JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 1275–1282

Received March 12, 2003
Accepted May 20, 2003
DOI 10.1211/0022357021620
ISSN 0022-3573

Influence of traditional Chinese anti-inflammatory medicinal plants on leukocyte and platelet functions

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Abstract

The enzymes 5-lipoxygenase and elastase are therapeutic targets in dermatological disorders such as psoriasis. Fifteen extracts from traditional Chinese medicinal plants used to treat topical inflammations were screened for their inhibitory effect on lipoxygenase, cyclooxygenase and elastase activity in intact leukocytes and platelets. *Astragalus membranaceus*, *Forsythia suspensa* and *Poria cocos* inhibited 5-lipoxygenase, with IC50 values of 141, 80 and 141 μg mL^{-1}, respectively. The latter two species, along with *Angelica dahurica* and *Angelica pubescens*, also inhibited elastase (IC50 values of 80, 123, 68 and 93 μg mL^{-1}, respectively), while *A. pubescens*, *Atractylodes macrocephala*, *Lentinus edodes*, *Rehmannia glutinosa* and *Paeonia lactiflora* selectively inhibited 12-(*S*)-HHTrE production, a valid marker of cyclooxygenase activity. The inhibition of phospholipase A_2 activity by *P. cocos* is discussed. Dehydrotumulosic and pachymic acids, which have been isolated from *P. cocos*, were shown to inhibit leukotriene B_4 release. The results indicate that both *P. cocos* and *F. suspensa* are potentially valuable species in the management of skin pathologies involving chronic inflammation.

Introduction

Psoriasis is one of the most important chronic pathologies of the skin. Apart from being usually recurrent, psoriasis can often be very debilitating, with 5–10% of all patients developing psoriatic arthritis, which causes inflammation and swelling in the hands, feet and large joints (DiSepio et al 1999). Conventional dermatology has made significant progress in the treatment of this disease, but the lack of a complete response often leads patients to try phytotherapy-based remedies.

Traditional Chinese medicine has built up a sophisticated system for treating skin disorders. However, herbal remedies are usually used in their native countries in accordance with protocols that are different from those used in Western countries. Therefore, it is necessary to assess the safety and efficacy of traditional medicinal plants from the rational perspective of Western medicine. We have previously shown that extracts obtained from medicinal plants used in traditional Chinese medicine have anti-inflammatory and antioxidant activity in different in-vivo and in-vitro experimental models (Ríos et al 1996; Cuéllar et al 1997, 1998, 2001; Schinella et al 2002).

The selection of medicinal plants by traditional Chinese medicine to treat dermatological diseases involving chronic inflammation is done on a complex, multifactorial basis. The diagnosis is usually what traditional Chinese medicine experts refer to as "kidney yin deficiency", which may be interpreted as a lack of endogenous cortisol. This compound is used clinically in Western medicine in the form of hydrocortisone or prednisone to help control many disorders, including acute inflammations, rheumatoid arthritis, allergies and many eruptic skin diseases. The unprocessed *Rehmannia glutinosa*, which is a cortisol-like substance, has the advantage of not suppressing, but rather enhancing, the immune system in many cases. *Angelica* species, traditionally used in the treatment of psoriasis, also act via the "kidney channel" and have been proven to alleviate pain (Amenta et al 2000). Since the spleen also plays a major role in immune function, Chinese medicine sometimes

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Acknowledgements and funding: The authors wish to thank Mr Carlos Llopis from Asia Natural Products S.L. for kindly supplying the plant material, and the Centre de Transfusions de la Comunitat Valenciana for supplying the buffy coats. This study was supported by the Spanish Government (DGESIC, PM98-0206). José M. Prieto was the recipient of a grant from the Spanish Ministerio de Educación y Cultura (FP96 20418957).

calls for the addition of "spleen dampness removing herbs" such as *Poria cocos*, as well as "spleen chi tonics" from species of the genera *Atractylodes* and *Astragalus*. According to traditional Chinese medicine, *Lentinus edodes* acts as a "liver-enhancing" herbal medicine, thus protecting the liver from damage associated with autoimmunity, inflammation, oxidation and infection. *Coptis chinensis*, *Paeonia lactiflora*, *Forsythia suspensa* and *Curcuma aromatica* provide analgesic and bacteriostatic properties, along with *Codonopsis pilosula*, which acts as a tonic (Tang & Eisenbrand 1992). Finally, *Phellodendron amurense* and *Scutellaria baicalensis* species are constituents of the traditional Chinese medicine prescription known as "Three Yellow Cleanser", which is commonly recommended for over 60% of all skin diseases.

In contrast to previous thinking, which maintained that leukotriene B_4 (LTB_4) is a simple chemotactic agent, the current understanding in Western medicine is that it is responsible for the long-term maintenance of topical inflammations (Rao et al 1994), making it an important target for the clinical management of psoriasis. Under experimental conditions, inhibitors of LTB_4 production have been shown to completely block hyperproliferation, oedema and neutrophil infiltration; these inhibitors are still in the pre-clinical developmental phase (DiSepio et al 1999). Because leukotriene formation and elastase release via neutrophil stimulation are simultaneously increased in a variety of inflammations and hypersensitivity-based diseases in humans (Bernstein et al 1994; Mayatepek & Hoffmann 1995), the putative antiphlogistic activity of extracts prescribed for such disorders may arise from their ability to block these two pro-inflammatory enzymes. The aim of our research was to assess the effect of selected extracts on the release and activity of LTB_4 and human elastase from intact human neutrophils, and to test their effects on the activity on cyclooxygenase 1 (COX-1) and 12-lipoxygenase in intact human platelets. We hoped to gain insight into the possible chemical mechanisms of these extracts.

Material and Methods

Biological material

The plants studied (Table 1) were commercial samples provided by Asia Natural Products (Amposta, Spain), with the exception of the commercial sample of fruiting bodies of *L. edodes* (voucher specimen DF-93-04), which was identified and kindly donated by Professor Peris from the Departament of Botany, University of Valencia, Burjassot, Spain. The medicinal plants tested were certified according to the Chinese Pharmacopoeia standards by the staff of the Traditional Chinese Medicine Institute of Amposta (Spain).

Buffy coats were obtained from the Centre de Transfusions de la Generalitat Valenciana (València, Spain).

Wistar rats, 12–14 weeks old, were provided by the animal facility of the Faculty of Pharmacy (University of València, Spain). The animals were maintained in standard nutritional and environmental conditions. Housing conditions and all in-vivo experiments were approved by the institutional Ethics Committee of the Faculty of Pharmacy, University of Valencia, in accordance with the guidelines established by the European Union Regulations on the Use of Animals for Scientific Purposes (CEE Council 86/609).

Extraction

The dry crude drugs were powdered. Extraction was performed by stirring plant material (50 g) with the solvent (500 mL) for 24 h, except for aqueous infusions, which lasted 10 min. The percentage yield is given in Table 1. After filtration, the marc was extracted twice under the same conditions. The solvents were removed under vacuum and the residues and water extract were lyophilized. Plant extracts were dissolved in dimethylsulfoxide (DMSO) by vigorous shaking for 30 min. Non-soluble parts were removed by centrifugation (10 min, 10 000 *g*) and solutions were adjusted to 40 mg mL^{-1}. The final

Table 1 Plant species tested.

Species	Part used	Solvent	Extraction yield (w/w)
Angelica dahurica (Fisch.)Benth et Hoocker (Apiaceae)	Root	Water	10.5
Angelica pubescens Maxim. f. (Apiaceae)	Root	EtOH 70%	22.5
Angelica sinensis (Oliv.) Diels (Apiaceae)	Stem	EtOH 70%	27.0
Astragalus membranaceus (Fisch.) Bunge (Leguminosae)	Root	EtOH 70%	16.5
Atractylodes macrocephala Koidzumi (Compositae)	Root	Water	32.5
Codonopsis pilosula (Franch) Nannfeldt (Campanulaceae)	Root	Water	25.0
Coptis chinensis Franch (Ranunculaceae)	Root	MeOH	17.5
Curcuma aromatica Salisb. (Zingiberaceae)	Rhizome	Water	8.0
Forsythia suspensa (Thunberg) Vahl. (Oleaceae)	Fruits	EtOH	11.5
Lentinus edodes Berk (Polyporaceae)	Fruiting bodies	Water	8.0
Paeonia lactiflora Pallas (Rutaceae)	Root	Water	7.0
Phellodendron amurense Ruprecht (Rutaceae)	Root bark	MeOH	19.5
Poria cocos (Schw.) Wolf (Polyporaceae)	Sclerotia	EtOH	1.4
Rehmannia glutinosa (Gaertnet) Liboschitz (Scrophulariaceae)	Root	EtOH	19.0
Scutellaria baicalensis Georgi (Lamiaceae)	Root	MeOH	22.0

concentration of DMSO was 0.5% in all incubations, including controls.

Chemicals

Boswellia serrata resin was obtained from H15 tablets (Gufic Chemicals, India), formononetin was obtained from Apin Chemicals Ltd (Abingdon, UK) and astragaloside I was a kind gift from Dr Li Junshan (Medical School of Beijing, Beijing, China). All other chemicals were of the highest analytical grade available and were purchased from Sigma (St Louis, MO, USA) or Merck (Darmstadt, Germany). Ultrapure high-performance liquid chromatography (HPLC) solvents were provided by Baker (Deventer, Holland).

Solid phase extraction (SPE) and analytical HPLC system

SPE was performed with Lichrolut columns C_{18}, 100 mg, 1 mL (Merck), attached to a 12-port vacuum manifold Visiprep (Supelco, St Louis, MO, USA). HPLC with diode array detection (HPLC-DAD) was performed on a Merck-Hitachi system (Intelligent Pump L-6200) equipped with an L-7455 Diode Array Detector and an L-7200 Auto Sampler, with an injection valve (Rheodyne, Rohnert Park, CA, USA) and a loop of 100 μL. A Lichrospher C_{18} precolumn (4 × 4 mm, 5 μm; Merck), Lichrospher C_{18} column (250 × 4 mm, 5 μm; Merck) and HSM-7000 software (Merck-Hitachi) was used. The elution profile was: 0–27 min, 100% A; 27.1–27.6 min, 0% A; 27.7–40 min, 100% A, the eluents being MeOH/H_2O (74:26) + trifluoroacetic acid (0.007%) (A) and MeOH (B). The flow rate was 1 mL min^{-1}.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The assay described by Mosmann (1983) was used as a test of cytotoxicity. The assay is based on the capacity of mitochondrial dehydrogenase enzymes to convert in living cells the yellow water-soluble substrate MTT into a dark blue formazan product that is insoluble in water. The coloured metabolite was dissolved in DMSO in an ultrasonic bath and measured using a Labsystem Multiskan MCC/340 plate reader (Labsystem, Helsinki, Finland) at 490 nm. Before carrying out the assay, human leukocytes (10^6 cells) were preincubated at 37 °C for 30 min with phosphate-buffered saline (PBS; pH 7.4) containing the extracts at 200 μg mL^{-1}. Controls received vehicle and corresponded to 100% viability.

Determination of 5-lipoxygenase activity

We followed the protocol established by Safayhi et al (1995). Briefly, cells were harvested by intraperitoneal injection of glycogen (1 mg g^{-1} bodyweight in 10 mL of Dulbecco's PBS, 37 °C) and washed twice by centrifugation (10 min, 300 *g*, room temperature). The cell viability of the elicited rat peritoneal leukocytes was assessed before each experiment using the trypan blue exclusion test. Only harvested cells with a viability greater than 95% were used. The cells (5×10^6) were finally resuspended in 1 mL PBS (with glucose, 1 g L^{-1}) and preincubated for 5 min with the test compounds at room temperature. Then, the reaction was started by addition of ionophore A23187 and Ca^{2+} (final concentrations 1.9 μM and 1.8 mM, respectively). After 5 min at 37 °C, the reaction was stopped with 1 mL of MeOH/1 M HCl (97:3), and 500 pmol of prostaglandin B_2 was added as an internal standard. After centrifugation (5 min, 10 000 *g*, 0 °C), the samples were applied to C_{18} SPE columns (100 mg), which were conditioned with 1 mL MeOH and 1 mL water. The columns were washed with 1 mL water and 1 mL 25% MeOH. The 5-lipoxygenase metabolites, namely 5-(*S*)-hydroxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid (5-(*S*)-HETE), LTB_4, 6-*trans*-LTB_4 and 6-*trans*-12-epi-LTB_4 (all-*trans*-isomers) were extracted with 300 μL of MeOH and analysed by HPLC-DAD.

LTB_4 enzyme immunoassay

Rat peritoneal leukocytes (95% viability, trypan blue exclusion test) were prepared and stimulated according to the previous protocol. The production of LTB_4, after purification by SPE, was determined by a specific enzyme immunoassay kit from Cayman Chemicals (Ann Arbor, MI, USA), employed according to the manufacturer's instructions.

Determination of COX-1/12-lipoxygenase activity

The assay was a modification of a protocol described by Laufer et al (1995). Cells were obtained by diluting human buffy coats with PBS (1:3) and centrifuging twice (10 min, 300 *g*), discarding the pellets and keeping the platelet-rich supernatants. After centrifuging, the resulting pellet was washed twice (10 min, 1000 *g*) and finally resuspended in Hank's Balanced Salt Solution (HBSS) with Ca^{2+} (1 mM) and Mg^{2+} (0.5 mM). The differential counting was done using a Sysmex D-800 coulter counter (Sysmex, Norderstedt, Germany). Semi-quantitative estimation of platelet viability was performed by fluorescence microscopy (Nikon, Japan), with acridine orange/ethidium bromide solution staining.

Aliquots of 80×10^6 platelets were preincubated for 5 min with the extract at room temperature. The reaction was started by addition of ionophore A23187 (final concentration 1.9 μM). After 1 min at 37 °C, the reaction was stopped with 1 mL of MeOH/1 M HCl (97:3), and 500 pmol of prostaglandin B_2 was added as internal standard. The platelets were subjected to the same processing as described for rat peritoneal leukocytes. The measured metabolites were 12-(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid (12-(*S*)-HHTrE) from the COX-1 pathway and 12-(*S*)-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-tetraenoic acid (12-(*S*)-HETE) from the 12-lipoxygenase pathway. 15-(*S*)-hydroxy-5*Z*,8*Z*,11*Z*, 13*E*-tetraenoic acid (15-(*S*)-HETE) could be also monitored.

Release and activity of human leukocyte elastase (HLE)

The effect of extracts on HLE activity and, indirectly, the degranulation process were assayed following the protocol described by Barret (1981). Human leukocytes were obtained by diluting human blood buffy coats with PBS (1:3) and centrifuging twice (10 min, 300 *g*), discarding the supernatants and keeping the leukocyte-rich pellets. After 10 min centrifugation at 300 *g* with lyse medium, the resulting pellet was washed twice and finally resuspended in HBSS with Ca^{2+} (1 mM) and Mg^{2+} (0.5 mM). Polymorphonuclear leukocytes (1.25×10^6) were suspended in 500 μL HBSS containing Ca^{2+} and Mg^{2+} and preincubated at 37 °C for 5 min. The cells were then incubated for 5 min in the presence of the extracts at 200 $\mu g\,mL^{-1}$. After 5 min, cells were stimulated by 5 μL of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (65 $\mu g\,mL^{-1}$). After 10 min, the mixture was placed on ice. It was then centrifuged at 3220 *g* and the supernatant (200 μL) was placed in a 96-well microtiter plate with the substrate *N*-*t*-Boc-L-alanine *p*-nitrophenyl ester. After incubation at 37 °C for 30 min, the enzyme activity was determined by colorimetry using a Labsystem Multiskan MCC/340 plate reader set to measure absorbance at 414 nm. In this assay, the interference of the extracts on the degranulation process and their inhibitory effect on HLE activity were accessed. Only the active extracts were assayed to determine their effect on elastase activity. Briefly, supernatants of TPA-stimulated leukocytes (1×10^6) were incubated at 37 °C for 15 min with extracts at a final concentration of 200 $\mu g\,mL^{-1}$ and the procedure described above was then followed.

Statistical analysis

Percentage inhibition is given as mean ± s.e.m. of three or more independent experiments, and every experiment was performed in duplicate. The inhibition of 5-lipoxygenase total activity is expressed as a percentage with respect to the control, which includes LTB_4, the all-*trans*-isomers of LTB_4 and 5-(*S*)-HETE. Inhibition of COX-1 and 12-lipoxygenase activity is expressed as a percentage with respect to the control of 12-(*S*)-HHTrE and 12-(*S*)-HETE, respectively. For HLE release/activity assays, absolute values of absorbance at 414 nm were used for the analysis. Background colours of the extracts were corrected.

Statistical evaluation was performed by analysis of variance followed by Dunnett's *t*-test for multiple comparisons using GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA, USA).

Results

None of the extracts had cytotoxicity in the MTT assay at a concentration of 200 $\mu g\,mL^{-1}$ (data not shown). The effect of extracts on the production of 5-lipoxygenase metabolites, 12-(*S*)-HHTrE and 12-(*S*)-HETE are summarized in Table 2, and the results of the HLE assays are presented in Table 3.

5-Lipoxygenase inhibition

We established the IC50 values for the total 5-lipoxygenase activity of three extracts, namely *Astragalus membranaceus* (126 $\mu g\,mL^{-1}$, r = 0.9850, $P < 0.05$), *F. suspensa* (80 $\mu g\,mL^{-1}$, r = 0.9848, $P < 0.05$) and *P. cocos* (141 $\mu g\,mL^{-1}$, r = 0.9887, $P < 0.05$). The extract of the reference, *B. serrata*, had an IC50 of 22 $\mu g\,mL^{-1}$ (r = 0.9915, $P < 0.05$). *Angelica sinensis*, *P. amurense* and *S. baicalensis* completely inhibited the production of 5-(*S*)-HETE at 200 $\mu g\,mL^{-1}$, but their effect on the total activity of the enzyme could not be measured due to interference between their secondary metabolites and the eicosanoids and/or the internal standard. In the case of *C. chinensis*, the IC50 for the inhibition of 5-(*S*)-HETE was 194 $\mu g\,mL^{-1}$ (r = 0.9977, $P < 0.05$).

Pachymic and dehydrotumulosic acids, isolated from *P. cocos* ethanolic extract (Giner-Larza et al 2000a), inhibited by 60% and 58%, respectively, the LTB_4 release by intact cells as measured by enzyme immunoassay at a dose of 100 μM (control: 336 ± 97 pg; dehydrotumulosic acid: 134 ± 19 pg, $P < 0.01$; pachymic acid: 140 ± 51 pg, $P < 0.01$). Astragaloside I and formononetin, representative secondary metabolites of *A. membranaceus*, were inactive.

COX-1 and 12-lipoxygenase inhibition

Only three extracts were able to inhibit both pathways at 200 $\mu g\,mL^{-1}$. They were *C. chinensis*, *P. amurense* and *P. cocos*. *Angelica pubescens*, *Astragalus membranaceus*, *Atractylodes macrocephala*, *C. pilosula*, *P. lactiflora* and *R. glutinosa* were selective inhibitors of COX-1 activity, but none of the extracts selectively inhibited 12-lipoxygenase activity. In the case of human platelets, we used piroxicam and nordihydroguaiaretic acid as reference compounds. Piroxicam inhibited 12-(*S*)-HHTrE with an IC50 of 7.8 μM (r = 0.9985, $P < 0.01$), and nordihydroguaiaretic acid inhibited both 12-(*S*)-HHTrE and 12-(*S*)-HETE production with IC50 values of 12.2 and 5.3 μM, respectively (r = 0.9981 and 0.9982, respectively, $P < 0.01$).

Phospholipase A_2 (PLA_2) inhibition

To determine the possible PLA_2 inhibition by *P. cocos* extract, we measured the 12-(*S*)-HHTrE and 12-(*S*)-HETE production by intact platelets in the presence of 200 $\mu g\,mL^{-1}$ of the extracts and 10 μM exogenous arachidonic acid (Figure 1). *P. cocos* inhibited 12-(*S*)-HHTrE production by 58 ± 11% (n = 3, $P < 0.01$), but failed to inhibit 12-(*S*)-HETE production (90 ± 9%, n = 4, $P > 0.05$). Under the same conditions, *A. membranaceus* had similar percentages of inhibition with or without exogenous arachidonic acid (27 ± 3%, n = 4, $P < 0.01$ for 12-(*S*)-HHTrE; 101 ± 15%, n = 4, $P > 0.05$ for 12-(*S*)-HETE).

Table 2 Effect of plant extracts ($200\,\mu g\,mL^{-1}$) on the production of 5-lipoxygenase, 12-lipoxygenase and cyclooxygenase 1 (COX-1) metabolites.

Species	5-Lipoxygenase			COX-1	12-Lipoxygenase
	LTB_4	5-(*S*)-HETE	Total	12-(*S*)-HHTrE	12-(*S*)-HETE
Control	100 ± 6	100 ± 9	100 ± 14	100 ± 5	100 ± 7
Angelica dahurica	89 ± 8	119 ± 22	98 ± 8	EI	EI
Angelica pubescens	110 ± 21	51 ± 10	85 ± 7	5 ± 4**	83 ± 11
Angelica sinensis	EI	Absence	ND	EI	EI
Astragalus membranaceus	51 ± 8*	36 ± 13*	37 ± 7**	28 ± 8**	101 ± 15
Atractylodes macrocephala	86 ± 4	90 ± 1	86 ± 4	56 ± 9**	110 ± 17
Codonopsis pilosula	65 ± 4	57 ± 18	62 ± 9	66 ± 8*	EI
Coptis chinensis	EI	44 ± 19*	ND	11 ± 11**	30 ± 9**
Curcuma aromatica	88 ± 1	86 ± 13	86 ± 5	111 ± 8	106 ± 18
Forsythia suspensa	12 ± 19**	60 ± 16	24 ± 10**	EI	119 ± 9
Lentinus edodes	103 ± 8	95 ± 8	104 ± 8	67 ± 11*	91 ± 25
Paeonia lactiflora	72 ± 9	72 ± 6	72 ± 6	30 ± 8**	96 ± 14
Phellodendron amurense	EI	Absence	ND	14 ± 8**	35 ± 6*
Poria cocos	42 ± 12**	38 ± 18*	39 ± 7**	25 ± 8**	44 ± 7*
Rehmannia glutinosa	107 ± 6	104 ± 10	104 ± 3	61 ± 9*	120 ± 18
Scutellaria baicalensis	EI	Absence	ND	EI	EI

Absolute amounts of eicosanoids were calculated by the corresponding molecular extinction factor. Amounts of 5-lipoxygenase products in absolute values (pmol product per 5×10^6 polymorphonuclear leukocytes per 5 min incubation; n = 12) were 480 ± 26 for leukotriene B_4 (LTB_4), 485 ± 45 for 5-(*S*)-HETE, and 1072 ± 88 for the sum of LTB_4, its all-*trans*-isomers and 5-(*S*)-HETE. The absolute amounts of COX-1 and 12-lipoxygenase products (pmol product per 80×10^6 platelets per 1 min incubation; n = 25) were 214 ± 10 and 360 ± 26 for 12-(*S*)-HHTrE and 12-(*S*)-HETE, respectively. Data are expressed as percentages with respect to the control (mean ± s.e.m., n = 3–12). *$P < 0.05$; **$P < 0.01$. EI, extract interferes with UV detection; ND, not determined; Absence, complete inhibition of synthesis of the metabolite.

Table 3 Effect of plant extracts ($200\,\mu g\,mL^{-1}$) on human leukocyte elastase (HLE) release and activity.

Species	HLE release	HLE activity
Control	80 ± 12	104 ± 1
Angelica dahurica	17 ± 4**	36 ± 13**
Angelica pubescens	31 ± 9**	22 ± 10**
Angelica sinensis	83 ± 5	ND
Astragalus membranaceus	49 ± 9	ND
Atractylodes macrocephala	90 ± 8	ND
Codonopsis pilosula	78 ± 9	ND
Coptis chinensis	EI	ND
Curcuma aromatica	85 ± 6	ND
Forsythia suspensa	9 ± 4**	28 ± 1**
Lentinus edodes	77 ± 6	ND
Paeonia lactiflora	EI	ND
Phellodendron amurense	EI	ND
Poria cocos	33 ± 8**	46 ± 9**
Rehmannia glutinosa	76 ± 8	ND
Scutellaria baicalensis	EI	ND

Only the active extracts in the HLE release test were assayed in the HLE activity test. EI, extract interferes with colorimetric detection; ND, not determined. HLE values are expressed as absorbance (mOD) at UV_{414} nm (mean ± s.e.m., n = 4–12). **$P < 0.01$.

HLE activity inhibition

Angelica pubescens ($IC50 = 123\ \mu g\,mL^{-1}$, $r = 0.9620$, $P < 0.05$), *Angelica dahurica* ($IC50 = 80\ \mu g\,mL^{-1}$, $r = 0.9946$, $P < 0.01$), *F. suspensa* ($IC50 = 68\ \mu g\,mL^{-1}$, $r = 0.9738$, $P < 0.05$) and *P. cocos* ($IC50 = 93\ \mu g\,mL^{-1}$, $r = 0.9955$, $P < 0.01$) inhibited HLE activity. *B. serrata*, an established dual 5-lipoxygenase/HLE activity inhibitor (Safayhi et al 1997) was used as a reference for the HLE assays. This extract had an IC50 of $14\ \mu g\,mL^{-1}$ ($r = 0.9923$, $P < 0.01$). The design of the release assay cannot distinguish between release inhibitors and direct enzyme inhibitors. In the present study, all the extracts giving a positive result in this assay were also positive as direct inhibitors of the enzyme. From these results, no specific inhibition of HLE release could be established.

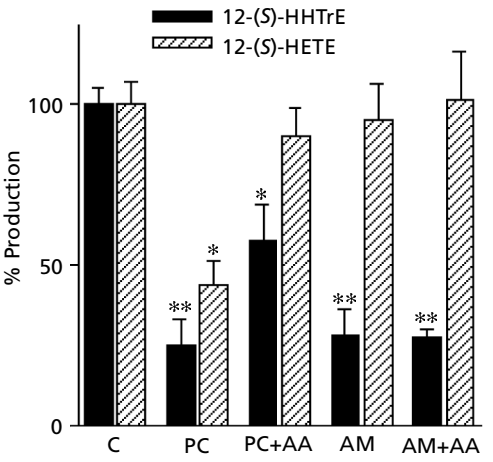

Figure 1 Effect of exogenous arachidonic acid (AA; 10 μM) on the production of eicosanoids by human platelets in the presence of *Astragalus membranaceus* (AM) and *Poria cocos* (PC) extracts at $200\ \mu g\,mL^{-1}$. C, control (dimethylsulfoxide). Data are expressed as percentages with respect to the control (mean ± s.e.m.). $*P < 0.05$; $**P < 0.01$.

Discussion

We report the effects of selected Chinese medicinal plants on 5-lipoxygenase, 12-lipoxygenase, COX-1 and HLE activity when tested in parallel on intact cells. Although data on the activity of some of these herbal medicines are available in the literature, both the extracts and the methodologies used vary to such an extent as to make any comparative evaluation of the efficacy of the extracts virtually impossible. Also, there has been very little work done on intact cells. We also report on 5-lipoxygenase activity, as measured by quantifying all the main metabolites released from the enzyme.

The ethanolic extract of *P. cocos* was the only extract that clearly inhibited not only the production of all the eicosanoids under study, but also HLE activity. In our ex-vivo models, the simultaneous inhibition of 12-(*S*)-HHTrE and 12-(*S*)-HETE in intact human platelets may be due to a direct inhibition of the corresponding enzymes or to a failure of the membranes to release endogenous arachidonic acid in reaction to inhibition of rat and human PLA_2. This particular extract is rich in lanostanes, which are proven PLA_2 inhibitors both in-vitro (Cuéllar et al 1996) and in-vivo (Giner-Larza et al 2000b). To determine the possibility of a direct inhibition of COX-1 and 12-lipoxygenase, we tested the extract with human platelets in the presence of 10 μM exogenous arachidonic acid (Figure 1), thus by-passing the PLA_2 to ensure that the substrate itself is not the limiting factor. Under these conditions, the extract only inhibited 12-(*S*)-HHTrE production, which suggests that this medicinal plant not only acts as a PLA_2 inhibitor, but that it also acts at the COX-1 level.

Pachymic and dehydrotumulosic acids have been isolated from *P. cocos* ethanolic extract (Giner-Larza et al 2000a). In enzyme immunoassays, both compounds were found to inhibit the LTB_4 release by intact cells by about 60% at a dose of 100 μM. This result underscores the important contribution of lanostanes to the activity of *P. cocos* in the treatment of topical inflammation and other processes involving the release of eicosanoids and neutrophil infiltration.

Despite great interest in the pharmacological properties of *A. membranaceus* (Ríos & Waterman 1997), the literature does not contain many references to its anti-inflammatory activity, although our group has previously studied the effects of *A. membranaceus* extract on several in-vivo models of topical inflammation (Cuéllar et al 1998). In the present study, we found that the extract of this species inhibited 5-lipoxygenase and COX-1 activity, but showed no effect on 12-lipoxygenase. These results rule out a direct inhibition of PLA_2 of intact human platelets under the assay conditions used. As the main secondary metabolites present in *A. membranaceus* are saponins and flavonoids, we tested two representative compounds of both chemical classes present in this species (astragaloside I and formononetin, respectively), but found that they did not inhibit eicosanoid release (data not shown). Further work on the isolation of the active principles of this species is in progress, but preliminary data has indicated that after a liquid/liquid partition of the crude extract with solvents of increasing polarity, the 5-lipoxygenase inhibitory activity remains in the dichloromethane fraction (Figure 2).

F. suspensa was the most potent inhibitor of both 5-lipoxygenase and HLE activity. The production of LTB_4 and its all-*trans*-isomers was decreased, while the inhibition of 5-(*S*)-HETE remained insignificant. Kimura et al (1987) have reported that the caffeoyl glycoside derivatives contained in this species are responsible for the inhibition of 5-(*S*)-HETE production in rat peritoneal leukocytes. In this case, the activity of the crude extract emphasizes the notion that all the main metabolites from 5-lipoxygenase must be quantified simultaneously and not individually. The effect on COX-1 human platelets could not be established due to the interference between the extract and the eicosanoids. In contrast, 12-lipoxygenase

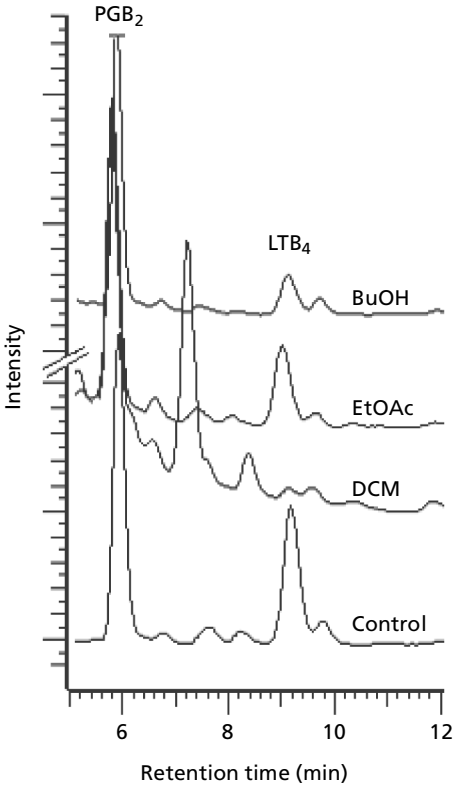

Figure 2 Effect of *Astragalus membranaceus* extracts on leukotriene B_4 (LTB_4) release from rat peritoneal neutrophils. Control: dimethylsulfoxide. DCM, dichloromethane extract; EtOAc, ethyl acetate extract; BuOH, *n*-butanolic extract; PGB_2, prostaglandin B_2.

activity was not affected, thereby ruling out an impairment of human platelet PLA_2 .

The effects of *A. membranaceus*, *F. suspensa* and *P. cocos* found in the present study, together with our previous results (Schinella et al 2002), allow us to make several interesting observations. Both *A. membranaceus* and *P. cocos* inhibit eicosanoid production with similar IC50 values and without any relevant antioxidant effect, thus supporting the theory of a direct, non-redox inhibition of the arachidonate cascade. As for *F. suspensa*, its selectivity towards lipoxygenases may be due to the existence of one or several secondary metabolites with direct non-redox 5-lipoxygenase inhibitors, together with potent antioxidants against enzymatic lipid peroxidation and superoxide production.

In our assays, *P. amurense* and *C. chinensis* showed a similar in-vitro profile: inhibition of 12-(*S*)-HHTrE, 12-(*S*)-HETE and 5-(*S*)-HETE release. Both these species contain a high amount of berberine, an alkaloid with a wide range of pharmacological activities (Tang & Eisenbrand 1992). Other plant extracts containing berberine, such as *Mahonia aquifolium*, also inhibit 5-lipoxygenase activity, but the alkaloid itself does not seem to be responsible for this effect (Müller & Ziereis 1994).

The obtained results with *A. macrocephala*, *C. pilosula* and *P. lactiflora* extracts are valuable as the literature contains little information about their pharmacological properties on intact cells (Wang & Zhu 1990; Tang & Eisenbrand 1992; Resch et al 1998). Our findings clearly demonstrate that they modulate the inflammatory process through a direct and specific inhibition of the COX-1 pathway in intact human platelets.

The extract from *A. pubescens* proved to be a potent and selective inhibitor of COX-1 activity on human platelets, inhibiting 12-(*S*)-HHTrE production by 95%. Liu et al (1998) had previously reported the inhibition of 5-(*S*)-HETE production by the dichloromethane extract of this species. Using the total extract, we observed not only a decrease in the 5-(*S*)-HETE production from endogenous arachidonic acid, but also a parallel increase of LTB_4 production as well as that of its all-*trans*-isomers. Under our assay conditions, the total extract does not seem to impair the 5-lipoxygenase activity completely. *A. pubescens*, along with *A. dahurica*, was thus shown to be a good inhibitor of HLE release/activity.

We have observed that when extracts were able to significantly inhibit the production of 12-(*S*)-HHTrE in intact human platelets, there was an accompanying rise in 15-(*S*)-HETE production. Under normal conditions, 15-lipoxygenase activity in these cells is a minor pathway, leading primarily to the formation of 12-(*S*)-HETE when stimulated by arachidonic acid or ionophore A23187 (Spector et al 1988). We thus propose that this effect is due to the channelling of the released endogenous arachidonic acid that is not metabolized by COX-1 to the 15-lipoxygenase pathway. One of the pharmacological effects of such an increase could actually be increased anti-inflammatory activity, since 15-(*S*)-HETE is itself an inhibitor of both 12-lipoxygenase and PLA_2 activities in human platelets. If we accept the release of 15-(*S*)-HETE as an indication of COX-1 inhibition in intact platelets, *A. pubescens* is active on this enzyme. Other extracts producing an increase of 15-(*S*)-HETE were *A. membranaceus, C. chinensis*, *F. suspensa*, *P. lactiflora*, *P. amurense* and *P. cocos*.

For some of the remaining extracts, only a low or moderate activity was observed, thus rendering them less interesting for the treatment of inflammations when used alone, although there may be complex synergistic actions that justify their inclusion in medicinal herbal formulations. In the case of *R. glutinosa* and *L. edodes* especially, the possible contribution of their immunomodulatory polysaccharides to the long-term in-vivo anti-inflammatory effects of Chinese herbal medicines should not be underestimated.

Conclusions

We have described the in-vitro effects of traditional Chinese medicinal plants used for topical inflammation. Their actions on relevant eicosanoid pathways and hydrolytic enzymes were monitored in parallel on intact cells, with special emphasis on both 5-lipoxygenase and HLE activity. The most interesting species in terms of LTB_4 inhibition were *A. membranaceus*, *F. suspensa* and *P. cocos*. Extracts of the latter two plant species were also found to inhibit HLE. If the blocking of both

5-lipoxygenase and HLE enzymes is considered a primary objective in dermatological disorders, then these two species appear to be valuable therapeutic tools. Our results were obtained with relevant and certified marketed medicinal plants, thus providing the basis for a rational clinical use of these medicinal plants and integration of traditional Chinese medicinal practices into Western medicine.

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