early and late phases. Drugs that act primarily on the central nervous system inhibit both phases equally, while peripherally acting drugs inhibit the late phase.\textsuperscript{21} Fractions AE and ME significantly inhibited both phases of the formalin test, but their effect was more pronounced in the late phase of the formalin test, which suggests that peripheral mechanisms are involved, and both fractions and also may exhibit an associated anti-inflammatory effect, since the anti-inflammatory drugs exhibited effect in this phase.

Another important finding was in the capsaicin test, which acts in the same earlier phase of the formalin test, but using a different pathway, with different mediators.\textsuperscript{22} In this test, both extracts, given intraperitoneally, demonstrated to be effective, particularly the AE, which significantly reduced the amount of time spent licking the injected paw. The calculated ID$_{50}$ value was 6.56 (mg/kg), with maximum inhibition of 66.4% at 10 mg/kg (Fig. 4). ME showed a calculated ID$_{50}$ value of 7.68 mg/kg, with maximum inhibition of 54.4% at the same dose (Fig. 4). The results show that the capsaicin-induced neurogenic paw licking response was similar to that of the first phase of the formalin test, and compounds with this action are good candidates for the treatment of neuropathic conditions, for which effective treatment is difficult.\textsuperscript{23} Sakurada and co-workers\textsuperscript{8} proposed the capsaicin-induced pain model for the study of compounds, which act on pain of a neurogenic origin. Capsaicin is a neurotoxic amine ex-

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**Fig. 2.** Effect of Extract AE, Administered Intraperitoneally at 5, 10 and 20 mg/kg, on Licking/Biting Response Induced by Intraplantar Injection of Formalin in Mice in Early Phase (0—5 min) (A) and Late Phase (15—30 min) (B)

Each group represents the mean of ten experiments. $^*p<0.05$ and $^{**}p<0.01$, compared with the corresponding control value. Data were analyzed by analysis of variance (ANOVA) or $t$-test and complemented by Dunnett’s or Newman–Keul’s post-hoc test.

**Fig. 3.** Effect of Extract ME, Administered Intraperitoneally at 5, 10 and 20 mg/kg, on Licking/Biting Response Induced by Intraplantar Injection of Formalin in Mice in Early Phase (0—5 min) (A) and Late Phase (15—30 min) (B)

Each group represents the mean of ten experiments. $^*p<0.05$ and $^{**}p<0.01$, compared with the corresponding control value. Data were analyzed by analysis of variance (ANOVA) or $t$-test and complemented by Dunnett’s or Newman–Keul’s post-hoc test.

**Fig. 4.** Effect of Extract AE (A) and ME (B), Administered Intraperitoneally at 3, 6 and 10 mg/kg, on Licking/Biting Response Induced by Intraplantar Injection of Capsaicin in Mice

Each group represents the mean of ten experiments. $^*p<0.05$ and $^{**}p<0.01$, compared with the corresponding control value. Data were analyzed by analysis of variance (ANOVA) or $t$-test and complemented by Dunnett’s or Newman–Keul’s post-hoc test.
tracted from red pepper which, when applied to the skin or injected into animals, produces irritation, a painful reaction and subsequent desensitization to chemically-induced pain.24) Studies have shown that capsaicin evokes the release of neuropeptides, excitatory amino acids (glutamate and aspartate) nitric oxide and pro-inflammatory mediators in the periphery, and transmits nociceptive information to the spinal cord.8)

The hot plate test is a technique that presents selectivity for drugs with analgesic effect “supraspinal” as opioid-derived analgesics.25,26) The analgesic effect involves forebrain structures as nucleus raphe magnus periaqueductal gray or nucleus accumbens.26) Both extracts AE and ME (Fig. 5), at high doses, 60 mg/kg, i.p., demonstrated to be effective in the hot-plate assay, increasing the latency time by 49.1% and 79.7%, respectively. Although both extracts were less active than the morphine in the same assay, the results appear to be related to the participation of opioid pathway, however, additional investigation with opioid antagonists is required to elucidate which mechanism of action is involved.

Considering the interesting antinociceptive profile shown by the extracts, two rare phenolic compounds, previously isolated from the acetonic extract of this plant and active against the writhing test,3) were evaluated against the formalin test at 10 mg/kg, i.p. Compound 2 inhibited both phases of pain (77.6%, first phase; 62%, second phase). On the other hand, compound 1 showed statistical effect only in the first phase, inhibiting the amount of time spent licking the injected paw by 70% (Fig. 6). These results suggest that these are acting by distinct mechanisms of action. Nonsteroidal anti-inflammatory drugs have antinociceptive effects solely in the second phase of the formalin test, such as indomethacin, which caused 33% of inhibition against the second phase at 10 mg/kg, i.p. (unpublished observation).

When tested in the capsaicin test, at 10 mg/kg, i.p., compounds 1 and 2 caused statistical reduction of the time of licking the injected paw, with inhibitions of 41.5% and 37.9%, respectively (Fig. 7). Another interesting finding is that the antinociception caused by the ME and AE could involve, at least in part, its ability to interact with excitatory amino acids, as demonstrated by the fact that it caused inhibition of 65 and 63% at 10 mg/kg, respectively, of the hyperalgesia induced by intraplantar injection of glutamate in mice (results not shown).

The isolated compounds showed similar effects of the extracts at 10 mg/kg, i.p., with inhibitions of 37.7% and 54.5% for compounds 1 and 2, respectively (Fig. 8). In general, compound 2, which possess a glucosyl group, is more active than its aglycone 1, suggesting its importance in relation to antinociceptive activity.

In summary, the results of the present study demonstrate that the extracts and compounds obtained from the aerial

![Fig. 5. Effect of Extract AE (A) and ME (B) and Morphine against Hot-Plate-Induced Antinociception in Mice](image1)

Each group represents the mean of ten experiments. *p < 0.05 and **p < 0.01, compared with the corresponding control value. Data were analyzed by analysis of variance (ANOVA) or t-test and complemented by Dunnett’s or Newman–Keul’s post-hoc test.

![Fig. 6. Effect of Compounds 1 and 2 at 10 mg/kg, i.p., on Licking/Biting Response Induced by Intraplantar Injection of Formalin in Mice in Early Phase (0—5 min) (A) and Late Phase (15—30 min) (B)](image2)

Each group represents the mean of ten experiments. *p < 0.05 and **p < 0.01, compared with the corresponding control value. Data were analyzed by analysis of variance (ANOVA) or t-test and complemented by Dunnett’s or Newman–Keul’s post-hoc test.

![Fig. 7. Effect of Compounds 1 and 2 at 10 mg/kg, i.p., on Licking/Biting Response Induced by Intraplantar Injection of Capsaicin in Mice](image3)

Each group represents the mean of ten experiments. **p < 0.01, compared with the corresponding control value. Data were analyzed by analysis of variance (ANOVA) or t-test and complemented by Dunnett’s or Newman–Keul’s post-hoc test.

![Fig. 8. Effect of Compounds 1 and 2, at 10 mg/kg, i.p., on Licking/Biting Response Induced by Intraplantar Injection of Glutamate in Mice](image4)

Each group represents the mean of ten experiments. **p < 0.01, compared with the corresponding control value. Data were analyzed by analysis of variance (ANOVA) or t-test and complemented by Dunnett’s or Newman–Keul’s post-hoc test.
parts of *P. glomerata* exhibit a marked analgesic effect against some classical models of pain in mice. In this study, we confirm the potent antinociceptive activity of two rare flavellagic acid derivatives. These findings encourage further pharmacological studies, to evidence the mechanism of action of these compounds, as well as to isolate the other active compounds present in ME.

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