Phytochemical and Analgesic Activity of Extract, Fractions and a 19-Hydroxyursane-Type Triterpenoid Obtained from Rubus rosaefolius (Rosaceae)

Márcia Kanegusuku, a Danúbia Sbors, a Eliza Stefanelo Bastos, a Márcia Maria de Souza, a Valdir Cechinel-Filho, a Rosendo Augusto Yunês, b Franco Delle Monache, a and Rivaldo Nierô*. a

a Programa de Mestrado em Ciências Farmacêuticas e Núcleo de Investigações Químico-Farmacêuticas (NIQFAQ)/CCS, Universidade do Vale do Itajaí (UNIVALI); 88.302–202, Itajaí, SC, Brazil; and b Curso de Pós-Graduação em Química, Universidade Federal de Santa Catarina (UFSC); 88.040–900, Florianópolis, SC, Brazil.

Received December 16, 2006; accepted February 8, 2007

The Rubus species has been in folk medicine to treat several ailments, including infectious and dolorous diseases. In this work we evaluate the phytochemical and analgesic activity of hydroalcoholic extract (HE), some fractions (hexane, dichloromethane, ethyl acetate and butanolic), as well as a pure compound denoted as 28-methoxytormentic acid (1) obtained from aerial parts of R. rosaefolius. The compounds were isolated and identified by chromatographic and spectroscopic analysis. The antinociceptive action was evaluated by two well known models of pain in mice: writhing and formalin induced-pain. The results showed that the HE, fractions and compound (1), exhibits potent and dose-related analgesic activity when evaluated in both models of pain. Compound (1), which seems to be the main active principle, showed promising analgesic effects, being several times more potent than aspirin and paracetamol, two well known analgesic and antiinflammatory drugs used as reference. In the writhing test, it showed an ID50 of 5.10 (3.64—7.14) mg kg⁻¹ and maximum inhibition (MI) of 64.22% When analyzed by formalin induced-pain test, this compound showed ID50 values of 9.98 (8.08—12.31) and 6.31 (5.07—7.98) mg kg⁻¹ and MI of 59.37 and 90.37% for the first and second phases, respectively. The results justify, at least partially the popular use of this plant for the treatment of dolorous processes, suggesting that 1 is one of the active principles of this plant.

Key words Rubus rosaefolius; antinociception; triterpene; formalin test; mice

Rosaceae consists of a large family of plants, represented by fruits such as plums, cherries, damson plums, quinces, strawberries, pears and peaches.1) The plants to the genus Rubus, have been traditionally used to treat different diseases, particularly diabetes.2—4) Chemical and pharmacological studies have shown that some species produce active principles that exert antigastropathic, antiinflammatory, antioxidant activity.5—7) Besides these activities, it has been reported that extracts of some species of this genus are potential antimicrobial and analgesic sources.8—10) Phytochemical studies carried out with these plants indicate the presence of steroids, triterpene and ellagic acid derivatives.8—11) Recently, we have shown that another species, R. imperialis, presents hypoglycaemic, cytotoxic and antinociceptive actions.9,11—13) The main isolated active compound, named niga-ichigoside F1, showed marked antinociception and its mechanism of action appears to be related to the dopaminergic, cholinergic, glutamatergic, and tachykinergic and oxiniregic systems.14)

Rubus rosaefolius, popularly known as “amora-do-mato” or “amora-vermelha” in Brazil, is a shrub measuring between 4 and 5 m, and is well distributed in the South of Brazil.1,15) The current study extends our previous work on the biological properties of the Rubus species, and describes its phytochemical analysis and in vivo antinociceptive activity against two pain models in mice (writhing and formalin tests) of the extract, fractions and a pure compound isolated from the aerial parts of R. rosaefolius.

MATERIAL AND METHODS

Plant Material Rubus rosaefolius (Rosaceae) was collected at Sao José – SC Brazil in January 2002 and identified by Dr. Ademir Reis (Department of Botany, UFSC). A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí – SC) under number V.C. Filho 035.

Phytochemical Analysis Air-dried material from R. rosaefolius (700 g) was cut into small pieces and macerated with methanol at room temperature for 7 d. After filtration, the solvent was removed by rotary evaporation under reduced pressure, yielding the respective methanolic extract (ME). Parallel to this, a hydroalcoholic extract (HE) was prepared through maceration of 100 g of plant with an ethanol–water (50 : 50) mixture and stored for use in the pharmacological test. The methanolic extract (35 g), were then suspended in a MeOH : water mixture (9 : 1) and successively partitioned with n-hexane, dichloromethane, ethyl acetate and n-butanol, to provide the respective fractions. The hexane and dichloromethane fractions (7.4, 4.2 g) were chromatographed on silica gel column eluted with a hexane : acetone mixture with increasing polarity. Similar fractions, which showed a positive reaction with anisaldehyde sulfuric reagents, were combined and rechromatographed as in the

Fig. 1. Molecular Structures of 28-Methoxytormentic (1) and Tormentic Acids (2) Obtained from R. rosaefolius

* To whom correspondence should be addressed. e-mail: niero@univali.br
previous case, giving a mixture of steroids and two pentacycles triterpenes. They were identified on the basis of their spectral data as 28-methoxytormentic acid (1), tormentic acid (2) (Fig. 1) stigmasteryl (3), campessterol (4), β-sitosterol (5) and its glycoside (6).

The purity of all the isolated substances was examined by thin layer chromatography (TLC) using Merck silica gel pre-coated aluminum plates 200 μm layer thickness, using several solvent systems of different polarity. Short wave UV light, anisaldehyde sulphuric and FeCl3 reagents were used to visualize spots.

**Animals** Swiss mice (25—35 g), housed at 22±2°C under a 12-h light/12 h dark cycle and with access to food and water *ad libitum*, were acclimatized to the laboratory for at least 1 h prior to testing. For each experiment, one group of animals was used. The experiments reported on here were carried out in accordance with the current ethical and care guidelines for the care of laboratory animals and the investigation of experimental pain in conscious animals.19 The experiments were approved by the local Ethics Committee of this Institution (113/2005-03 UNIVALI). The number of animals (6—8 for group of treatment) and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

**Pharmacological Assays** Writhing Test: The abdominal constriction induced by intraperitoneal injection of acetic acid (0.6%), was carried out according to the procedures described previously, but with minor modifications.17 Male Swiss mice (25—30 g) were pretreated with extract, fractions or compound 1 (3—10mg kg⁻¹, i.p.) 30 min prior to injection with acetic acid injection. The control animals received a similar volume of 0.9% NaCl (10 ml kg⁻¹, i.p.). All the experiments were carried out at 23±2°C. After the challenge, pairs of mice were placed in separate boxes and the number of contractions of the abdominal muscles together with stretching, were counted cumulatively over a 20-min period. The antinociceptive activity was expressed as the reduction in the number of abdominal contractions between the control and the pretreated animals. Although this test is a non-specific model, it is widely used for analgesic screening and involves local peritoneal receptors (cholinergic and histamine receptors) and the mediators of acetylcholine and histamine.

Formalin-Induced Test: The procedure used was similar to that described previously.18 Animals from the same strain were used, and 20 μl of 2.5% formalin solution (0.92% formaldehyde), made up in a phosphate-buffered solution (NaCl 137.0 mM, KCl 2.7 mM and phosphate buffer 10 mM), was injected intraplantarly in the right hindpaw. After injection, the time spent licking the injected paw was timed and considered as indicative of pain. The initial nociceptive scores normally peaked 5 min after formalin injection (first phase) and 15—30 min after formalin injection (second phase), representing neurogenic and inflammatory pain, respectively. In order to investigate the possible antinociceptive action 3—10 mg kg⁻¹, i.p. of the extract, fractions and compound were used.

**Statistical Analysis** The results are represented as a mean±S.E.M., except the ID₅₀ (i.e., the dose of extracts that reduced responses by 50% relative to the control values) are presented as geometric means accompanied by their respective 95% confidence limits. The ID₅₀ were determined by linear regression GraphPad. Statistical significance between groups was calculated by means of analysis of variance followed by Newman–Kuels’ multiple comparison tests. *p*-values less than 0.05 (*p*<0.05) were considered as indicative of significance.

**RESULTS AND DISCUSSION**

Recently, many plants have received special attention as sources of new antinociceptive agents.3,18,20 Despite the absence of experimental studies concerning the analgesic properties of *Rubus rosaefolius*, other plants of the *Rubus* genus have exhibited important antinociceptive effects in mice.6,12 Table 1 shows that the hydroalcoholic extract and any fractions caused a pronounced effect when analysed against writhing model in mice. As can be observed, the HE as well as the hexane, dichloromethane, ethyl acetate and butanolic fractions, caused activity, with MI values of 97.9, 93.4, 88.0, 93.8 and 51.4%, respectively. The ID₅₀ calculated values were 2.4 (1.5—6.6), 27.3 (22.9—32.5), 21.0 (15.7—28.0), 54.4 (37.0—78.8), and 24.0 (13.1—43.8), respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ID₅₀ (mg kg⁻¹)</th>
<th>MI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroalcoholic extract</td>
<td>2.4 (1.5—6.6)</td>
<td>97.9</td>
</tr>
<tr>
<td>Hexane fraction</td>
<td>27.3 (22.9—32.5)</td>
<td>93.4</td>
</tr>
<tr>
<td>Dichloromethane fraction</td>
<td>21.0 (15.7—28.0)</td>
<td>88.0</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>1.9 (1.3—4.8)</td>
<td>93.8</td>
</tr>
<tr>
<td>Butanolic fraction</td>
<td>54.4 (37.0—78.8)</td>
<td>51.4</td>
</tr>
<tr>
<td>Aspirin26)</td>
<td>24.0 (13.1—43.8)</td>
<td>35.0</td>
</tr>
<tr>
<td>Paracetamol26)</td>
<td>18.8 (15.7—22.6)</td>
<td>38.0</td>
</tr>
</tbody>
</table>

Table 2. Antinociceptive Activity of Hydroalcoholic Extract and Fractions of *Rubus rosaefolius* against Acetic Acid Induced Abdominal Constrictions in Mice (Comparison with Aspirin and Paracetamol)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phase I</th>
<th>Phase II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ID₅₀ (mg kg⁻¹)</td>
<td>MI (%)</td>
</tr>
<tr>
<td>Hydroalcoholic extract</td>
<td>7.0 (4.2—11.6)</td>
<td>62.8</td>
</tr>
<tr>
<td>Hexane fraction</td>
<td>Inactive</td>
<td>ND</td>
</tr>
<tr>
<td>Dichloromethane fraction</td>
<td>Inactive</td>
<td>ND</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>Inactive</td>
<td>ND</td>
</tr>
<tr>
<td>Butanolic fraction</td>
<td>Inactive</td>
<td>ND</td>
</tr>
<tr>
<td>Aspirin26)</td>
<td>Inactive</td>
<td>17.0</td>
</tr>
<tr>
<td>Paracetamol26)</td>
<td>Inactive</td>
<td>11.0</td>
</tr>
</tbody>
</table>

ND, not determinate.
With regard to the formalin test, Table 2 shows that the HE, dichloromethane and ethyl acetate caused marked inhibition, particularly in the second phase by the systemic route. The calculated ID$_{50}$ values were 2.3 (1.2—8.8), 10.0 (6.7—17.6) and 6.4 (4.2—12.7) mg/kg, with maximum inhibition of 98.2, 87.9 and 83.5%, respectively.

Considering that the dichloromethane fraction demonstrated a better chromatographic profile and a pronounced antinociceptive effect, it was chromatographed on silica gel columns eluted with hexane : EtOAc gradient, monitored by TLC. Given 28-methoxytormentic acid (1), tormentic acid (2), stigmasterol (3), campesterol (4), β-sitosterol (5) and its glycoside (6), which were directly compared with authentic samples and spectroscopic data (IR, $^1$H- and $^{13}$C-NMR). It is well documented that compounds 2—6 present pharmacological potential, particularly antiinflammatory.$^{21—25}$ However, the antinociceptive profile of 1, to our knowledge, is being evaluated here for the first time. Figures 2 and 3 show the result for compound (1), analyzed against the writhing and formalin models. As can be noted, in the writhing test, 1 significantly inhibited dose-dependent manner with a calculated ID$_{50}$ value of 0.8 (0.6—1.2) $\mu$mol kg$^{-1}$ and maximum inhibition of 64.22% (Fig. 2). When analyzed in the formalin test, I inhibited, in a dose-dependent manner, both the first and second phases of pain, by the systemic route (Fig. 3). The calculated ID$_{50}$ values were 8.3 (7.6—9.3) and 6.4 (5.8—7.3) $\mu$mol kg$^{-1}$, [19.7 (15.9—24.5) and 12.5 (9.9—15.7) $\mu$mol kg$^{-1}$] with maximal inhibition of 59.3 and 90.4% for the first and second phases, respectively. It is interesting to note that the reference drugs practically prevented only the inflammatory effects (second phase). However, 1 was about 10-fold more active in inhibiting neurogenic pain (first phase) than pain of an inflammatory origin (second phase).

CONCLUSIONS

In summary, our results show that the compound isolated (1) was active in these experimental models together with other fractions, suggesting that other active principles are present in minor concentrations or the existence of a possible synergic effect. Studies are currently in progress to determine the other constituents responsible for the antinociceptive properties of $R$. rosaefolius.

Acknowledgments The authors are grateful to Prof. Dr. Ademir Reis for the botanical identification and to CNPq, ProPPEC/UNIVALI and FAPESC-SC, Brazil, for financial support.

REFERENCES


Fig. 2. Effect of 1 (3—10 mg kg$^{-1}$, i.p.) against Acetic Acid Induced Abdominal Constrictions in Mice

Each column represents mean±S.E.M. of six experimental values. Significance levels, when compared with the control group. *p<0.05; **p<0.01.

Fig. 3. Effect of 1 (3—10 mg kg$^{-1}$, i.p.) against Formalin Induced Pain in Mice

(A) The first phase (0—5 min) and (B) second phase (15—30 min). Each column represents mean±S.E.M. of six experimental values. Significance levels, when compared with the control group. *p<0.05; **p<0.01.