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INHIBITION OF 5-LIPOXYGENASE AND CYCLO-OXYGENASE IN LEUKOCYTES BY FEVERFEW

INVOLVEMENT OF SESQUITERPENE LACTONES AND OTHER COMPONENTS

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Abstract—Leaves or infusions of feverfew, *Tanacetum parthenium*, have long been used as a folk remedy for fever, arthritis and migraine, and derived products are widely available in U.K. health food shops. Previous reports have suggested interactions with arachidonate metabolism. Crude chloroform extracts of fresh feverfew leaves (rich in sesquiterpene lactones) and of commercially available powdered leaves (lactone-free) produced dose-dependent inhibition of the generation of thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) by ionophore- and chemoattractant-stimulated rat peritoneal leukocytes and human polymorphonuclear leukocytes. Approximate IC_{50} values were in the range 5–50 μg/mL, and inhibition of TXB_2 and LTB_4 occurred in parallel. Isolated lactones (parthenolide, epoxyartemorin) were also inhibitory, with approximate IC_{50} values in the range 1–5 μg/mL, as were crude extracts treated with cysteine (to neutralize reactive α-methylene butyrolactone functions of the sesquiterpenes). Inhibition of eicosanoid generation appeared to be irreversible but not time-dependent. We conclude that feverfew contains a complex mixture of sesquiterpene lactone and non-sesquiterpene lactone inhibitors of eicosanoid synthesis of high potency, and that these biochemical actions may be relevant to the claimed therapeutic actions of the herb.

The aromatic herb feverfew (*Tanacetum parthenium*) has been extensively documented by herbalists in Britain since the sixteenth century for the treatment of fevers, aches and pains [1, 2]. Latterly, sufferers from arthritis and migraine have taken to eating fresh feverfew leaves grown domestically [3] or tablets made from dried powdered feverfew leaves which are widely available in health food shops in the U.K. [4]. Although the use of this plant for most indications is largely anecdotal and unsupported by rigorous clinical evidence, two well conducted clinical trials of feverfew as prophylactic against migraine gave encouraging results [5, 6].

These facts have prompted the testing of extracts obtained from feverfew plants in a variety of models which might be relevant to its putative anti-inflammatory analgesic and anti-migraine actions. Thus it was established that feverfew extracts inhibit the generation of prostaglandins from exogenous arachidonic acid in a cell-free system [7] and evidence was obtained suggesting an action at the level of phospholipase A_2 [8, 9], as well as the direct inhibition of cyclo-oxygenase [10]. Extracts of feverfew leaves were also shown to inhibit the aggregation [8] and secretion of 5-hydroxytryptamine from platelets [11] and to inhibit secretion of granular constituents from human polymorphonuclear leukocytes (PMN§) [11]. Moreover it was shown that feverfew can inhibit the secretion of histamine from activated mast cells [12]. Further work demonstrated that various sesquiterpene α-methylene butyrolactones present in feverfew, such as parthenolide and canin [13, 14], were themselves capable of exerting inhibitory actions on platelets [15], and it was proposed that they do so by covalent modification of sulphydryl groups in the target cells [16]. We recently suggested a similar mechanism to explain the irreversible inhibition of vascular smooth muscle contractility caused by fresh parthenolide-containing feverfew extracts [17], and noted that preparations obtained from commercial dried powder which lack α-methylene butyrolactones did not exert these actions [18].

This suggested that it would be timely to investigate the action of feverfew extracts and purified components on both 5-lipoxygenase and cyclo-oxygenase pathways of endogenous (rather than exogenous) arachidonate metabolism using a whole cell system relevant to the inflammatory process, and in which both pathways are expressed. For this reason, we chose rat peritoneal leukocytes that have been shown to be useful for investigating the pharmacological control of eicosanoid metabolism [19]. An additional aim of the work was to compare the activities of extracts of fresh feverfew with those of the commercially available powder.

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§ Abbreviations: PMN, polymorphonuclear; TXB_2, thromboxane B_2; LTB_4, leukotriene B_4; PLA_2, phospholipase A_2; fMLP, *n*-formyl-methionyl-leucyl-phenylalanine.

MATERIALS AND METHODS

Preparation and incubation of leukocytes. Mixed peritoneal leukocytes were elicited from 200–300 g

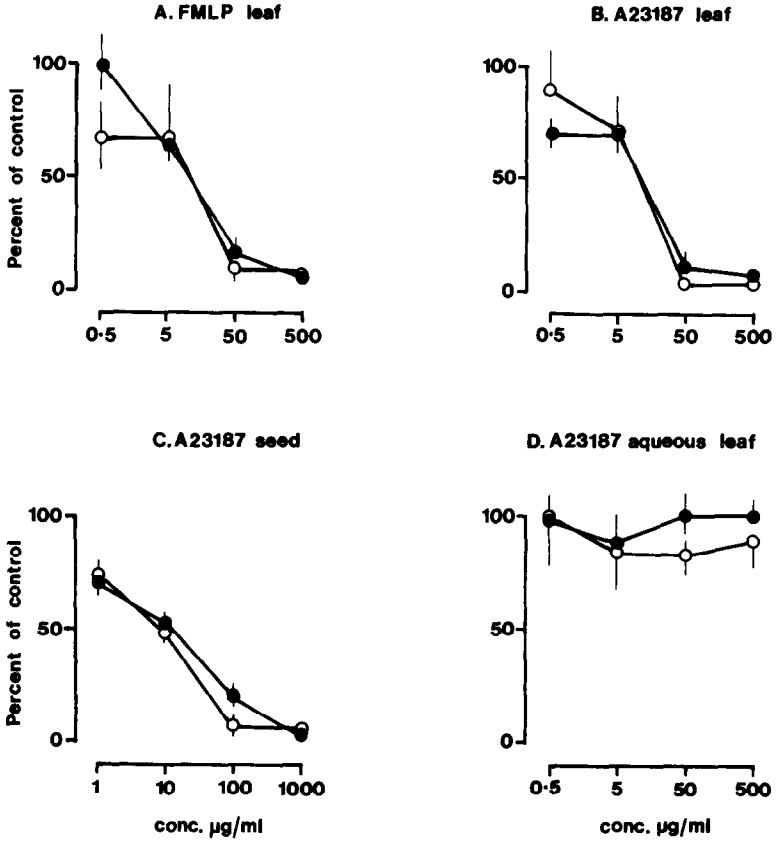

Fig. 1. Inhibition by feverfew extracts of the generation of TXB_2 (○, stable hydrolysis product of TXA_2 and a marker for the cyclo-oxygenase pathway of arachidonic acid metabolism) and LTB_4 (●, marker for 5-lipoxygenase pathway). Extracts of fresh feverfew leaves (A,B,D) or seeds (C) were prepared by steeping the material in chloroform (A,B,C) or water (D). Samples of extract were incubated with rat peritoneal leukocytes as described in Materials and Methods and eicosanoids measured by radioimmunoassay. Cells were stimulated with 10^{-5}M fMLP (A) or 10^{-6}M A23187 (B,C,D). Amounts of eicosanoids generated were: fMLP: TXB_2 13.5 ± 1.0 ng/mL, LTB_4 8.2 ± 0.6 ng/mL; A23187: TXB_2 19.0 ± 2.6 ng/mL, LTB_4 59.3 ± 4.3 ng/mL. Results expressed as per cent of amount generated in control tubes containing methanol vehicle, and are mean values of three tests, each assayed in duplicate. The bars show SEM.

Wistar rats of either sex using i.p. glycogen, and the cells (approx. 85% PMNs and 15% mononuclear cells) were prepared essentially as described by Moroney *et al.* [19]. Suspensions of human PMNs (approx. 97%) were obtained from informed non-medicated volunteers (according to guidelines provided by local ethics committee) using conventional differential sedimentation procedures with Histopaque 1077 [20]. Both types of leukocytes were resuspended in Hank's balanced salt solution containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+} at 5×10^6 cells/mL and 0.5 mL aliquots were taken for incubation. After 5 min pre-incubation at 37° with 5 μL methanol vehicle or different concentrations of feverfew extracts resuspended in methanol, eicosanoid generation by the cells was initiated by addition of 10^{-6} M calcium ionophore A23187 or 10^{-5} M chemoattractant peptide fMLP for 10 min. These stimulants were added in 5 μL dimethyl sulphoxide, with equivalent volumes of solvent in controls. After pelleting the cells, the supernatants were kept for direct radioimmunoassay without extraction of thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) (sensitivities 20 and 10 pg, inter-assay coefficients of variation 12.0 and 8.4%, respectively; values based on 10 successive assays), taken as indicating activity of the cyclo-oxygenase and 5-lipoxygenase pathways of arachidonate metabolism in these cells. Further details may be found in Ref. 19.

Extracts of fresh and dried feverfew plants; preparation of parthenolide. Crude extracts of *Tanacetum parthenium* (L.) Sch. Bip., Compositae, were prepared by stirring freshly gathered leaves or ground seeds in 20 vol. chloroform, using plants grown at Castle Donington, Leicestershire (see Refs 14 and 21 for details). These plants were authenticated in the Department of Botany, University of Nottingham, and voucher specimens are deposited in the herbarium of the Department of Pharmacy, King's College, London. After removing solvent, samples of the viscous dark green oil were stored at

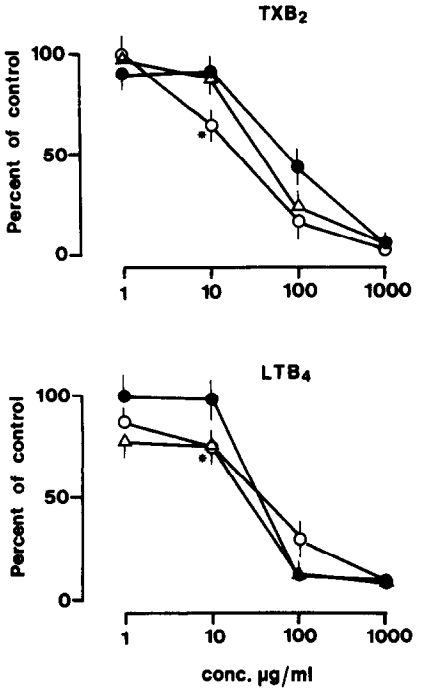

Fig. 2. Effect of pre-incubation on inhibition by feverfew leaf extract of A23187-induced eicosanoid generation by rat peritoneal leukocytes. The ionophore was added immediately after the feverfew extract (●), or 10 min (△) or 60 min later (○). Other details as described in Fig. 1B. Student's unpaired *t*-test was used to evaluate the statistical significance of differences between the 10 and 60 min preincubations and the control (non-preincubated samples): * indicates a significant difference, $P < 0.05$.

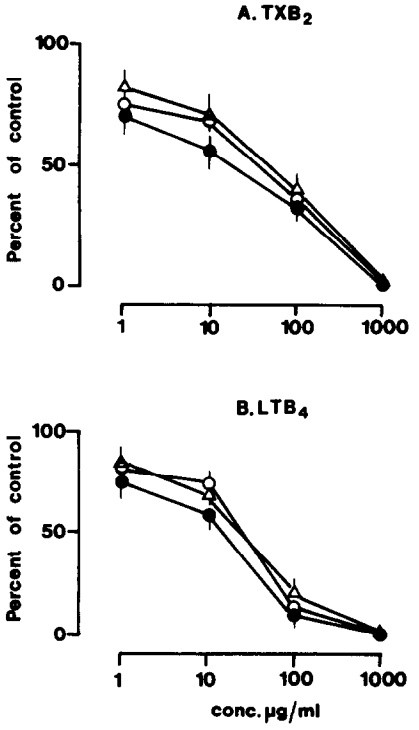

Fig. 3. Inhibition of eicosanoid generation by feverfew is essentially irreversible after dilution of the inhibitor. Rat leukocytes were incubated for 10 min at 37° with a range of feverfew concentrations (chloroform extract of fresh leaves) and then stimulated by adding 10^{-6}M A23187 without further treatment (control, ●), or after pelleting and resuspending the cells under identical conditions once (○) or twice (△). The buffers used for resuspension did not contain feverfew. Each concentration was tested three times, and results are expressed as per cent of control, mean ± SEM. Application of Student's unpaired *t*-test showed that there were no significant differences compared to the control inhibitor treatments.

−20° and when necessary portions were resuspended in methanol (with brief sonication in a bath, Jencons Scientific Ltd) for addition to the leukocyte incubations.

The sesquiterpene lactone content of the crude extracts was determined using the procedure described by Dolman *et al.* [21] in which adducts formed from reaction of the lactones with 9-thiomethylanthracene were quantified by HPLC analysis using anthracene as standard. The crude extracts appeared to be chemically and pharmacologically stable for at least 6 months if stored solvent-free at −20°.

Aqueous extracts were prepared by steeping macerated leaves in hot water and after cooling and filtration, lipophilic material was removed by back extraction into chloroform and water removed by lyophilization. The brown residues from aqueous and organic phases were dissolved in methanol. Extracts of dried powdered leaves of feverfew (supplied by Slater and Frith, Norwich, U.K.) were prepared by stirring a weighed quantity of the powder with chloroform for 1 hr, followed by filtration, drying with $MgSO_4$ and evaporation of solvent. Samples of the brown residue were redissolved in methanol with brief sonication prior to testing.

Parthenolide, epoxyartemorin and tanaparthin-α-peroxide were purified from fresh feverfew leaves by back extraction of aqueous extracts or by chloroform extraction, followed by extensive column chromatography over silica gel as described [14]. Semi-purified fractions were assayed for lactone content either by the chemical-HPLC method described above (Ref. 21), or by proton NMR of samples dissolved in $CDCl_3$. Repeat column chromatography, guided by TLC and NMR, eventually led to the pure materials.

In certain experiments, lactones were neutralized by Michael addition reaction between the α-methylene function and sulphydryl groups. This was achieved by stirring crude extracts in 95% methanol–5% water with excess cysteine for 3 hr at room temperature. Evaporation of solvent gave a treated extract which when assayed after derivatization by HPLC showed no peaks at 369 nm, indicating that all the α-methylene butyrolactone functions had indeed been blocked.

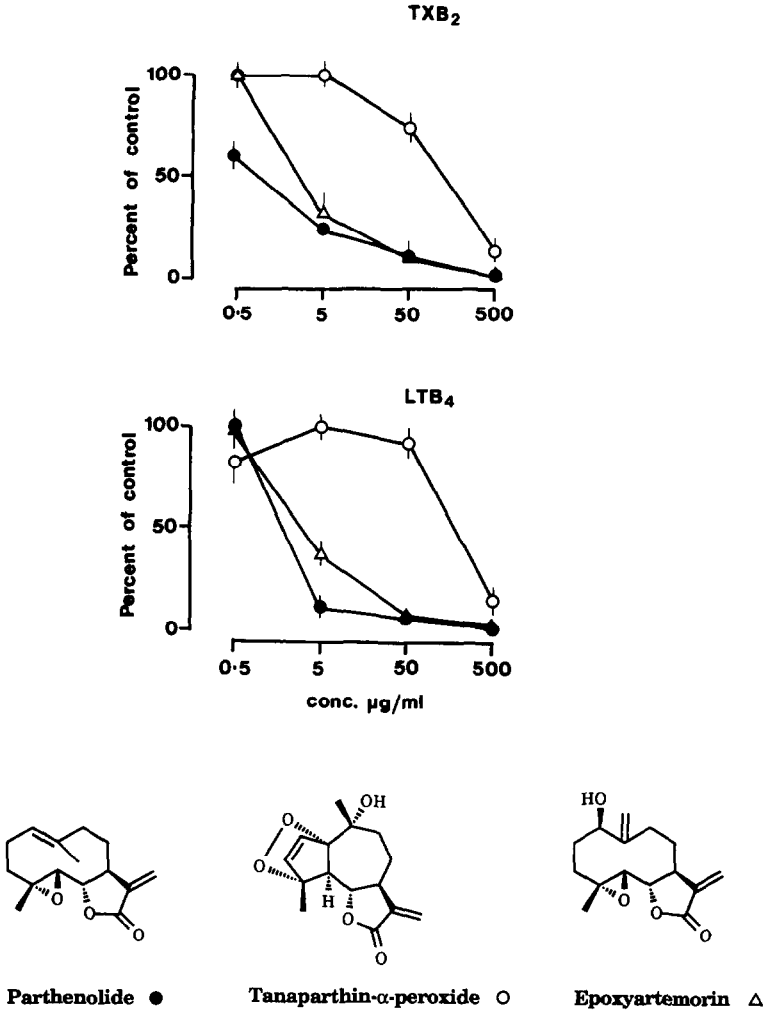

Fig. 4. Inhibition of rat leukocyte TXB_2 and LTB_4 generation by parthenolide (●), epoxyartemorin (△) and tanaparthin-α-peroxide (○). Three tests at each concentration; results expressed as per cent of amounts generated in response to A23187 in control tubes containing methanol vehicle (mean values ± SEM).

RESULTS

Preincubation of intact rat and human leukocytes for 10 min with crude chloroform extracts of fresh feverfew leaves caused a dose-dependent and potent inhibition of their capacity to generate eicosanoid products of the 5-lipoxygenase and cyclo-oxygenase pathways. Figure 1 shows some representative experiments in which rat peritoneal leukocytes were stimulated with calcium ionophore A23187 and the chemoattractant peptide fMLP. As shown previously [22], these agents stimulate both pathways of arachidonate metabolism but in different proportions, reflecting differences in the activation process (A23187: amounts of LTB_4 greater; fMLP: amounts of TXB_2 greater, see legend to figure). Despite these differences, inhibition by the chloroform leaf extract was remarkably similar (Fig. 1A and B), with both TXB_2 and LTB_4 inhibited in parallel fashion. Approximate IC_{50} values for both eicosanoid products were between 5 and 50 μg/mL and at 500 μg/mL inhibition was 90% or greater.

Similar results were obtained when testing chloroform extracts of feverfew seeds (Fig. 1C), whereas an aqueous leaf extract (from which all lipophilic material had been removed by back extraction into chloroform) was essentially inactive over the same concentration range (Fig. 1D). This extraction procedure effectively removes sesquiterpene lactones such as parthenolide as well as many other lipophilic secondary metabolites present within the feverfew.

We investigated whether inhibition of eicosanoid metabolism is time dependent by incubating the rat peritoneal leukocytes with various doses of feverfew inhibitor for 0, 10 and 60 min before adding A23187 to initiate the generation of TXB_2 and LTB_4. The action of A23187 is very rapid, with >80% of the products being released within 10 min [22], so the

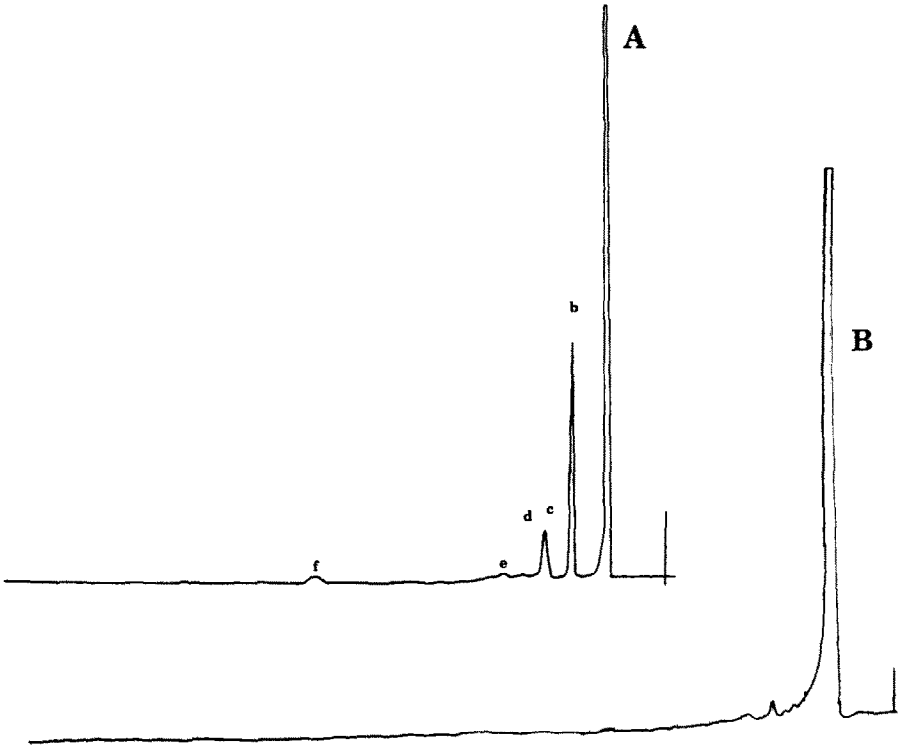

Fig. 5. Removal of sesquiterpene lactones from feverfew by treatment with cysteine. (A) Shows HPLC trace following 0.1 μL injection of material prepared by chloroform extraction of 0.8 g fresh leaves of *Tanacetum parthenium* and derivatization as described in Ref. 21. Identification of peaks is as follows: a, excess 9-thiomethylanthracene; b, parthenolide; c, tanaparthin-β-peroxide; d, tanaparthin-α-peroxide; e, reagent impurity; f, 3β-hydroxycostunolide. Trace (B) (performed under the same conditions) shows results for injection of 2.0 μL of the material used in (A) but after treatment with cysteine in methanol for 2 hr at room temperature. The single large peak represents excess 9-thiomethylanthracene. The lactones have been effectively removed so that derivatization cannot occur.

protocol used here should reveal whether the preincubation with inhibitor enhances its effectiveness, with any such effect shown as a time-dependent leftwards shift of the dose-inhibition curves. The results (Fig. 2) demonstrate that there was a slight trend towards an increased effectiveness of inhibition with preincubation. However, this only reached statistical significance for the effects of 10 μg/mL inhibitor preincubated for 60 min. Overall, we conclude that the inhibitory action of leaf extract is not to any substantial extent enhanced in a time-dependent manner.

To establish whether the inhibitory action of feverfew is reversible, we incubated rat leukocytes with various doses of inhibitor and then exposed them to ionophore either without further treatment (control, conditions similar to those in all previous tests), or after washing the cells once or twice by centrifugation and resuspension. Reversible inhibition would be indicated by rightwards shifts of the dose-inhibition curves as the inhibitor is removed by dilution. The results of this experiment (Fig. 3) show a small trend towards a reduction of the inhibitory effect of feverfew on both TXB_2 and LTB_4 generation following washout by dilution, but the differences were not significant. We conclude that the inhibition is effectively irreversible under these experimental conditions, although they do not indicate whether this is a mechanistic effect of the active compound(s) or due to their inherent high lipophilicity and slow dissociation from the biophase.

The possible involvement of parthenolide and other sesquiterpene lactones in inhibiting eicosanoid metabolism was investigated in two ways. In the first experiment, the purified lactones parthenolide, epoxyartemorin and the lactone endoperoxide tanaparthin-α-peroxide [14] were tested directly. The results (Fig. 4) show that all three compounds caused dose-dependent inhibition of the production of both TXB_2 and LTB_4, although the endoperoxide was much less potent. Approximate IC_{50} values expressed as micromolar are: parthenolide 6, 12 (value against TXB_2 given first), epoxyartemorin 17, 15 and endoperoxide 880, 1050. These data show that sesquiterpene lactones within the crude feverfew extracts may account substantially for their inhibitory properties, but they do not show whether they account for all the activity.

In another approach, we took advantage of the fact that α-methylene butyrolactones are vulnerable to nucleophilic attack by sulphydryl reagents, leading to loss of the crucial methylene function. Treatment of feverfew extracts with cysteine either during the initial aqueous extraction procedure or after

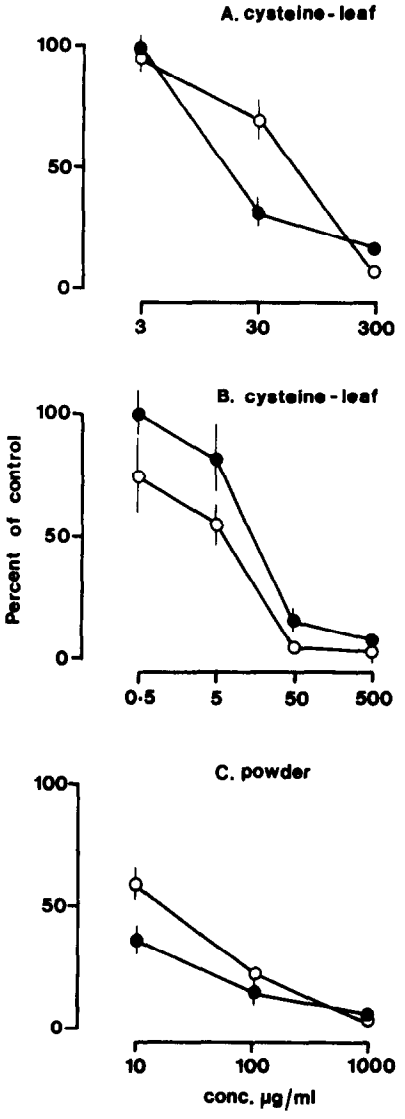

Fig. 6. Inhibition of eicosanoid generation by extracts which do not contain parthenolide. (A) Cysteine-treated feverfew leaf extract (cysteine added during extraction procedure). (B) Resuspended feverfew extract in methanol treated with cysteine. (C) Chloroform extract of commercially available powdered leaves which does not contain butyrolactones. Rat peritoneal leukocytes stimulated with A23187. (○) TXB_2; (●) LTB_4. Three tests at each concentration of inhibitor, values show mean ± SEM.

redissolving the extract in methanol prior to testing led to covalent modification of the lactones and disappearance of the lactone-thiomethylanthracene peaks when the samples were assayed using the chemical-HPLC analysis, as expected (Fig. 5). However, these modified feverfew extracts remained potent as inhibitors of leukocyte eicosanoid generation, as shown in Fig. 6A and B. Moreover, a chloroform extract prepared from commercially available powdered feverfew leaves, and which according to our assay no longer contained measurable quantities of α-methylene butyrolactones, demonstrated potent dose-dependent inhibition of both TXB_2 and LTB_4 generation (Fig. 6C). Thus, it appears that the extracts contain non-butyrolactone components capable of inhibiting eicosanoid generation in the leukocyte system.

In a further set of experiments (Fig. 7), human PMN leukocytes were used as cell source in order to establish the generality of the inhibitory effects of feverfew. These cells make large amounts of LTB_4 in response to ionophore A23187, but very little TXB_2 (see legend to Fig. 7), and as such are not so useful for concomitant studies of the two pathways of arachidonate metabolism. Nevertheless, the results show that, as expected, lactone-rich chloroform extracts of fresh feverfew leaves produce dose-dependent inhibition of both leukotriene and thromboxane pathways in parallel fashion with an approximate IC_{50} value of 10 μg/mL, whereas an aqueous extract free of lactones was not active (cf. Fig. 1D).

The effects on cell viability of feverfew extracts dissolved in methanol and the vehicle itself were tested under identical incubation conditions. The cells were examined by microscope and stained with trypan blue, and the extent of cytosolic lactate dehydrogenase release was measured as described [20]. There was no evidence from these experiments of any toxic effect of the feverfew extracts on the leukocytes.

DISCUSSION

Our results represent the first detailed analysis in a relevant inflammatory cell model of the effects of feverfew and derived products on the two major routes of arachidonic acid metabolism via the cyclo-oxygenase and 5-lipoxygenase pathways. They show that the extracts and two derived sesquiterpene lactones inhibit both of the pathways strongly, with complete suppression of eicosanoid generation at concentrations of 500–1000 μg/mL and IC_{50} values in the range 5–50 μg/mL. The inhibition appears to be irreversible but not markedly time dependent.

In all the examples studied so far it was evident that the concentration-dependent inhibition of both LTB_4 and TXB_2 generation occurs in a parallel fashion. This points to phospholipase as a major site of action, most likely phospholipase A_2 (PLA_2) because this enzyme is of major importance in the initial arachidonate-releasing steps of eicosanoid synthesis in the neutrophil [23–25]. Indeed, we have found that chloroform extracts of feverfew seeds and leaves and of the commercial powder all strongly inhibit human recombinant secretory PLA_2 activity with a potency on a weight basis equal to or greater than mepacrine (J.R.S. Hoult, I. Lobo and D.J. Masters, unpublished experiments). These findings therefore extend earlier work showing that feverfew extracts can inhibit phospholipase [8, 9] as well as the generation of cyclo-oxygenase and 5-lipoxygenase metabolites from labelled exogenous arachidonic acid [26], and that inhibition of prostaglandin biosynthesis in microsomes by feverfew is not due to inhibition of cyclo-oxygenase [7]. However, based on our data we cannot rule out direct actions

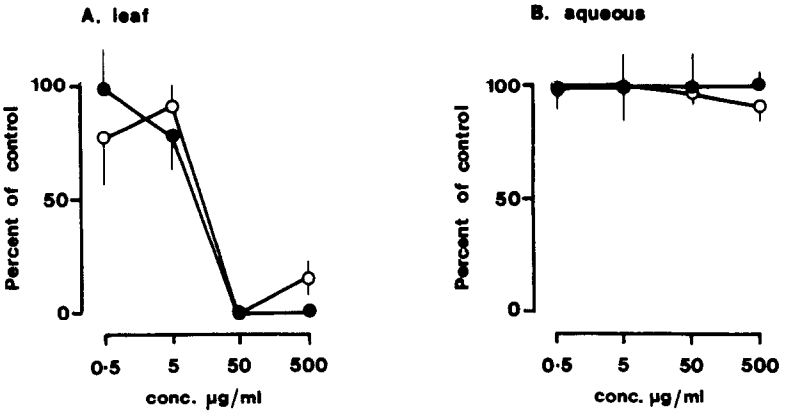

Fig. 7. Effect of feverfew on eicosanoid generation by human peripheral PMN leukocytes stimulated by 10^{-6}M A23187. (●) LTB_4 (control yield: 156 ± 22 ng/mL); (○) TXB_2 (control: 3.1 ± 0.3 ng/mL). Feverfew extracts used were (A) chloroform extract of fresh leaves, (B) aqueous extract (parthenolide-free), both as in Fig. 1.

on both the cyclo-oxygenase and 5-lipoxygenase enzymes, which could be investigated by incubating the leukocytes with radiolabelled arachidonic acid. Nevertheless, the present experimental design is more physiological in that it concerns the activation of endogenous arachidonate metabolism in intact cells.

Our results also suggest strongly that the crude feverfew extracts contain at least two entities or groups of compounds responsible for the eicosanoid inhibition.

First, we found that three purified α-methylene butyrolactones also inhibited thromboxane and leukotriene generation and that two of them (parthenolide and epoxyartemorin) had high potency. These compounds exert other inhibitory biological effects such as on cellular secretory mechanisms [11, 12, 15], cytostatic/anti-tumour activity [27, 28] and have a potent anti-inflammatory profile [27, 29, 30], and in all cases it is thought that the mechanism involves covalent reaction with sulphydryl groups. However, the inhibition of eicosanoid generation seen here may not conform to this pattern as it was not appreciably time dependent (Fig. 2), as would be predicted from such a mechanism. Instead, it may be that the epoxy function (present in parthenolide and epoxyartemorin but not tanaparthin-α-peroxide) is responsible. In this context it is interesting that in the only other study that we know of related to sesquiterpene lactones and leukotriene/thromboxane metabolism it was found that scandenolide (an epoxide-containing α-methylene butyrolactone) was inhibitory whereas coronopilin (a non-epoxide α-methylene butyrolactone) was not [31].

The second reason for suspecting additional non-butyrolactone inhibitors of eicosanoid generation derives from the results in Fig. 6. This showed that cysteine-treated extracts (from which the lactones had been removed) and extracts of commercially available powdered leaves (which do not contain appreciable amounts of butyrolactones) were both active as inhibitors. We do not yet know what are the active ingredient(s), and subfractionation of the crude material is necessary. However, these results contrast very sharply to those we obtained using smooth muscle bioassays: fresh leaf extracts and derived butyrolactones are potent and irreversible inhibitors of aortic contractility, whereas the butyrolactone-depleted powdered leaf extracts are not inhibitory [17, 18].

In summary, *Tanacetum parthenium* contains at least two types of compounds capable of strongly suppressing the generation of eicosanoids, at a site presumed to be PLA_2. There may be possibilities for developing new PLA_2 inhibitors based on these compounds, and these actions may be relevant to the claimed therapeutic properties of the plant.

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