

# PROSTAGLANDINS LEUKOTRIENES AND ESSENTIAL FATTY ACIDS

Prostaglandins Leukotrienes and Essential Fatty Acids (1994) 50, 279–284  
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## The Fern *Polypodium Decumanum*, Used in the Treatment of Psoriasis, and its Fatty Acid Constituents as Inhibitors of Leukotriene B<sub>4</sub> Formation

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**ABSTRACT.** The fern *Polypodium decumanum*, commonly called Calaguala, has a clinically documented use in South America and Spain in the treatment of psoriasis. One of the inflammatory mediators isolated in abnormally high quantities in the psoriatic skin is leukotriene B<sub>4</sub> (LTB<sub>4</sub>). Calaguala was tested in an in vitro model using human leukocytes for its ability to inhibit the LTB<sub>4</sub> formation. The inhibition was found to be caused by the polyunsaturated fatty acids (PUFAs) linoleic, linolenic and arachidonic acid. IC<sub>50</sub> values were determined for the isolated acids and compared to a group of closely related acids also commonly found in nature. The IC<sub>50</sub> values for most acids tested were of the same magnitude (20–60 μM) except for arachidonic acid which showed stimulatory activity and 8(R) hydroxylinoleic acid which gave 30% inhibition with the highest dose tested (120 μM). The amounts of PUFAs in different Calaguala extracts were quantitatively analysed and it is concluded that the fatty acid constituents of Calaguala may contribute to the clinical effects of the extract.

### INTRODUCTION

Calaguala is the common name of the fern *Polypodium decumanum* (Willd.) which in South America is used in the treatment of the skin diseases psoriasis and atopic dermatitis (1, 2). The clinical effects of the extract prepared from the leaves and rhizoma of the plant have been confirmed and documented in clinical trials performed both in Honduras and Spain. The extract is given orally to the patients and only minor side effects, such as slight gastric disturbances, have been observed (3, 4).

Psoriasis is a widely spread chronic hyperproliferative and inflammatory disease with symptoms mainly from the skin and with a probable immunological aetiology (5). Abnormalities in the amounts and function of several inflammatory mediators have also been documented (6).

In previous works we have reported the effect of Calaguala in a skin transplantation model in mice (7) indicating a possible immunosuppressive effect. We have also studied Calaguala's activity in two in vitro models for the inflammatory mediator PAF (Platelet Activating Factor) and confirmed a dose-dependent inhibition of the extract in both assays (8).

Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) has been isolated in elevated amounts in the skin lesions from psoriatic patients (9). LTB<sub>4</sub> has several effects on leukocytes, including chemotaxis, aggregation, degranulation and superoxide generation. In addition, it is capable of inducing keratinocyte mitogenic activities and thereby able to induce cutaneous inflammation characterized by neutrophil accumulation and epidermal hyperproliferation. These events are both typical for psoriasis (10). Furthermore, the administration of benoxaprofen which is considered to be an inhibitor of LTB<sub>4</sub>, has been shown to improve the course of the disease (11). In another study (12) topical application of lonapalene, a LTB<sub>4</sub> inhibitor, was able to produce a clinical improvement with concomitant decrease in the content of LTB<sub>4</sub> in the skin.

The aim of this study was to investigate the effect of Calaguala on the synthesis of LTB<sub>4</sub>. The in vitro bio-assay was then used as a guide in the fractionation of the extract and isolation of the active components in the plant. Furthermore, since the active compounds isolated turned out to be polyunsaturated fatty acids (PUFAs), a study was performed in order to investigate which structural features were essential for the effect that fatty acids have on the LT biosynthesis. As a final step a high performance liquid chromatography (HPLC) method was set up to quantitatively analyse the amount of fatty acids in the Calaguala extract.

Date received 14 July 1993  
Date accepted 5 November 1993

## EXPERIMENTAL

### Plant material

Leaves of *P. decumanum* Willd. (Polypodiaceae) were collected in October 1988 (for the quantitative analysis of the different batches the collection times were 1 January 1988, 2 October 1988, 3 April 1989 and 4 November 1988) from a cultivation at Lago de Yogo, Honduras. The plant was identified by Dr Robert Stolze, Dept of Botany, Field Museum of Natural History, Illinois, USA, where a voucher specimen is kept.

### Preparation and fractionation of the Calaguala extract

The dried and ground leaves (6.6 kg) were extracted with 3 × 35 l methanol (puriss) overnight, under stirring at room temperature. The extract was filtered, concentrated in vacuo, and lyophilized (yield: 267 g). According to the Scheme, 50 g of the methanolic extract was then dissolved in 400 ml water and extracted with 4 × 600 ml chloroform (yield: 16 g). 15 g of the chloroform extract was dissolved in a minimum amount of chloroform, absorbed on 50 g of Silica 60 (Mesh 70–230) and applied on a column packed with 350 g of Silica 60. A flow rate of 2 ml/min was used and fractions of 20 ml were collected. The elution started with chloroform and then continued with a 6-step gradient of methanol in chloroform (2.5%, 5%, 10%, 20%, 40%, 80%, 100%, 1.5 l of each step). Every fourth fraction was spotted on thin layer chromatography (TLC) plates (Silica gel 60, Merck, layer thickness 0.25 mm) and fractions of similar

composition were combined resulting in 8 fractions which were tested in the LT assay.

The main activity was found in fraction 2 which was then washed through a BondElut C18 column using methanol as solvent. The fraction obtained was then further purified by reversed phase HPLC (Nucleosil C18, 4 × 200 mm, 5 μm, acetonitrile/water 7/3 as mobile phase, flow rate 1 ml/min, detection:UV at 210 nm). Three major peaks (A, B, C in the order of elution from the column) were collected for further analysis.

### GC-MS analyses

The three compounds obtained from HPLC were analysed by GC-MS as methylester derivatives (13). A capillary GC with a non-polar column (30 m; DB-5, J&W Scientific film, 25 μm; diameter, 0.25 mm; carrier He, 15 psi) and an ion trap mass spectrometer (ITS40, Finnigan MAT) were used. After splitless injections, the GC was programmed from 60–200°C with 40°C/min and then from 200–275°C with 28°C/min and then kept at 275°C. The three compounds were identified as A: linoleic acid (c,c18:2ω6), B: alpha-linolenic acid (all c18:3ω3) and C: arachidonic acid (all c20:4ω6). The identification was confirmed by comparison with authentic samples on HPLC.

### The LT assay

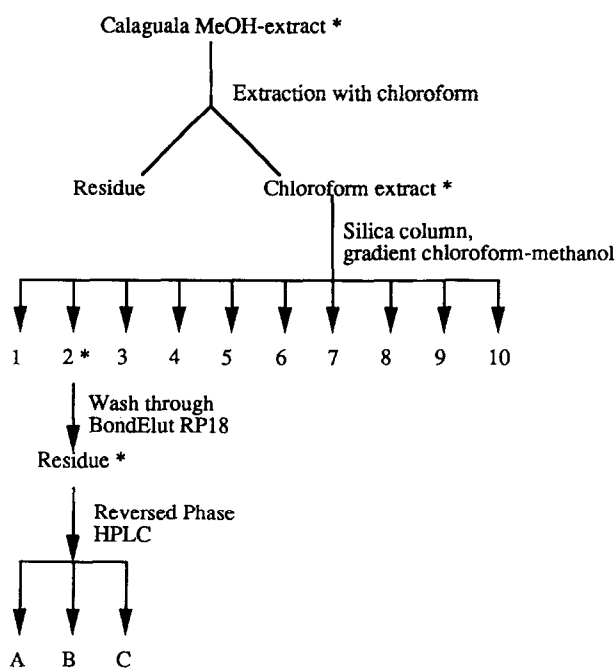
The bio-assay was performed with minor modifications according to the principles published by Tornhamre et al (14).

### MATERIALS

Synthetic LTB<sub>4</sub>, Ca ionophore A23187 and the fatty acids (99% purity) were purchased from Sigma. The 8(R)-hydroxylinoleic acid (15) was kindly provided by Professor Ernst Oliw, Division of Biochemical Pharmacology, Uppsala University. Dextran T-500 and sulfasalazine were purchased from Kabi Pharmacia, Uppsala, Sweden.

The ionophore was dissolved in ethanol in a concentration of 1 mM (stock solution) and with a final concentration in the test solutions of 1 μM for sulfasalazine and Calaguala methanolic extract and 2 μM for the fractions and pure acids. As solvents for the inhibitors phosphate buffered saline (PBS) were used for the Calaguala extract and sulfasalazine, 1% DMSO in PBS for the fractions and 10% DMSO for the pure acids, the final concentration of DMSO never exceeding 1%.

*Preparation of cell suspension.* Concentrated suspensions of human leukocytes in a CPD-adenine solution were obtained from the University Hospital (Uppsala, Sweden). After sedimentation with 2% Dextran T-500 solution containing 0.9% NaCl for 30 min at 4°C the supernatant was removed and centrifuged at 200 × g for 10 min. The sediments were treated with one volume of ice-cold water for 21 s followed by addition of 9 volumes of Ca<sup>2+</sup>



**Scheme** Isolation of fatty acids from the methanolic extract of Calaguala with bioactivity guided fractionation. \*Indicates a minimum of 40% activity in the LTB<sub>4</sub> assay.

and  $Mg^{2+}$  free buffer in order to lyse the remaining erythrocytes. After centrifugation (500g for 10 min at 4°C) the lysis was repeated once. The leukocytes (and remaining platelets) were then suspended in PBS (PBS with  $Ca^{2+}$  and  $Mg^{2+}$ ) and the cell count was adjusted to  $30 \times 10^6$  leukocytes/ml.

**Incubation procedure.** 900  $\mu$ l portions of the leukocyte suspension were preincubated with the inhibitor (final volume 1 ml) for 15 min at 37°C. For control incubations the different solvents were added in corresponding composition. The incubation was started with the addition of 1–2  $\mu$ l ionophore A23187 and terminated after 5 min by the addition of 5 volumes of ethanol.

**Purification and analysis of  $LTB_4$ .** Before HPLC the samples were centrifuged, evaporated to dryness, dissolved in the mobile phase (see below) and filtered. The samples were analysed by RP-HPLC using a LKB 2150 pump and a Nucleosil 120–3 C18 column eluted with acetonitrile/methanol/water/acetic acid (28:18:54:1, v/v/v/v; apparent pH 5.6) at a flow rate of 0.4 ml/min. Eluted  $LTB_4$  was detected with a LKB 2158 Uvicord SD UV detector at 280 nm. It was identified by comparing the HPLC retention time with that of a synthetic standard. The components of the fractions eluted close to the solvent front and were well separated from the eicosanoid. The inhibition of the  $LTB_4$  formation was calculated as the relative decrease in the absorbance peak for the LT as compared to the vehicle.

### Quantitative determination of fatty acids in Calaguala

A reversed phase HPLC system was used to analyse the fatty acid content of the Calaguala methanolic extract. The system used was Prepsil ODS 200–8 C18 column, UV detection at 210 nm, mobile phase: 80% acetonitrile–20% aq phosphoric acid pH 2, flow rate 1 ml/min. As internal standard oleic acid was used. For the standard curve the ratio of the area under curve for the acids analysed to the internal standard was spotted against the amount of acids and equation for the line obtained was used to calculate the amounts of PUFAs present in the extracts. Four different batches (different collection times, see Table 1) of Calaguala were analysed 3–4 times. Prior to HPLC injection the extract passed through a BondElut  $NH_2$  column using diethylether containing 2% acetic acid as solvent. This system selectively elutes free fatty acids (16).

## RESULTS

### The $LTB_4$ assay

The effect of the Calaguala methanolic extract and the positive control sulfasalazine on the formation of  $LTB_4$  by A23187 stimulated leukocytes is shown in the Figure.

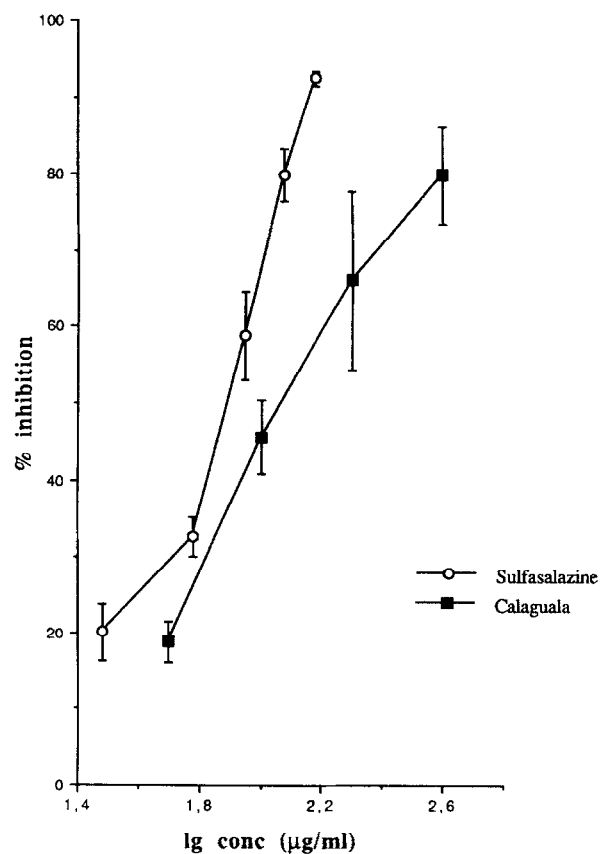
Both Calaguala and sulfasalazine caused a concentration-dependent inhibition of  $LTB_4$  formation with an  $IC_{50}$  of 130  $\mu$ g/ml and 68  $\mu$ g/ml (170  $\mu$ M), respectively.

### Fractionation of the Calaguala extract

In order to isolate the compounds in Calaguala responsible for the LT activity, fractionation of the extract was monitored by the  $LTB_4$  assay. According to the Scheme, the activity was completely retained in the chloroform-soluble part and after further fractionation using a Silica gel column located in fraction 2. Subsequent chromatographic purification (BondElut, HPLC) led to the isolation of three compounds. The mass spectrum of peak A,

**Table 1** Concentrations of the PUFAs in Calaguala as % of the total methanolic extract. The batch numbers refer to plant material collected at different times at the cultivation at Lago de Yogo, Honduras. The times are: 1 January 1988, 2 October 1988, 3 April 1989, 4 November 1988

Fatty acid	Calaguala batches			
	1	2	3	4
Linoleic	0.009	0.22	0.2	0.23
Alpha-Linolenic	0.027	0.18	0.39	0.3
Arachidonic	0.0027	0.04	0.062	0.057



**Figure** Effect of Calaguala and Sulfasalazine on the formation of  $LTB_4$  by A23187 stimulated leukocytes. Each value represents the mean  $\pm$  SEM of 4–7 experiments, all performed in duplicate.

after methylation, showed major signals at  $m/z$  294( $M^+$ ), 263 ( $M^+-31$ ), 245 ( $M^+-49$ ) and in the lower mass range at  $m/z$  109, 95 and 81, which strongly indicated that it contained linoleic acid. The results from peak B with signals at  $m/z$  292 ( $M^+$ ), 261 ( $M^+-31$ ), 95 and 81 and peak C with signals at  $m/z$  318 ( $M^+$ ), 287 ( $M^+-31$ ) and 269 ( $M^+-49$ ) indicated that they consisted of alpha-linolenic and arachidonic acid, respectively.

### The effect of fatty acids on $LTB_4$ formation

To confirm that the isolated fatty acids were responsible for the inhibitory effect of Calaguala on  $LTB_4$  formation, they were incubated in the test system in different concentrations (10–100  $\mu M$ ). Linoleic acid and alpha-linolenic acid showed a dose-dependent inhibition with  $IC_{50}$  values of approx. 30  $\mu M$ . However, the concentration range for the inhibitory activity was found to be very narrow; only 3 points in the curve were obtained with both acids leading to some uncertainty with the  $IC_{50}$  values. With lower concentrations, stimulatory activity was recorded. Arachidonic acid showed stimulatory activity over the whole concentration range tested (0.2–200  $\mu M$ ).

In order to study if a specific structural feature of the fatty acid was crucial for the inhibitory effect, several other unsaturated fatty acids were incubated in a similar way. The acids chosen are: (Table 2) oleic acid (c18:1 $\omega$ 9), elaidic acid (t18:1 $\omega$ 9), eicosapentaenoic acid (c20:5 $\omega$ 3), ricinoleic acid (12(R) hydroxy-c18:1 $\omega$ 9) and 8(R) hydroxylinoleic acid (8(R) hydroxy-cc18:2 $\omega$ 6). The specific structural features studied include chain length, number of double bonds and stereochemistry. The  $IC_{50}$  values obtained are oleic acid 21  $\mu M$ , ricinoleic acid 41  $\mu M$ , elaidic acid 54  $\mu M$  and eicosapentaenoic acid 57  $\mu M$ . For 8(R) hydroxylinoleic acid the inhibitory activity never exceeded 30% in the doses tested (up to 120  $\mu M$ ). For several of the acids stimulatory activity was obtained with lower concentrations.

**Table 2** Effect of the PUFAs on  $LTB_4$  formation by human leukocytes. Leukocytes were incubated with 3–5 different concentrations of the acids in the presence of Ca ionophore (2  $\mu M$ ). Each value was determined 3–6 times in duplicate, a dose-response curve was prepared and regression analysis was used to calculate the figures shown

	$IC_{50}$ ( $\mu M$ )	% inhibition obtained by 70 $\mu M$ of the acids
Ricinoleic acid	41	73
12(R)OH c18:1 $\omega$ 9		
Oleic acid c18:1 $\omega$ 9	21	89
Elaidic acid t18:1 $\omega$ 9	54	57
Linoleic acid cc18:2 $\omega$ 6	31	97
Linolenic acid		
All c18:3 $\omega$ 3	42	100
8(R)OH linoleic acid		
8ROH-cc18:2 $\omega$ 6	nd	30
Eicosapentaenoic acid		
All c20:5 $\omega$ 3	57	62

nd = not determined.

### Quantitative determination of fatty acids in Calaguala

Four different batches with collection times 1 January 1988, 2 October 1988, 3 April 1989 and 4 November 1988 were analysed for their contents of linoleic, alpha-linolenic and arachidonic acid by using RP-HPLC. The concentrations found varied from 0.009 to 0.39% for linoleic acid, from 0.027 to 0.23% for linolenic acid and 0.0027 to 0.062% for arachidonic acid (Table 1).

### DISCUSSION

There is considerable evidence to support the role of different lipoxygenase products in psoriasis. One of them, the  $LTB_4$ , has been suggested to be an important mediator of the inflammatory and hyperproliferative changes in the disease (17). Two inhibitors of  $LTB_4$  formation, benoxaprofen and lonapalene, have in clinical trials been shown to improve the course of psoriasis (11, 12).

In recent clinical studies the antiinflammatory drug sulfasalazine, which is a  $LTB_4$  inhibitor, has successfully been employed in the treatment of psoriasis (18, 19). Sulfasalazine is frequently used in the treatment of ulcerative colitis, but the mechanism of its action is not fully understood. One of its documented pharmacological effects is, however, inhibition of the formation of lipoxygenase products (15).

In the present study we have investigated the fern, *P. decumanum* which is used in the treatment of psoriasis, for its ability to inhibit  $LTB_4$  formation by human leukocytes and isolated the active principles responsible for the activity.

The crude methanolic extract of *P. decumanum* showed, considering the complexity of the extract, a significant and dose-dependent activity in the assay. The potency of Calaguala in the assay used was found to be of the same magnitude as sulfasalazine, which was used as a positive control.

In the subsequent fractionation of the extract the LT activity was well separated from the non-active parts and as a result of column chromatography three compounds were identified as  $LTB_4$ -active in the plant: the PUFAs linoleic, alpha-linolenic and arachidonic acid. When tested in pure form, linoleic and alpha-linolenic acid dose-dependently inhibited the formation of  $LTB_4$ , whereas arachidonic acid had stimulatory activity.

Linoleic and alpha-linolenic acids are known inhibitors of  $LTB_4$  formation, with reported  $IC_{50}$  values for linoleic acid of 45  $\mu M$  in human neutrophils (20) and 5.8  $\mu M$  in rat peritoneal macrophages for linolenic acid (21).

The five other PUFAs, that were chosen in the order to study the effect of chain length, number of double bonds and stereochemistry in the test system; oleic, elaidic, eicosapentaenoic, ricinoleic and 8(R) hydroxylinoleic acid, all showed a dose-dependent inhibition of the  $LTB_4$  formation. The differences in the  $IC_{50}$  values for the acids were small, the only exception being the 8(R) hydroxylinoleic acid. To be able to draw any con-

clusions about the structural features that are important for the inhibition, additional fatty acids should be tested in a similar way.

All acids tested showed stimulatory activity when tested in low concentrations in the assay. A possible explanation to this is the likely presence of fatty acid hydroperoxides as impurities in the acid solutions. Low concentrations of hydroperoxides which can be expected in the dilute acid solutions are known to activate lipoxygenase enzymes (22).

To determine if the fatty acids in Calaguala extract, which is given orally in doses up to 100 mg/kg/day, could explain the clinical effects reported, a quantitative determination of the fatty acid content was performed. The concentrations varied between the different batches analysed, possibly depending on seasonal variation of components of plants. According to the analysis, the doses of linoleic and alpha-linolenic acid administered, could reach approximately 200 mg/day which is a lower dose than reported for several of the clinical trials with PUFAs (23–25).

Evidence that PUFAs can affect immune function (26–28) and inflammatory reactions has led to great interest in the effects of dietary manipulation of a variety of chronic diseases, such as psoriasis (29), rheumatoid arthritis and ulcerative colitis (30). In most cases the main interest has been focused on substituting the saturated fatty acids of animal origin in the Western diet with fish oil containing big amounts of eicosapentaenoic acid. In vitro eicosapentaenoic acid has been shown to decrease the LTB<sub>4</sub> formation (31). Eicosapentaenoic acid can serve as a substrate for lipoxygenase instead of arachidonic acid and the product formed, LTB<sub>5</sub>, has a significantly lower biological activity than LTB<sub>4</sub> (32).

In clinical studies (28, 33), where psoriatic patients were put on a low-fat diet supplemented with fish oil, mild to moderate improvement was observed in about 60% of the cases indicating a possible role of eicosapentaenoic acid as a complement to conventional treatment. The role of vegetable oils in clinical improvement of psoriasis has also recently been indicated by several authors. In a study performed by Kragballe (25) moderate to excellent improvement was observed in the majority of patients receiving a combination of eicosapentaenoic, linoleic and gamma-linolenic acids. The importance of vegetable oils containing linoleic acids is also pointed out by Ziboh (24). The importance of vegetable PUFAs in normal skin function is also indicated by the fact that one of the most prominent symptoms that experimental animals exhibit when suffering from essential fatty acid deficiency is scaly skin. This symptom, which is caused by transepidermal water loss, can be reversed by the use of linoleic acid (34).

The mechanism underlying the inhibition of LTB<sub>4</sub> by PUFAs is unknown. One possible mechanism of action may, however, be the induction of ATP depletion of the cell, since 5-lipoxygenase activity is dependent on intact energy supply (20).

Another explanation could be that linoleic and dihomogammalinolenic acid have also been shown to stimulate the formation of 15-lipoxygenase products from arachidonic acid and the products formed show a significantly more potent inhibition of 5-lipoxygenase than the PUFAs themselves (19).

In the test model used arachidonic acid which serves as a substrate for LT formation was found to cause an increased formation of LTB<sub>4</sub>. This is contradictory to reports where decreased LTB<sub>4</sub> formation has been observed when incubating rat peritoneal macrophages (22) or human leukocytes (35) in the presence of exogenous arachidonic acid. Considering all this, it seems possible that PUFAs have a therapeutic effect in inflammatory diseases in which LTs are suspected to play a pathogenic role, including psoriasis. Despite the fact that lower concentrations of fatty acids than in most published reports were found in Calaguala, it is possible that they may be part of the explanation to the observed clinical improvements of the skin disease psoriasis in patients treated with the extract.

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