Analgesic–antiinflammatory properties of *Proustia pyrifolia*

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**Abstract**

The antiinflammatory (per os and topical) and analgesic (per os) properties of the aerial part of *Proustia pyrifolia* a species in danger of extinction were investigated, and the major compounds of two of its active extracts were isolated. In addition, the evaluation of cytotoxicity in three tumoral cell lines and the acute toxicity of the crude methanol extract were also assayed, together with the antioxidant activity for the different extracts of this species. The results of the evaluation of the topical antiinflammatory activities induced by arachidonic acid, and phorbol 12-myristate 13-acetate of the different extracts showed that this species possesses active constituents that could diminish cyclooxygenase and lipoxygenase activities, the enzymes that allow the synthesis of proinflammatory endogenous substances as prostaglandin E2 and leukotrienes, respectively. Our results corroborate the antiinflammatory and analgesic effects of *Proustia pyrifolia*, and could justify its use in folk medicine for the treatment of rheumatic and gout illnesses. From bio-active extracts /sitosterol, quercetin and dihydroquercetin were obtained, and these compounds could explain in part the antiinflammatory, analgesic and antioxidant activities of this species. The crude methanol extract did not present acute toxicity or cytotoxic activity, however only this extract exhibited antioxidant activity.

**Keywords**: *Proustia pyrifolia*; Analgesic–antiinflammatory-toxicity; Steroids–flavonoids

**Abbreviations**: A, antiinflammatory activity; AA, arachidonic acid; ADR, adriamycin; AL, allopurinol; An, analgesic effect; A-549, human lung carcinoma; CC, column chromatography; C control, median writhes reached in control animals which received only the vehicle; CH2CH2, dichloromethane; C sample, median writhes reached in sample-treated animals; DCE, dichloromethane extract; Et2O, ethyl ether; GME, crude methanol extract; HCI, hydrochloric acid; HE, hexane extract; HT-29, human colon carcinoma; I, inhibition of xanthine oxidase; Ic, median inflammation reached in the control group; IC50, inhibitory concentration; Is, median inflammation in the sample-treated animals; IND, indomethacin; INF, aqueous extract; IR, infrared; LD50, lethal dose; L, median pain; ME, methanol extract; NM, nimesulide; P, mean pain; PGE2, prostaglandin E2; P-388, lymphoid neoplasm from DBA/2 mouse; S.E.M., standard error medium; SQF, Herbarium of the Escuela de Química y Farmacia; T, dermal antiinflammatory activity; TLC, thin layer chromatography; TPA, phorbol 12-myristate 13-acetate; Vi, initial paw volume; Vf, final paw volume; XO, xanthine oxidase; ΔWc, difference median values of the weights of the right and the left ear sections of the control animals; ΔWs, difference median values of the weights of the right and the left ear sections of the treated animals

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1. Introduction

Different species of *Proustia* genus have been frequently used as antiinflammatory and analgesic to treat gout and rheumatic illnesses, however, there is little information about their efficacy and acute toxicity (Muñoz et al., 1981). This genus accumulates sesquiterpene α-tocedrene derivatives that are typical for the subtribus Nassauviinae of the family Asteraceae (Zeder et al., 1986), and a guaianolide β-D-glucopyranoside has been previously isolated from *Proustia ilicifolia* (Bittner et al., 1989).

The objective of this research was to validate the use of a native shrub, *Proustia pyrifolia* DC. (Asteraceae) for folklore medicine, therefore, we explored its acute toxicity and its antiinflammatory and analgesic properties. For the in vivo assays oral administration was used, the same as in folklore medicine. The correlation among the different in vivo assays, allowed us to suggest the probable mechanism of action of the metabolites isolated from two bioactive extracts. We report the results obtained with crude methanol (GME), hexane (HE), dichloromethane (DCE), methanol (ME) and aqueous extracts (INF) in the biological assays. Antioxidant activity was also studied as it can be related with the antiinflammatory properties (Das and Maulik, 1994). To investigate other pharmacological activities not described by the folklore medicine, we evaluate GME cytotoxicity against three tumoral cell lines in search of potentially useful compounds that might help in cancer research.

2. Materials and methods

2.1. General experimental procedures

$^1$H and $^13$C NMR were recorded in CDCl$_3$ at 400 MHz for $^1$H and 100 MHz for $^13$C; internal standard TMS. $^1$D($^1$H, $^13$C) and $^2$D (COSY, HMQC, HMBIC and ROESY) experiments were performed using the standard Bruker DISNMR pulse program.

Column chromatography (CC) was run using silica gel 60G (Merck 7734). TLC was performed on silica gel GF254 (Merck 5554); spots were detected under UV light, or after spraying Liebermann–Burchard reagent and then heating for about 5 min at 120°. IR spectra were made in KBr; melting point was determined on a Kofler hot stage microscope and is uncorrected.

2.2. Plant material

The aerial part of *Proustia pyrifolia* DC was collected at Cuesta La Dormida, Chacabuco, Chile, in January, and identified by Prof. Sebastian Teiller. A voucher specimen is kept at the Herbarium of the Escuela de Química y Farmacia (SQF 22143), Universidad de Chile.

2.3. Extraction and isolation

Air dried and powdered material (0.8 kg) of plant material was extracted with methanol at room temperature. After removing the solvent under vacuum this crude methanol extract (GME, 72.7 g) was used for additional pharmacological assays.

A separate portion of the powdered material (6.0 kg) was extracted successively at room temperature with n-hexane, CH$_2$Cl$_2$ and MeOH, yielding after removal of the solvents in vacuo. 131 g of HE, 73 g of DCE and 400 g of ME, respectively. Part of this last extract (20.0 g) was submitted to CC on silica gel with n-hexane/Et$_2$O gradient (0, 10, 20, 50 and 100% Et$_2$O) yielding two fractions of increasing polarity (1–2). Fraction 1 was applied on Sephadex LH-20 with aceton/n-hexane/CH$_2$Cl$_2$ 1:1 yielding 1 (95.0 mg) and a rest fraction. The latter was rechromatographed on a chromatotron with n-hexane/aceton 5:4 yielding 2 (30 mg). Identification of these compounds was performed through comparison of the NMR data with those reported in the literature.

An amount of 100 g HE was subjected to repeated columns chromatography on silica gel and eluted with mixtures of n-hexane–CH$_2$Cl$_2$ (v/v), CH$_2$Cl$_2$–MeOH (v/v) and finally MeOH. Fractions of 500 mL were collected. Fractions eluted with n-hexane–CH$_2$Cl$_2$ (25:75 v/v) were joined and dried (25 g) and subjected to a second column chromatography over silica gel, and fractions of 200 mL were collected and monitored by TLC. From fractions (13–16) eluted with n-hexane–CH$_2$Cl$_2$ (50:50 v/v) and 40:60 (v/v) 281 mg of the compound 3 were obtained. Identification of this compound was performed by direct comparison of the melting point, chromatographic (TLC) and spectroscopic (IR) data with an authentic reference compound.

2.4. In vivo assays animals

Pirbright guinea pigs (220–300 g) of both sexes were used for the per os antiinflammatory study. CF-1 mice of either sex (20–25 g) were used to assess the analgesic and topic antiinflammatory effects, and acute toxicity. Animals under standard conditions from the Chilean Public Health Institute were fasted overnight before the day of the experiments.

2.5. Acute toxicity

For each dose, groups of 10 mice of both sexes were allowed free access to water. GME suspended in saline Arabic gum, 5%, were orally administered via a gastric catheter. They were weighed daily for a week to detect physiological alterations. In case of death of the animals, the LD$_{50}$ is determined by the Morgan Scoring method (Morgan, 1992).

2.6. Cytotoxicity assays

A screening procedure was used to assess the cytotoxicity of GME against the following cell lines: P-388 (lymphoid...
neoplasm from DBA/2 mouse, ATCC CCL-46), A-549 (human lung carcinoma, ATCC HTB-38) and HT-29 (human colon carcinoma, ATCC HTB-38). Cells were seeded into 16 mm wells (multi-dishes) (NUNC 42001) at concentrations of 1 × 104 cells/well (P-388), 2 × 104 cells/well (A-549) (HT-29), respectively, in 1 mL aliquots of MEM 10% FCS medium containing the compound to be assessed at the concentrations assayed. In each case, a set of control wells was incubated in the absence of sample and counted daily. After 4 days at 37 °C, under a 10% CO2, 98% humid atmosphere, P-388 cells were observed through an inverted microscope and the degree of inhibition was determined by comparison with the control, whereas A-549 and HT-29 were stained with crystal violet before examination (San Feliciano et al., 1993). Adriamycin (ADR) as reference drug was used.

2.7. Xanthine oxidase activity

Both xanthine and xanthine oxidase (XO) from cow’s milk were purchased from Sigma Co. and the standard inhibitor allopurinol (AL) was obtained from Laboratorios Saval, Chile; GME, HE, DCE and ME were evaluated at 50 mg/mL and those having an inhibition >50% were further tested for IC50 determination (Schmeda-Hirschmann et al., 1992). The inhibition of XO activity using xanthine as the substrate was spectrophotometrically measured in relation to the production of uric acid, which was determined at 290 nm using a UNICAM spectrophotometer. The assayed mixture consisted of 1.0 mL of test solution, 2.9 mL of phosphate buffer (Na2HPO4/KH2PO4, pH = 7.5), and 0.1 mL of enzyme solution. After preincubation of the mixture at 25 °C for 15 min, the reaction was initiated by adding 2.0 mL of substrate solution. The assayed mixture was incubated at 25 °C for 30 min. The reaction was stopped by adding 1.0 mL of 1 M HCl and the absorbance was measured. The inhibition percent of xanthine oxidase activity (%I) was calculated as:

\[
\%I = \left(1 - \frac{A}{B}\right)\left(1 - \frac{C-D}{B-C}\right) \times 100;
\]

where A is the activity of XO without test material (total uric acid), B the blank of 0 at XO, C the enzyme activity with test material (residual uric acid) and D the blank of C without the enzyme.

The IC50 determination of allopurinol was 0.035 mg/mL (0.267 mM). For XO activity, the significance of the drug-induced changes was estimated using the Wilcoxon test for independent data (Holland et al., 1973).

2.8. Antiinflammatory activity per os

For each per os dose, the antiinflammatory activity was evaluated in groups of eight mice and 16 control subjects, using a intraperitoneal injection of 0.5 mL of 0.6% acetic acid (Delporte et al., 2002). The analgesic effects were calculated by comparing the number of abdominal writhes of the treated and the control group, which only received the vehicle. The number of abdominal writhes of each mouse was counted for 30 min, beginning 5 min after acetic acid administration (Delporte et al., 2002).

The following equation was used to calculate the mean pain percentage: %P = \left(\frac{C_{\text{sample}} - C_{\text{control}}}{C_{\text{control}}}\right) \times 100; where C_{\text{sample}} is the median writhes reached in sample-treated animals and C_{\text{control}} (41.6 ± 3.79) is the median writhes reached in control animals which received only the vehicle (Delporte et al., 2002).

The analgesic effect, An, was calculated according to the following equation: %An = 100 – %P.

In antinflammatory and analgesic assays, the dry extracts (GME, INF, HE, DCE and ME) were orally administered 1 h before κ-carrageenan, or acetic acid, respectively, by means of an intragastric catheter, suspended in saline Arabic gum. The dosages of extracts used in each assay were selected according to previous work on the same biological activity (Delporte et al., 1998).

For all in vivo pharmacological assays, the drug-induced changes were statistically estimated using the Wilcoxon test for independent data (Holland and Wolfe, 1973). The effects were considered significant for p < 0.05. The S.E.M. values were calculated for the mean %I and %C values, for the mean writhes constriction and for the mean weight of ears in treated and untreated animals in each assay.
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Sodium naproxen, obtained from Laboratorios Saval, Chile, was used as reference drug in per os analgesic and antiinflammatory assays and was suspended in the same vehicle. A-carrageenan was obtained from Sigma. For the dermal antiinflammatory activity, nimesulide (AA-induced oedema) and indomethacin (TPA-induced oedema) from Laboratorio Chile and Laboratorio-Madex, respectively, were used as reference drugs at the dose of 1 and 0.5 mg/20 μL/ear, respectively (Delporte et al., 2002).

3. Results and discussion

3.1. Phytochemical study

Two compounds were isolated and identified from ME, quercetin 1, and dihydroquercetin 2. These flavonoids were identified by their 1 HNMR and 13 C NMR data (Mabry et al., 2003).

3.2. Acute toxicity and cytotoxicity activity

GME did not show acute toxicity per os up to the maximum dose of 2 g/kg and the weight of the mice had a normal variation after the 7 days of observation. Common side effects such as, mild diarrhea, loss of weight and depression were not recorded. It is important to carry out toxicological studies in other animal species in order to demonstrate its lack of toxicity. Table 1 shows the IC50 of the different extracts and reference drug (AL).

3.3. Antioxidant activity

The antioxidant properties of GME, HE, DCE, and ME were evaluated as their abilities to inhibit XO. Table 2 shows the IC50 of the different extracts and reference drug (AL).

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC50 (mg/mL)</th>
</tr>
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<tbody>
<tr>
<td>GME</td>
<td>46.5</td>
</tr>
<tr>
<td>DCE</td>
<td>&gt;60</td>
</tr>
<tr>
<td>ME</td>
<td>&gt;60</td>
</tr>
<tr>
<td>AL</td>
<td>0.005</td>
</tr>
</tbody>
</table>

IC50, inhibitory concentration; GME, crude methanol extract; DCE, dichloromethane extract; ME, methanol extract; AL, alliponinol.

GME showed activity, but this activity is lower than the reference drug, however, both DCE and ME presented a weak effect and HE lack of effect. The low activity of ME could be explained by the weak inhibitory activity of the major compounds 1 and 2 against XO (Iio et al., 1986).

3.4. Antiinflammatory (per os and topic) and analgesic properties

Analgesic and antiinflammatory response to sodium naproxen (SN) is doses-related (Delporte et al., 2002). Table 3 shows the results for the pharmacological assays of the various extracts, together with the maximum effect of SN for the per os antiinflammatory and analgesic activities, and the antiinflammatory dermal maximum effect of nimesulide (NM) and indomethacin (IND) (Delporte et al., 2003).

In the assays carried out per os GME, HE and ME exhibited the strongest analgesic activities similar to the reference drug (SN). In relation to the results obtained in per os antiinflammatory studies, ME showed the strongest effect, and was similar to the reference drug (SN); HE did not present significant antiinflammatory activity.

It is important to point out the general correlation observed between (per os) antiinflammatory and analgesic activities found for the GME, DCE and ME. This could be explained in terms of the presence of compounds with a similar mechanism for both activities, as for example inhibition of the synthesis of prostaglandin E2 (PGE2) by the activation of the cyclo-oxygenase enzyme, the level of PGE2 increases markedly, and its production provokes inflammation and pain (Dannhardt and Kiefer, 2001). Therefore, we assume that some active metabolites of these extracts could inhibit cyclooxygenase activity.

In the assays carried out topically, the AA-induced oedema response is rapid in onset with a short duration. In contrast, TPA produces a longer lasting response with a delayed onset. The TPA model seems to be dependent mainly on leukotrienes, which are synthesized by the lipooxygenases enzymes, whereas the AA model is mainly related to PGE2 (Lloret and Moreno, 1995).

For AA and TPA induced oedema, GME showed significant effect only against AA assay and its mechanism of action might be explained by cyclooxygenase inhibition.
On the contrary, HE and ME presented important activities only against TPA. Therefore, we can conclude that these extracts present active metabolites whose antiinflammatory effect might be explained by the inhibition of the synthesis of the leukotrienes.

DCE was active in both AA and TPA models, therefore, it must contain active compounds inhibitors of the synthesis of the leukotrienes and PGE2.

In relation to the phytochemical study, our results indicate that the major component present for the HE was 3, in a previous studies this steroid has been reported as antiinflammatory, analgesic and antipyretic when administered per os (Santos et al., 1995; Villaseñor et al., 2002). Also it has been demonstrated that 3 is an effective topical antiinflammatory agent mainly in acute inflammation induced by TPA; its effect on leukocyte migration to the inflamed site might be an important aspect of its mechanism of action (Gómez et al., 1999).

Therefore, this steroid is one of the responsible compounds for the HE antiinflammatory effect against TPA-induced oedema and per os analgesic activity.

The phytochemical analysis showed for ME the presence of a high percentage of flavonoids, two of them were identified as 1 and 2; these compounds have been previously reported to have antiinflammatory, antioxidant and antiallergic effects (Pathak et al., 1991; Pelzer et al., 1998). The ability of quercetin to inhibit nitric-oxide synthase, 5-lipoxygenase, phospholipase A2 and C and cyclooxygenase-2, all proinflammatory enzymes has been reported (Lee et al., 1982; Rao et al., 1985; Chiesi and Schwaller, 1995; De Pascual-Teresa et al., 2004). Therefore, the per os and topical antiinflammatory activities of ME might be attributed in part to 1 and 2.

In summary, 3 as well as 1 and 2 could contribute, at least in part, to the analgesic and antiinflammatory properties observed for HE and ME, respectively.

4. Conclusions

Our pharmaco-toxicological results corroborate that Proustia pyrifolia present analgesic and antiinflammatory effects and absence of acute toxicity. These results support scientifically the use of Proustia pyrifolia in popular medicine for the treatment of rheumatic and gout illnesses. Since all the extracts showed pharmacological activities, we assume that different active secondary metabolites are present in crude extracts and perhaps some of these compounds may operate in a synergistic manner.

The flavonoids quercetin and dihydroquercetin, and the steroid β-sitosterol were isolated, these compounds are responsible in part of the pharmacological effects observed.

Acknowledgment

The authors thank Dr. Julio Maldonado of the Chilean Public Health Institute for the experimental animals and for their collaboration.

References


Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose</th>
<th>%A ± S.E.M.</th>
<th>%An ± S.E.M.</th>
<th>%TA-AA ± S.E.M.</th>
<th>%TA-TPA ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>INF</td>
<td>0.4 mL/25 g</td>
<td>51.2 ± 11.5</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>INF</td>
<td>4.0 mL/kg</td>
<td>33.3 ± 7.2</td>
<td>34.9 ± 3.5</td>
<td>24.5 ± 4.8</td>
<td>n.t.</td>
</tr>
<tr>
<td>GME</td>
<td>3.0 mg/kg</td>
<td>33.6 ± 5.1</td>
<td>64.5 ± 9.7</td>
<td>26.2 ± 2.8</td>
<td>66.2 ± 12.6</td>
</tr>
<tr>
<td>GME</td>
<td>600 mg/kg</td>
<td>11.8 ± 3.0</td>
<td>60.5 ± 6.4</td>
<td>55.8 ± 10.0</td>
<td>63.6 ± 10.1</td>
</tr>
<tr>
<td>HE</td>
<td>3.0 mg/kg</td>
<td>38.1 ± 8.0</td>
<td>42.1 ± 10.5</td>
<td>8.7 ± 3.0</td>
<td>49.4 ± 8.9</td>
</tr>
<tr>
<td>HE</td>
<td>600 mg/kg</td>
<td>49.6 ± 7.0</td>
<td>64.8 ± 12.2</td>
<td>81.8 ± 20</td>
<td>70.0 ± 4.0</td>
</tr>
<tr>
<td>ME</td>
<td>3.0 mg/kg</td>
<td>54.6 ± 0.8</td>
<td>70.0 ± 4.0</td>
<td>75.5 ± 4.0</td>
<td>48.8 ± 4.0</td>
</tr>
<tr>
<td>ME</td>
<td>600 mg/kg</td>
<td>49.6 ± 7.0</td>
<td>64.8 ± 12.2</td>
<td>n.t. n.t.</td>
<td>n.t. n.t.</td>
</tr>
<tr>
<td>SN</td>
<td>12.5 mg/kg</td>
<td>70.0 ± 4.0</td>
<td>75.5 ± 4.0</td>
<td>48.8 ± 4.0</td>
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<tr>
<td>SN</td>
<td>40.0 mg/kg</td>
<td>54.6 ± 0.8</td>
<td>70.0 ± 4.0</td>
<td>75.5 ± 4.0</td>
<td>48.8 ± 4.0</td>
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<tr>
<td>SN</td>
<td>12.5 mg/kg</td>
<td>70.0 ± 4.0</td>
<td>75.5 ± 4.0</td>
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<tr>
<td>SN</td>
<td>40.0 mg/kg</td>
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<td>70.0 ± 4.0</td>
<td>75.5 ± 4.0</td>
<td>48.8 ± 4.0</td>
</tr>
</tbody>
</table>

Without asterisk, p > 0.05; n.t.: non tested; A, antiinflammatory effect per os; An, analgesic effect; TA-AA and TP-TPA, topical antiinflammatory effects induced for AA and TPA, respectively; INF, aqueous extract; GME, crude methanol extract (or crude methanol extract); HE, hexane extract; DCE, dichloromethane extract; ME, methanol extract; SN, sodium naproxen (2-3-6-methoxy-2-naphthyl) propionic acid); NM, nimesulide (4-nitro-2-phenoxymethanesulfonanilide); IND, indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid).


