In Vitro Anti-Inflammatory Activity of Iridoids and Triterpenoid Compounds Isolated from Phillyrea latifolia L.

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Two iridoids, oleuropeisode and ligustroside, and two triterpenoid compounds, oleanolic acid and ursolic acid, have been isolated from the leaves of Phillyrea latifolia L. (Oleaceae). These compounds were tested for interactions with the cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways of arachidonate metabolism in calcium ionophore-stimulated mouse peritoneal macrophages and human platelets, and for their effect on cell viability. Structure-activity relationships obtained for in vitro screening results were discussed. These compounds are capable of exerting inhibitory actions on enzymes of the arachidonate cascade. All compounds assayed showed a significant effect on prostaglandin E2 (PGE2) release, with inhibition percentages similar to the reference drug indomethacin (IC50=0.95 μM). The IC50 values of the active compounds are: oleuropeisode 47 μM, ligustroside 48.53 μM, oleuropein acid 23.51 μM and ursolic acid 60.91 μM. In the leukotriene C4 (LTC4)-assay, only oleanolic acid showed a significant effect (IC50=16.79 μM). We also investigated the action of compounds on thromboxane B2 (TXB2), release induced by calcium ionophore in human platelets. Of all the tested compounds, only ligustroside (IC50=122.63 μM) and ursolic acid (IC50=50.21 μM) showed a significant effect, although with less potency than the reference drug ibuprofen (IC50=1.27 μM). Thus, our compounds possess an array of potentially beneficial anti-inflammatory properties which may, along with other constituents, contribute to the claimed therapeutic properties of the plant from which they are derived.

Key words  Phillyrea latifolia; iridoid; triterpenoid; PGE2; LTC4; TXB2

There is growing evidence in the literature that Olea europeae L. (Oleaceae), the well-known olive leaves traditionally used as an herbal remedy and spice for thousands of years, possesses an array of interesting pharmacological actions. These pharmacological effects can be attributed to iridoids, which occur in a great variety in plants of the family Oleaceae. Clinical data on the beneficial effects of olive leaves in the treatment of hypertensive disease have been available since the 1950s. Oleuropeisode, which is the major iridoid with clinical relevance in olive leaves, increases coronary blood flow and shows antiarrhythmic and spasmylic effects. Olive leaf is also used in folk remedies as an antidiabetic. Oleuropeisode shows a hypoglycemic effect and increased tolerance of orally administered glucose. This compound has also been shown to be antimicrobial and a potent antioxidant endowed with antiinflammatory properties. Many species of the Oleaceae family are known to produce large amounts of oleuropeisode. Although members of the Oleaceae have been studied extensively and have been seen to contain a profusion of substances with marked bioactivity, there are, on the other hand, abundant members of this family which are poorly studied. We have been interested in examining the phytochemistry and biological activity of the Phillyrea genus from the Oleaceae. Members of the Phillyrea are widely represented in the Mediterranean and Iberian flora, and consist of a group of plants, many of which are used in traditional medicine. Different classes of natural products with interesting biological properties have been isolated from these species, including flavonoids and oleuropein derivatives.

In Spanish traditional medicine, Phillyrea latifolia L. is used as a folk remedy against a variety of diseases such as for the treatment of ulcers and mouth inflammations, and as an astringent and diuretic agent. In the course of our research on antiinflammatory activity from medicinal plants, we have studied the phytochemistry of P. latifolia and have isolated four compounds. This paper led to the isolation of two iridoids, oleuropeisode and ligustroside, and two triterpenoid compounds, oleanolic acid and ursolic acid. In considering the bioactivity of oleuropeisode, it is important to take into account the fact that P. latifolia also contains oleuropeisode. The presence of this compound increases the possible real value of this species, because we now report that it is a potential source of oleuropeisode with an overall yield of approximately 4%.

Some of these compounds contained in P. latifolia have been reported to have bioactivities. Ligustroside, iridoid present in P. latifolia together with oleuropeisode, has been shown to possess enzyme inhibitory and antioxidant activities. Recently, both compounds have been shown to have a potent cytotoxic effect on several tumor cell lines. Oleanolic acid and ursolic acid are triterpenoid compounds that exist widely in food, medicinal herbs and other plants. Many medicinal plants of repute contain these compounds and their derivatives as major aglycones. Both oleanolic acid and ursolic acid are effective in protecting against chemically induced liver injury in laboratory animals. Oleanolic acid has been reported to exhibit low toxicity and, as such, it is marketed in China for human liver disorders. The mechanism of hepatoprotection by these two compounds may involve the inhibition of toxicant activation and the enhancement of the body's defense system. Oleanolic acid and ursolic acid have also shown antihyperlipidemic, antiulcer, antinfungal, antiviral, hypoglycemic activity, as well as antitumor-promoting effects. Recently, both compounds have been noted for their antiinflammatory activity, which is stimulating addi-
tional research in this field. Oleanolic acid and ursolic acid were reported to inhibit acute experimental edema in animals. There are also many reports on in vitro biological actions of both compounds, which could help to understand the mechanisms of their actions. It is, however, remarkable that many observations reported were obtained in studies using exorbitantly high concentrations, thus the interpretation of the results must be critically reconsidered in order to evaluate the practical implications of a probable use in vivo.

As an extension of our phytochemical study of Oleaceae plants, we have studied the chemical constituents and the in vitro antinflammatory activity of compounds of *P. latifolia*. In the present paper, we present the isolation of oleuropeoside, ligustroside, oleanollic acid and ursolic acid from *P. latifolia*, and we test them for their interactions with the generation of eicosanoids in cellular systems. Our work concerns the effects of compounds on prostaglandin E2 (PGE2)- and leukotriene C4 (LTC4)-release from ionophore-stimulated mouse peritoneal macrophages and on the release of thromboxane A2 (assayed as TXB2) from ionophore-stimulated human platelets. We also propose a relationship with their structure in order to establish the possible real value of this kind of compounds as antinflammatory agents.

**MATERIALS AND METHODS**

**Materials** Indomethacin, nordihydroguaiaretic acid (NDGA), ibuprofen, A23187 calcium ionophore, dimethylsulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA) and 3-(4,5-dimethylthiazolol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (U.S.A.). The culture media and serum were purchased from Life Technologies (Barcelona, Spain). Enzyme-linked immunoabsorbent assay (ELISA) kits for the determination of PGE2, LTC4 and TXB2 were provided by Cayman Chemical Co. (U.S.A.).

**Extraction, Isolation and Identification** The powdered leaves of *P. latifolia* (800 g) was carried out with acetone at room temperature. The extract (46 g) was chromatographed on Sephadex LH-20 and eluted by MeOH to afford four fractions (F1—F4). Fraction F2 (24 g) was fractionated on a silica gel column (G-60, 26—40 μm, Merck) using a linear gradient of CHCl3-MeOH 97:3; 95:5; 93:7; 90:10; 85:15 to afford ten main groups of fractions (A1—A10). Fractions A1 and A2 yielded ursolic acid (83 mg) and oleanolic acid (136.4 mg), respectively (Fig. 1). Fraction A6 (557.4 mg) was purified by flash chromatography on silica gel and eluted successively by CHCl3, CHCl3-MeOH 98:2 and MeOH, affording ligustroside (23 mg) (Fig. 1). Fraction A8 (1.9 g) on further flash chromatography on silica gel using as eluents CHCl3, CHCl3-MeOH 98:2 and MeOH gave oleuropeoside (404 mg) (Fig. 1). Identification and assignment of the isolated compounds were performed by a comparison of their spectroscopic data with those of authentic samples and/or previously reported data.

**Biological Assays: Preparation of Test Samples** Test compounds were dissolved in DMSO and assayed at concentrations ranging from 25 to 100 μM. Indomethacin (25—100 μM), NDGA (6.25—25 μM) and ibuprofen (25—100 μM) were used as reference compounds (PGE2, LTC4 and TXB2, respectively).

**Cell Viability** To assess the correlation between cytotoxicity and suppression of eicosanoid release by test compounds, preliminary cytotoxicity studies were performed. Cell viability was assessed using a MTT-based colorimetric assay. 3 × 105 cells diluted with Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS) were pipetted into 96-well microtiter plates, and were incubated overnight at 37°C and 5% CO2. The cells were exposed to various concentrations of samples for 3 h under the same conditions of incubation. A 20 μM MTT solution (5 mg/ml in phosphate buffered saline) was added and further incubated for 3 h at 37°C. After carefully aspirating the medium 100 μl of DMSO was added for the dissolution of formazan crystals. The absorbance of each well was read at 520 nm using a microplate reader. The values of the maximum non-toxic concentration obtained were then used in PGE2, LTC4 and TXB2 determinations.

**Assay of PGE2 and LTC4 Release in Activated Macrophages** Macrophages from NMRI male mice were collected by peritoneal lavage with phosphate buffered saline. The cells were suspended in DMEM supplemented with 10% FCS, and seeded into 24-well plates at a concentration of 5 × 105 cells/well. After adhering to plates (24 h at 37°C in an atmosphere of 5% CO2), nonadherent cells were washed off, and the cells received fresh DMEM (without FCS). Cells were pretreated for 1 h at 37°C with the test compounds or vehicle, and then stimulated for a further 2 h by adding calcium ionophore A23187 (final concentration 10−6 M). Controls contained only DMSO (basic level of released eicosanoids) or reference compounds (total inhibition of eicosanoid release). The contents of all dishes were frozen at −20°C and retained for analysis for PGE2 and LTC4.

**Assay of TXB2 Release in Activated Platelets** Venous blood was obtained from healthy donors of both sexes and anticoagulated with EDTA. Blood was centrifuged at 900 rpm for 20 min at 20°C, and the upper straw-coloured suspension of platelet-rich plasma (PRP) retained. Aliquots of PRP (1.5 × 108 platelets/sample) were pretreated for 2 min at 37°C with the test compounds or vehicle, and then stimulated for a further 20 min at 25°C by adding calcium ionophore A23187 (final concentration 1.8 × 10−6 M). Con-
trols contained only DMSO (basic level of released eicosanoid) or reference compounds (total inhibition of eicosanoid release). After centrifugation at 3000 rpm for 10 min at 4 °C to stop the cellular response, the supernatants were frozen at −20 °C and retained for analysis by ELISA for TXB₂.

**Enzyme-Linked Immunosorbent Assay for Eicosanoids**

Imuneactive eicosanoids were assayed by standard sandwich ELISA. In brief, 96-well microtiter plates were coated with goat anti-mouse polyclonal antibodies and culture supernatants were added to each well. They were then mixed with eicosanoid conjugated with acetycholinesterase (eicosanoid tracer) and specific antiserum. After an overnight incubation at 4 °C, the plates were washed to remove unbound reagents and Ellman’s reagent was added as an enzyme substrate. Optical density was measured at 412 nm in a microplate reader.

**Statistics**

PGE₂, LTC₄, and TXB₂ concentrations were assessed directly from a standard curve. The results are expressed as mean±S.D. of groups of five culture dishes of at least duplicate and in most cases triplicate determinations. IC₅₀ values were calculated from four concentrations as described. Statistical analysis was performed using Student’s t-test for unpaired data, and a p value of ≤0.05 was considered to indicate a significant difference.

**RESULTS**

Ligustroside, oleuropeaside, and ursolic acid did not show a cytotoxic effect on cells up to a concentration of 100 µM. Of all the test compounds, oleancolinic acid was only weakly toxic at 100 µM.

**Effects of Drugs on PGE₂ and LTC₄ Release**

As shown in Tables 1 and 2, addition of the calcium ionophore A23187 to mouse peritoneal macrophages causes the generation of nanogram amounts of eicosanoids via both cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways. This effect was measured in terms of immunoassayable PGE₂ and LTC₄, respectively. Validation of this system for the identification of inhibitors of the two divergent pathways of arachidonate metabolism was performed using indomethacin, a well-characterized COX inhibitor (91.47% inhibition of PGE₂) (Table 1), and NDGA, a known inhibitor of 5-LOX, which produced 99.75% inhibition of LTC₄ (Table 2). The compounds tested showed a considerable activity as inhibitors of eicosanoid release from ionophore-stimulated mouse peritoneal macrophages. All compounds assayed showed a significant effect on PGE₂ release, with inhibition percentages similar to the reference drug indomethacin (IC₅₀=0.95 µM) (Fig. 2A). Inhibition was more evident with ligustroside (IC₅₀=48.53 µM) (Fig. 2C) and oleuropeaside (IC₅₀=23.51 µM) (Fig. 3A), with inhibition percentages of around 90% at the highest non-cytotoxic dose of 100 µM and 50 µM, respectively. Ursolic acid (IC₅₀=60.91 µM) (Fig. 3B) and oleuropeaside (IC₅₀=47 µM) (Fig. 2B), at the highest non-cytotoxic dose of 100 µM, showed an inhibition rate of around 80%. In the LTC₄-assay, only oleancolinic acid showed a significant effect (IC₅₀=16.79 µM) (Fig. 4B), with an inhibition percentage slightly lower than the reference drug, NDGA (IC₅₀=0.08 µM) (Fig. 4A). Ligustroside, oleuropeaside, and ursolic acid had no significant effect on LTC₄-release.

**Effects of Drugs on TXB₂ Release**

We also investigated the action of compounds on TXB₂-release induced by calcium ionophore in human platelets. As shown in Table 3, activation of human platelets with calcium ionophore A23187 also causes the generation of nanogram amounts of eicosanoids via COX pathway. This was measured in terms of immunoassayable TXA₂ (assayed as TXB₂), which is the main-end product of arachidonate metabolism produced through a COX-1 mechanism in blood activated platelets. Validation of this system for the identification of inhibitors was obtained using as reference drug, ibuprofen, which produced 98.9% inhibition of TXB₂. Of all the tested compounds, only ligustroside (IC₅₀=122.63 µM) (Fig. 5B) and ursolic acid (IC₅₀=50.21 µM) (Fig. 5C) showed a significant effect on TXB₂-release, although with less potency than the reference drug, ibuprofen (IC₅₀=1.27 µM) (Fig. 5A). Oleancolinic acid and oleuropeaside had no significant effect on TXB₂-release.

**DISCUSSION**

Phytochemical and biological studies aimed at the discovery and development of novel antiinflammatory agents from natural sources have been conducted in our laboratories for a number of years. Antiinflammatory compounds can act on many steps of pathophysiological processes. Thus, to evaluate the potential and the therapeutic index of a new compound, qualified information on the mechanism and the selectivity of its action is needed. One of the major pathogenetic and pathophysiological areas which are recognized today as possible sites for drug intervention is arachidonic acid (AA) metabolism. Thus, both the COX (constitutive COX-1 and inducible COX-2) and 5-LOX pathways of arachidonate cascade are targets for the development of new antiinflammatory agents.

To find new COX and LOX inhibitors from medicinal plants, we have selected the leaves of *P. latifolia*, which have
been used for the treatment of inflammation in traditional medicine. Four compounds, oleuropeoside, ligustroside, oleanolic acid and ursolic acid were isolated as active constituents from the acetonic extract of this plant. These compounds are present in *P. latifolia* as the major secondary metabolites, suggesting an important role for them in the traditional preparation.

Oleanolic acid and ursolic acid are capable of exerting inhibitory actions on enzymes of the arachidonate cascade. Thus, ursolic acid exerts a preferential effect on the COX pathway (giving rise to reduced PGE_{2} levels in activated mouse macrophages, and to a lesser extent reduced TxB_{2} levels in human platelets). In contrast, oleanolic acid produces reductions in generation of both COX and 5-LOX eicosanoid metabolites, but effects were greater against LTC_{4}. Oleanolic acid exerts particularly potent inhibition of 5-LOX with less effect on COX (giving rise to reduced PGE_{2}}
Fig. 4. Effect of NDGA (A) and Oleanolic Acid (B) on LTC4 Release from Calcium-Ionophore Stimulated Mouse Peritoneal Macrophages
Inhibition percentages are expressed as mean values ± S.D. of 3 experiments. Significance *p<0.05; **p<0.01.

Table 3. Inhibition of TXB2-Release from Human Platelets Stimulated with Calcium Ionophore A23187 (1.8×10-6 M) by Compounds Isolated from Pityrea latifolia

<table>
<thead>
<tr>
<th>Compound</th>
<th>TXB2 (ng/ml)</th>
<th>% Inhibition</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±0.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cells alone</td>
<td>5.00±0.3</td>
<td>—</td>
<td>1.12 µM</td>
</tr>
<tr>
<td>Ibuprofen (100 µM)</td>
<td>1.10±0.2 ±a)</td>
<td>98.90</td>
<td>1.27 µM</td>
</tr>
<tr>
<td>Ligustroside (100 µM)</td>
<td>58.0±0.7 ±a)</td>
<td>42.00</td>
<td>122.63 µM</td>
</tr>
<tr>
<td>Ursolic acid (100 µM)</td>
<td>51.0±0.2 ±a)</td>
<td>49.00</td>
<td>50.21 µM</td>
</tr>
</tbody>
</table>

(a) All values are mean ± S.E.M.  (b) Statistical significance from control (p<0.01)
(c) Statistical significance from control (p<0.05).

Fig. 5. Effect of Ibuprofen (A), Ligustroside (B) and Ursolic Acid (C) on TXB2-Release from Calcium-Ionophore Stimulated Human Platelets
Inhibition percentages are expressed as mean values ± S.D. of 3 experiments. Significance *p<0.05; **p<0.01.
levels in activated mouse macrophages because of PG-synthase inhibition, but it has no significant effect on TXB$_2$ in human platelets).

Our results identify some features concerning the inhibition of arachidonate metabolism by these two compounds. If the antiinflammatory effect is compared with chemical structure, some relationships can be observed which are consistent with previous data on the effect of these triterpenoids on other inflammatory conditions in vivo and in vitro. The common feature of these triterpenoids is a 3-hydroxy, a polar distal residue (e.g., a 28-carboxy and additional methyl substituents in the body of the molecule) and a non-polar triterpenoid nucleus. These results indicate that this chemical feature, polar residues separated by a non-polar spacer, seems to be essential to show the activity. However, the magnitude of the inhibitory effect depends on both the substrate and the substituents on the pentacyclic ring system. The presence of a 30-methyl at position 20 of the triterpenoid nucleus, which was intact in the two molecules, was recognized to be significant for PG-synthase activity in mouse macrophages. Furthermore, enhancement of the inhibitory activity was found in oleancic acid, which presented an additional 29-methyl at position 20, while that at position 19 as occurred in ursoic acid led to reduction of the PG-synthase activity. However, this substitution with a 29-methyl at position 19 of the triterpenoid nucleus is a positive chemical feature for TX synthase activity in human platelets, as is apparent when the activity of ursoic acid is compared with oleancic acid, which is inactive on TX synthase. These facts suggest that the double methylation at position 20 is one of the most positive characters for PG-synthase activity, but it is pernicious for TX-synthase activity. However, since the synthesis of TXB$_2$ from AA depends on both the activity of COX and TX synthase, independent suppression of TXB$_2$ release may also be explained by the inhibition of COX due to ursoic acid.

Our results also identify or confirm some features concerning the inhibition of 5-LOX metabolism by triterpenoids, which are consistent with previous studies in vivo. The double methylation at position 20 of triterpenoid nucleus was recognized to be significant for the 5-LOX activity in mouse macrophages, as can be deduced from the data on oleancic acid compared with that of ursoic acid, with which no 5-LOX inhibition was observed.

The two iridoid glycosides found in *P. lutea*, oleurpoeoside and ligustroside, are also capable of exerting inhibitory actions on enzymes of the arachidonate cascade, although they did not interfere in the 5-LOX pathway in our cellular systems. These compounds exert a preferential effect on the COX-1 pathway. Thus, ligustroside gives rise to reduced PGE$_2$ levels in activated mouse macrophages, and to a lesser extent reduced TXB$_2$ levels in human platelets, while oleurpoeoside only has significant effect on PG synthase activity. Our results also identify some chemical features concerning the inhibition of arachidonate metabolism by our iridoids, although they appear to be at variance with previous investigations in vivo. In previous work, we suggested that selective inhibition of TXB$_2$ release is the primary target of action of some iridoids of plant origin, but an inhibitory effect on the PG-synthase pathway in our cellular systems is unlikely. However, if the magnitude of the antiinflammatory effect is compared with chemical structure of iridoids tested, some relationships can be observed which are consistent with previous studies in vivo.

As occurs in the iridoids tested in the present experiment, the opening of the cyclopetanone ring of the iridoid molecule, and derivatization to a dihydroxyphenylethanol ester (oleuropeoside), both of which decreased in vivo topical activity, are accompanied by a decrease in TXB$_2$ activity, but are two of the most positive chemical features for PG-synthase activity. Furthermore, enhancement of the COX-1 inhibitory activity was found in ligustroside, with loss of hydroxy functionality at the C-5’ position of oleuropeoside. It is also worth noting the ability of compounds to penetrate intact macrophages and platelets, which depends on their liposolubility. The interaction observed between compounds and model membranes can be largely explained in terms of a fluidifying effect due to the introduction of lipophilic molecules in the ordered structure of the lipid bilayer. Although the carbohydrate chain played an important role in the membrane activity of the iridoid glycosides, the presence of different substituents in the backbone structure has been clearly demonstrated to modulate their incorporation or interaction with lipids in the membrane. It is possible that the rate of the iridoid effect depends on the glycoside level in the cell membrane, as can be deduced from the data on the iridoids tested in the present experiment.

Regarding these results, selective inhibition of the COX pathway, with greater PG-synthase than TX-synthase activity, is suggested to be the primary target of action of compounds isolated from *P. lutea*. It is unlikely that the activity of oleancic acid and oleuropeoside is associated with an inhibitory effect on the TX-synthase pathway in our cellular systems. However, since the synthesis of PGs involves two stages, cyclo-oxygenation followed by isomerization, suppression of PGE$_2$ formation may also be explained by the inhibition of PG-isomerase due to these two compounds. Moreover, oleancic acid can be classified as a “dual inhibitor,” since it was evident that both COX and LOX “arms” of arachidonate metabolism were inhibited by this compound, with greater LOX than COX activity. In conclusion, our compounds possess an array of potentially beneficial anti-eicosanoid properties which may, alongside other constituents, contribute to the claimed therapeutic properties of the plant from which they are derived. The extent to which chemical features may account for the actions of compounds on the two pathways of arachidonate metabolism considered in this paper, and the relationships with their protective effect in vivo, requires further rigorous analysis.

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