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The CO_2-SFE crude lipid extract and the free fatty acid extract from *Perna canaliculus* have anti-inflammatory effects on adjuvant-induced arthritis in rats

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Abstract

The anti-inflammatory (AI) activity of a supercritical fluid extract (CO_2-SFE) of tartaric acid-stabilised *Perna canaliculus* mussel powder, and of the free fatty acid (FFA) class separated from the CO_2-SFE extract by column chromatography, was investigated in the rat adjuvant arthritis model. Administration of the CO_2-SFE extract (100 mg/kg BW/day s.c.) for 15 days post-adjuvant inoculation significantly reduced rear paw swelling by 34% and the deterioration in total body condition by 52% in arthritic rats, compared to vehicle controls. These observations were accompanied by a decreased serum ceruloplasmin oxidase activity, and reduced inflammatory response of the spleen. The mussel FFA extract given at one third of the dose (30 mg/kg BW/day s.c.) and for a shorter treatment period (5 days during the inflammatory phase) achieved an even greater AI activity, and was equipotent to piroxicam (2 mg/kg BW/day s.c.). Preliminary toxicology assessment using both arthritic and non-arthritic (healthy) rats revealed no significant differences between the mussel treatment groups and respective vehicle controls in either organ weights, tissue histology or selected biochemical parameters. These results indicate the CO_2-SFE crude lipid extract and its FFA components from stabilised *P. canaliculus* mussel powder contain biologically significant AI activity *in vivo*, with no apparent adverse side effects.

Keywords: Adjuvant arthritis; Anti-inflammatory; *Perna canaliculus*; ω-3 PUFA

1. Introduction

Human rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by synovial membrane inflammation and hyperplasia that leads to joint destruction (Lee and Weinblatt, 2001). Interest in the pathogenesis of the synovitis has focussed in part on the cyclooxygenase (COX) and 5-lipoxygenase (5-LO) pathways of lipid metabolism. These enzymes catalyse the oxygenation of polyunsaturated fatty acids (PUFA), preferably arachidonic acid (AA), to form the biologically active prostaglandin (PG) and leukotriene (LT) metabolites. COX-2 and 5-LO are co-expressed and up-regulated in the rheumatoid synovium (Claria and Romano, 2005), and simultaneous inhibition of both enzymes is proposed as a safe an effective treatment modality in RA (Charlier and Michaux, 2003).

Recent studies have examined ways in which dietary ω-3 PUFA may influence the production of ω-6 AA-derived PG and LT mediators of inflammation (Stamp et al., 2005). One area of investigation is the anti-inflammatory (AI) activity of lipid extracts from the marine mollusc *Perna canaliculus* (Bivalvia: Mytilidae), commonly known as the green-lipped mussel of New Zealand. Certain crude lipid extracts of tartaric acid-stabilised *P. canaliculus* mussel flesh have exhibited significant anti-COX and anti-5-LO activity *in vitro* (McPhee et al., 2007; Treschow et al., 2007). The greatest activity is found in the free fatty acid (FFA) component of *P. canaliculus*, and a homologous series of novel, long-chain ω-3 PUFA with enhanced AI activity (as determined

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by an *in vitro* leukotriene-inhibition assay) has been isolated and characterised (Treschow et al., 2007). The *in vitro* AI effect of the *P. canaliculus* lipid extracts has been explained on the basis of modulation of AA metabolism, with the formation of less bioactive PG and LT analogues (McPhee et al., 2001, 2007).

In the present study, a CO_2-SFE crude lipid and FFA extract of tartaric acid-stabilised *P. canaliculus* mussel flesh was investigated for AI activity *in vivo* by using the Complete Freund's adjuvant polyarthritis rat model. Because of the many deleterious side effects of AI agents in current usage, a preliminary toxicology study utilising both inflamed (arthritic) and uninflamed (healthy, non-arthritic) rats was also performed.

2. Materials and methods

2.1. Chemicals

Solvents for all chromatographic procedures were of analytical grade quality and obtained from E. Merck (Darmstadt, Germany). Column chromatography utilised Kieselgel 60 particle size 230–400 mesh (E. Merck), and the silver-ion TLC utilised Kieselgel 60 G particle size 30–40 μm (E. Merck). Lipid standards for TLC were obtained from Nu-Chek Prep Inc. (Elysian, MO, USA). Squalane, piroxicam and sodium pentobarbital were obtained from Sigma Chemical Co. (St Louis, MO, USA), and the olive oil used as the vehicle for *in vivo* treatments was purchased from a local pharmacy retailer (Amcal). All other chemicals used for anti-inflammatory and toxicology analyses were obtained from Sigma, except U–^{14}C Palmitic acid (859 mCi/mmol) which was obtained from Amersham (Buckinghamshire, England).

2.2. Preparation of *P. canaliculus* lipid extracts

Tartaric acid-stabilised freeze-dried *P. canaliculus* mussel powder (McFarlane Marketing (Aust) Pty Ltd, Melbourne, Australia) was extracted for lipids by the procedure of super-critical fluid extraction (SFE), utilising CO_2 as the extracting medium (Treschow et al., 2007). The extract was obtained as a concentrated oil, and stored under N_2 at −20 °C in amber vials to minimise autoxidation. The mussel FFA class was separated from the CO_2-SFE crude lipid by normal-phase open column chromatography and used in the adjuvant arthritis study (Treschow et al., 2007). The mussel FFA class was further separated by preparative silver-ion TLC to isolate an enriched fraction containing the novel ω-3 PUFA component, which was identified by GC-MS analysis and used in the *in vitro* assay (Treschow et al., 2007).

2.3. Induction of adjuvant arthritis in rats and treatment with lipid extracts

All experiments involving animals were approved by RMIT University Animal Ethics Committee (Approval no. AEC 9411) and were conducted in compliance with the Australian National Health and Medical Research Council Guidelines on the care and use of laboratory animals. All animals received humane care throughout the course of this study. Adjuvant arthritis was elicited in male Long Evans rats (150–200 g) by subcutaneous (s.c.) tail base injection of heat killed, dried *Mycobacterium tuberculosis* dispersed in squalane (10 mg/mL) at a final concentration of 2 mg/kg body weight (BW) (McColl et al., 1987). The severity of the disease was quantified using three variables: (i) rear paw size (measured by a micrometer) (ii) daily body weight changes; and (iii) subjective disease activity in both the fore and rear paws (determined using the categories in Table 1). Subjective disease activity also considered the total body condition of the animal, including fur, eyes and vasodilation of the ears. Evaluation was performed by an independent observer unaware of treatment protocols on days 10 and 15 after adjuvant injection (day 0).

P. canaliculus lipid extracts were dissolved in olive oil for s.c. administration to adjuvant arthritic rats (see treatment schedule

Table 1
Antiarthritic activity of *P. canaliculus* lipid extracts during the *in vivo* rat polyarthritis assay

Group[1]	Treatment[2]	Dose[3]	Mean change in arthritic signs		Subjective disease activity			Ceruloplasmin[8]
			Δ rear paw size[4]	Δ BW[5]	Arthritis score[6]		Total body condition[7]	
		(mg/kg BW)	(mm)	(g)	Fore paws	Rear paws		
I	Olive oil control	850	1.98±0.17	−16.2±1.9	1.4±0.1	2.5±0.4	2.5±0.2	0.70±0.03
II	Mussel FFA	30	0.52±0.40[a]	−12.9±5.8	0.7±0.3	1.2±0.7	1.2±0.4[e]	0.44±0.04[f]
III	Mussel crude lipid	50	1.76±0.38	−14.3±3.1	1.0±0.4	1.9±0.7	1.7±0.5	0.52±0.03[e]
IV	Mussel crude lipid	100	1.91±0.27	−12.9±3.5	1.0±0.3	1.9±0.2	1.6±0.3	0.56±0.03[e]
V	Piroxicam	2	0.57±0.37[a]	−10.5±2.9	0.6±0.1[c]	1.2±0.9	1.2±0.3[e]	0.43±0.1[f]
VI	Olive oil control	850	3.12±0.27	−17.3±6.0	2.0±0.0	3.2±0.3	3.1±0.3	0.64±0.04
VII	Mussel crude lipid	100	2.06±0.41[b]	−20.2±7.0	0.8±0.3[d]	1.3±0.6	1.5±0.3[d]	0.39±0.05[g]

[1]Data are expressed as mean±SEM (n=8 rats/group, non-responders omitted). [2]Day 0 = day of adjuvant inoculation (2 mg/kg BW); all treatments were dosed for 5 days s.c. (days 10–14) except for treatment groups VI and VII where animals were dosed for 15 days s.c. (days 0–14). [3]Olive oil control (I and VI) dose=850 mg/kg BW s.c. [4]Mean changes in rear paw size (both left and right rear paws) was calculated by: rear paw diameter on day 15 minus rear paw diameter on day 10. [a]$p<0.05$ significant difference from adjuvant control (I) after statistical analysis by Dunnett's test. [b]$p<0.10$ significant difference from adjuvant control (VI) by Student's t-test. [5]Body weight change was calculated by: BW (day 15) minus BW (day 10); minus sign indicates BW loss. [6]Subjective arthritis score of 0–4 points based on the extent of arthritic symptoms: 0, no localised articular lesion or swelling; 1, localised articular lesion or ankle swelling; 2, localised articular lesion and ankle swelling; 3, moderate generalised ankle and foot swelling; 4, gross generalised ankle and foot swelling. [c]$p<0.10$ significant difference from adjuvant control (I) after statistical analysis by Kruskal–Wallis test. [d]$p<0.10$ significant difference from adjuvant control (VI) by Mann–Whitney test. [7]Subjective disease activity also considered the total body condition of the animal (0–4 points), including fur, eyes and vasodilation of the ears. [8]Serum ceruloplasmin levels of uninflamed (no adjuvant) olive oil control rats=0.34±0.01 absorbance units/30 min. [e]$p<0.10$ significant difference from adjuvant control (I) after statistical analysis by Dunnett's test. [f]$p<0.001$ significant difference from adjuvant control (I) by Dunnett's test. [g]$p<0.001$ significant difference from adjuvant control (VI) by Student's t-test.

Table 2
Preliminary toxicology assessment: effects of *P. canaliculus* lipid extracts on inflamed (arthritic) rats

Group[1]	Treatment[2]	Dose[3]	Δ BW[4]	SDH	β-oxidation	Serum TG
		(mg/kg BW)	(g)	(IU/L)	(pmol/min/mg protein)	(mg/dL)
I	Olive oil control	850	−16.2±1.9	57.4±27.4	37±7	35±3
II	Mussel FFA	30	−12.9±5.8	16.9±5.2	35±2	34±5
III	Mussel crude lipid	50	−14.3±3.1	23.9±4.5	46±9	28±8
IV	Mussel crude lipid	100	−12.9±3.5	20.3±3.8	35±1	32±5
VI	Olive oil control	850	−17.3±6.0	13.8±4.0	44±6	47±13
VII	Mussel crude lipid	100	−20.2±7.0	11.8±1.5	39±3	44±5

[1]Data are expressed as mean±SEM or percentage of respective control (n=4 rats/group, non-responders omitted). [2]Day 0 = day of adjuvant inoculation (2 mg/kg BW); all treatments were dosed for 5 days s.c. (days 10–14) except for treatment groups VI and VII where animals were dosed for 15 days s.c. (days 0–14). [3]Olive oil control (I and VI) dose=850 mg/kg BW s.c. [4]Minus sign indicates BW loss.

Table 1). For groups I–V, the arthritis was allowed to develop for 10 days before daily treatment with the extracts commenced (days 10–14). For groups VI and VII, treatments were administered daily for 15 days starting from the day of adjuvant inoculation (days 0–14). Groups I and VI were administered olive oil-vehicle only and acted as the respective arthritic controls, whilst the therapeutic NSAID piroxicam (dissolved in 0.154 M NaCl and equimolar NaOH) was administered to group V arthritic rats as the positive control. All injection volumes were 1 mL/kg BW, and the s.c. injection sites used for the five day treatments were left hind leg, right hind leg, scruff (neck region), right back and left back, respectively. For the 15 day treatments the same injection sites were used but on a rotational basis.

All animals that responded to the inflammogen were sacrificed on day 15, the day following the final treatment day. The animals were anesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg BW), and blood was removed from the abdominal aorta. Liver, kidney, spleen, heart, brain and testes were excised, weighed and examined for any gross morphological changes. Sections of liver, kidney and spleen were fixed in 10% formalin saline for histological analysis. Serum was obtained by centrifugation of the clotted blood at 10 000 *g* for 10 min. Serum and representative samples of liver were frozen in liquid N_2 and stored at −80 °C for biochemical assays. The method of Schosinsky et al. (1974) was employed to measure serum oxidase activity of the acute phase response protein, ceruloplasmin.

2.4. In vitro leukotriene-inhibition assay

The enriched fraction of the mussel FFA class containing novel ω-3 PUFA that was purified by preparative silver-ion TLC was diluted in methanol and tested *in vitro* for inhibition of leukotriene biosynthesis by calcium ionophore A23187-stimulated human neutrophils (5 min, 37 °C) (Treschow et al., 2007). This assay monitors the production from AA of four of the 5-LO pathway products, namely LTB_4, 5-HETE and two non-enzymic isomers, 6-*trans* LTB_4 and 6-*trans*, 12-*epi* LTB_4.

2.5. Preliminary toxicology assessment

Preliminary subacute repeat-dose toxicology studies were performed in inflamed animals with adjuvant-induced arthritis (see treatment schedule Table 2), as well as in uninflamed (healthy, non-arthritic) animals (see treatment schedule Table 3). The uninflamed treatment groups were sacrificed on day 6 (the day following the final treatment day) by the same method used for the inflamed groups. Liver, kidney, spleen, heart, brain, stomach and testes were excised, weighed and examined for any gross morphological changes. Sections of liver, kidney and spleen were fixed in 10% formalin saline for histological analysis. Serum was obtained and frozen with representative samples of liver as described in Section 2.3.

Ethoxyresorufin *O*-deethylase (EROD) and ethoxycoumarin *O*-deethylase (ECOD) enzyme activities in uninflamed rat livers were determined using the method of Lake (1987). Liver microsomes were prepared as described by Fowler et al. (1996), and microsomal protein content was determined by the method of Markwell et al. (1978). EROD and ECOD are markers for cytochrome P450 activity in liver microsomes (Cho et al., 2003).

Serum sorbitol dehydrogenase (SDH) activity was measured in both inflamed and uninflamed groups using Sigma Diagnostics® Sorbitol Dehydrogenase Kit (50-UV).

Mitochondrial β-oxidation was measured in the liver of both inflamed and uninflamed groups using the method of Freneaux et al. (1990). Liver mitochondria were prepared as described by McGarry et al. (1978). An aliquot was used to determine

Table 3
Preliminary toxicology assessment: effects of *P. canaliculus* lipid extracts on uninflamed (healthy, non-arthritic) rats

Group[1]	Treatment[2]	Dose[3]	Δ BW[4]	EROD	ECOD	SDH	β-oxidation	Serum TG
		(mg/kg BW)		(pmol/min/mg protein)	(pmol/min/mg protein)	(IU/L)	(pmol/min/mg protein)	(mg/dL)
A	Olive oil control	850	+17.9±1.2	2.94±0.13	85±2	24.2±13.0	25±5	85±15
B	Mussel FFA	30	+13.5±2.6	2.54±0.16	84±2	9.6±2.3	29±5	85±6
C	Mussel crude lipid	50	+14.9±2.3	2.41±0.20	92±2	16.7±3.5	29±2	95±12
D	Mussel crude lipid	100	+16.4±0.5	2.22±0.20	84±1	17.3±3.6	38±5	111±16

[1]Data are expressed as mean±SEM or percentage of respective control (n=4 rats/group). [2]The FFA component and crude lipid extract of *P. canaliculus* were dissolved in olive oil; all treatments were dosed for 5 days s.c. [3]Olive oil control dose=850 mg/kg BW s.c. [4]Plus sign indicates BW increase.

mitochondrial protein content by the method of Markwell et al. (1978).

Serum TG levels were assayed in duplicate using Sigma Diagnostics® Triglyceride Kit (336-10) in both inflamed and uninflamed treatment groups.

2.6. Statistical analysis

For multiple groups (I to V), one way ANOVA (followed post-hoc by Dunnett's test) was used to analyse parametric data whereas the Kruskal–Wallis test was used for non-parametric data (e.g. disease activity scores). For two groups only (groups VI and VII), the Student's *t*-test was used for parametric data and the Mann–Whitney test was used for non-parametric data.

3. Results

3.1. Preparation of P. canaliculus lipid extracts

The maximum yield of CO_2-SFE extractable crude lipid from *P. canaliculus* freeze-dried mussel powder was 4.76% w/w. The mussel extract was orange–amber with a viscous waxy appearance at ambient temperature. Analysis of the extract by TLC afforded several lipid classes including sterol esters, triglycerides, free fatty acids, diglycerides, sterols, phospholipids and monoglycerides. The percentage yield of the mussel FFA class was 39% w/w of the CO_2-SFE crude lipid. The novel PUFA isolated from the mussel FFA class by preparative silver-ion TLC constituted 3.4% w/w of the mussel FFA class (and 1.32% of the CO_2-SFE crude lipid extract).

3.2. Inhibition of adjuvant-induced arthritis in Long Evans rats

When compared to their respective olive oil-arthritic control groups, the crude lipid material (50 and 100 mg/kg BW/day) administered for 5 days did not significantly alter rear paw swelling (Table 1). However, the administration of the crude lipid (100 mg/kg BW/day) for 15 days significantly reduced rear paw swelling by 34%. By contrast, administration of the FFA class (30 mg/kg BW/day/5 days) significantly reduced rear paw swelling by 74%, and was equipotent to piroxicam treatment (2 mg/kg BW/day/5 days s.c.).

A subjective 0–4 scale was used to assess the overall arthritis score and total body condition of the animals (Table 1). When

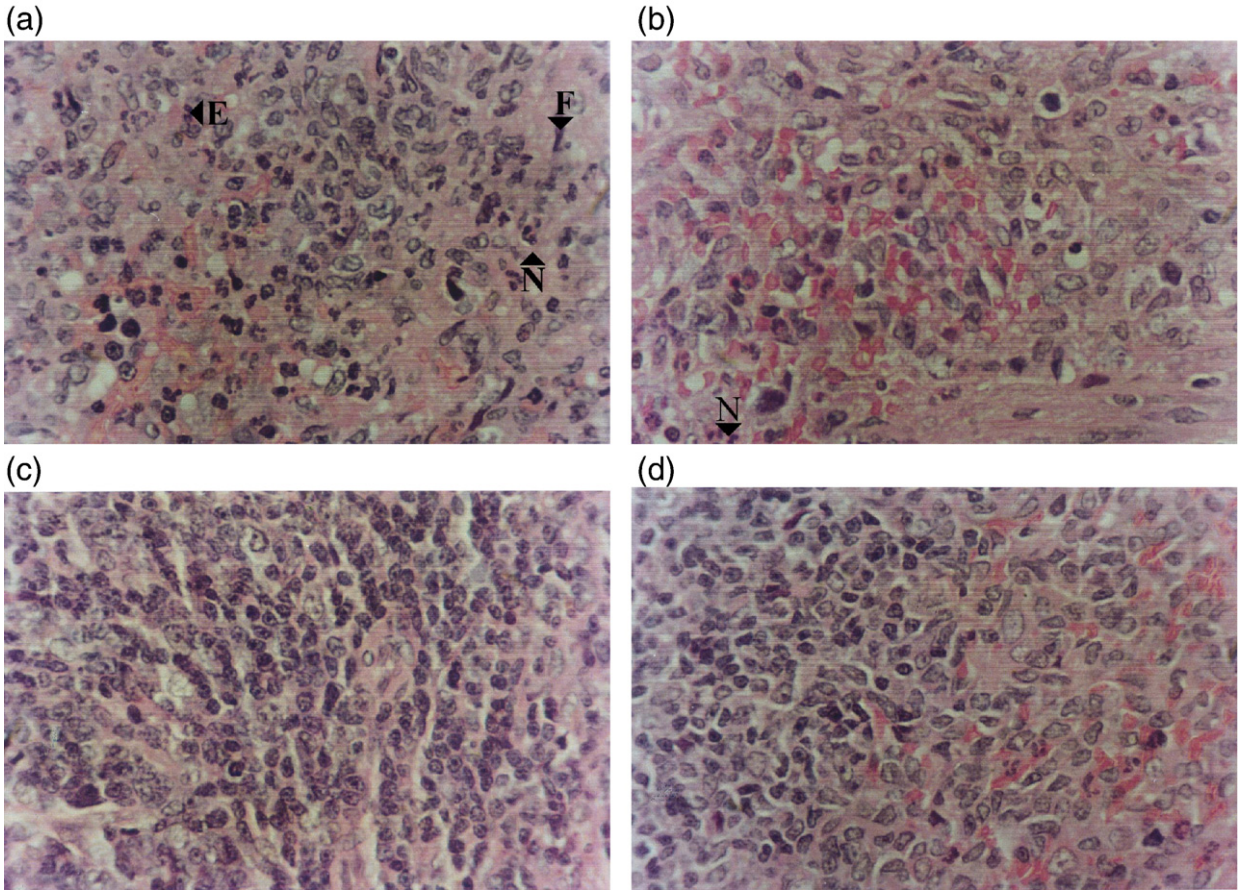

Fig. 1. Hematoxylin- and eosin-stained sections of spleen from treated and untreated arthritic rats (×320 magnification). (a) Chronic inflammation-induced changes in the spleen red pulp from the olive oil-vehicle arthritic control is represented by a marked cellular infiltration of chronic inflammatory cells, including multinucleated eosinophils (E) and neutrophils (N), and deposition of fibrin by fibroblasts (F). The spleens of arthritic rats treated with (b) crude lipid material (100 mg/kg BW/day/15 days) had moderate infiltration by neutrophils (N) and some fibrillar deposits, but overall less inflammation-induced changes compared to the arthritic control. (c) Mussel FFA (30 mg/kg BW/day/5 days) and (d) piroxicam (2 mg/kg BW/day/5 days) treated spleens no significant inflammation-induced changes and near-normal morphology.

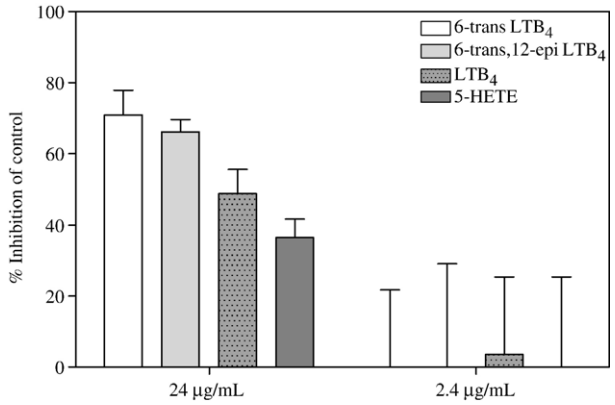

Fig. 2. Percent inhibition of neutrophil synthesis of 5-LO metabolites by the novel PUFA class of *P. canaliculus*. FFA extract was tested at 24 µg/mL of neutrophils and 2.4 µg/mL of neutrophils. Exogenous AA substrate was present at 3 µg/mL of neutrophils.

compared to their respective olive oil-arthritic control groups, fore paw inflammation and deterioration of total body condition was significantly reduced by 60% and 52%, respectively, following administration of crude lipid extract (100 mg/kg BW/day) for the longer dosage period of 15 days, but not for the shorter 5 day period. The FFA class (30 mg/kg BW/day) was able to significantly reduce the deterioration of total body condition by 52% following the 5 day treatment period, and was equipotent to piroxicam treatment (2 mg/kg BW/day/5 days s.c.).

All adjuvant inoculated rats lost BW over the time-course of the study (Table 1). Treatment with the mussel extracts or piroxicam did not significantly alter the extent of BW loss.

Adjuvant-induced changes in the spleen red pulp from the olive oil-vehicle arthritic control was characterised by a marked cellular infiltration of chronic inflammatory cells including multinucleated eosinophils and neutrophils, and by an early deposition of fibrin by fibroblasts (Fig. 1). Independent assessment of histopathology revealed that the spleens of arthritic rats treated with crude lipid material (100 mg/kg BW/day/15 days) had a moderate infiltration by neutrophils and some fibrillar deposition, but overall less inflammation-induced changes compared to the olive oil-arthritic control group. The spleen after mussel FFA treatment (30 mg/kg BW/day/5 days) and piroxicam treatment (2 mg/kg BW/day/5 days) showed no significant inflammation-induced changes and near-normal histology.

All mussel treatments and piroxicam significantly reduced the serum ceruloplasmin activity when compared to their respective olive oil-arthritic control groups (Table 1).

3.3. In vitro leukotriene-inhibition assay

The novel ω-3 PUFA class of *P. canaliculus* was screened *in vitro* by the leukotriene-inhibition assay. Inhibition of production of leukotriene metabolites of between 35–70% was observed at 24 µg/mL of neutrophils (Fig. 2).

3.4. Preliminary toxicology assessment

No significant differences were observed between inflamed or uninflamed groups and their respective olive oil-vehicle controls for both BW changes (Tables 2 and 3) and organ somatic indices (Tables 4 and 5). Organ somatic indices for inflamed animals were generally higher than for the uninflamed groups, as the inflamed animals lost BW whilst their organ weights remained unchanged.

Hepatic mitochondrial β-oxidation of fatty acids and serum TG levels were used as biomarkers of hepatic lipid metabolism and are shown in Table 2 (inflamed) and Table 3 (uninflamed), respectively. No significant differences were seen between the mussel extract-treatment groups and their respective olive oil-vehicle controls, in either the inflamed or uninflamed animals. Hepatic microsomal metabolism, measured as EROD and ECOD activity (Table 3), and the specific biomarker for liver damage, serum SDH activity (Tables 2 and 3) were not significantly changed between the mussel extract-treated groups and respective olive oil-vehicle controls.

Independent histopathology assessment was performed on representative liver and kidney specimens from both inflamed and uninflamed treatment groups. Features which were assessed included the extent of changes in fat deposits, hydropic changes and cellular necrosis. These assessments did not reveal any significant changes in histology of the mussel extract-treated groups (both CO_2-SFE and FFA extracts) when compared to

Table 4
Effects of *P. canaliculus* lipid extracts on organ somatic indices of adjuvant arthritic rats

Group[1]	Treatment[2]	Dose[3]	Organ somatic indices[4]					
		(mg/kg BW)	Liver	Spleen	Kidney	Heart	Brain	Testes
I	Olive oil control	850	4.83±0.51	0.68±0.09	0.95±0.04	0.56±0.02	0.71±0.06	1.09±0.03
II	Mussel FFA	30	5.43±0.43	0.60±0.09	0.96±0.04	0.58±0.06	0.84±0.02	1.24±0.07
III	Mussel crude lipid	50	4.91±0.35	0.58±0.06	0.94±0.02	0.54±0.03	0.77±0.03	1.10±0.05
IV	Mussel crude lipid	100	5.47±0.62	0.69±0.09	0.99±0.05	0.48±0.07	0.77±0.03	1.26±0.05
V	Piroxicam	2	5.05±0.25	0.58±0.09	0.98±0.05	0.57±0.04	0.74±0.03	1.2±0.10
VI	Olive oil control	850	5.32±0.52	0.67±0.11	0.98±0.02	0.61±0.05	0.71±0.02	1.19±0.07
VII	Mussel crude lipid	100	5.13±0.31	0.61±0.11	0.87±0.04[a]	0.54±0.01	0.72±0.05	1.26±0.04

[1]Data are expressed as mean±SEM (n=4 rats/group, non-responders omitted). [2]Day 0 = day of adjuvant inoculation (2 mg/kg BW); all treatments were dosed for 5 days s.c (days 10–14) except for treatment groups VI and VII where animals were dosed for 15 days s.c (days 0–14). [3]Olive oil control dose=850 mg/kg BW s.c. [4]Organ somatic index was calculated by: organ wet weight (g) divided by body weight (g)×100. Somatic indices for uninflamed (non-arthritic) olive oil control rats are: liver 4.84±0.03; spleen 0.24±0.01; kidney 0.84±0.03; heart 0.33±0.02; brain 0.58±0.01; testes 1.07±0.03. [a]$p<0.05$ significant difference from adjuvant control (VI) by Student's t-test.

Table 5
Effects *P. canaliculus* lipid extracts on organ somatic indices of uninflamed (healthy, non-arthritic) rats

Group[1]	Treatment[2]	Dose[3]	Organ somatic indices[4]					
		(mg/kg BW)	Liver	Spleen	Kidney	Heart	Brain	Testes
A	Olive oil control	850	4.84±0.03	0.24±0.01	0.84±0.03	0.33±0.02	0.58±0.01	1.07±0.03
B	Mussel FFA	30	4.79±0.20	0.24±0.02	0.85±0.02	0.34±0.01	0.65±0.04	1.08±0.04
C	Mussel crude lipid	50	4.95±0.45	0.25±0.02	0.83±0.07	0.33±0.02	0.60±0.02	1.04±0.05
D	Mussel crude lipid	100	4.73±0.11	0.24±0.01	0.86±0.06	0.34±0.02	0.65±0.02	1.04±0.02

[1]Data are expressed as mean±SEM (n=4 rats/group). [2]All treatments were dosed for 5 days s.c. [3]Olive oil control dose=850 mg/kg BW s.c. [4]Organ somatic index was calculated by: organ wet weight (g) divided by body weight (g) ×100.

respective olive oil-vehicle controls (micrographs not shown). Furthermore, there was no evidence of treatment-induced inflammatory responses as the mussel extract-treated spleens of uninflamed (non-arthritic) rats showed no significant cellular infiltration by chronic inflammatory cells nor fibrin deposition when compared to the uninflamed (non-arthritic) olive oil controls (micrographs not shown). Preliminary histopathology of representative stomach sections from mussel extract-treated rats was also performed to evaluate the potential for gastric irritation of anti-inflammatory treatments. Features which were assessed included the extent of gastric mucosal damage, however no histological changes relating to mucosal damage were seen (micrographs not shown).

4. Discussion

The AI activity of stabilised lipid extracts isolated from the green-lipped mussel of New Zealand, *P. canaliculus*, was investigated in this study. The tartaric acid-stabilised freeze-dried powder of the mussel flesh was subjected to supercritical-CO_2 fluid treatment, and the resulting crude lipid extract was immediately fractionated to isolate the FFA class. The mussel FFA class was further separated to provide an enriched fraction containing novel ω-3 PUFA. The novel PUFA were identified as 5, 9, 12, 15-octadecatetraenoic acid (C18:4); 5, 9, 12, 16-nonadecatetraenoic acid (C19:4); 7, 11, 14, 17-eicosatetraenoic acid (C20:4) and 5, 9, 12, 15, 18-heneicosapentaenoic acid (C21:5) by GC-MS analysis; and this profile of the novel PUFA concurs with that previously obtained by Treschow et al. (2007). The ω-3 PUFA fraction was subjected to an *in vitro* assay for AI activity, using a sensitive HPLC procedure for the quantification of leukotriene biosynthesis by stimulated human neutrophils. In this study, formation of the 5-LO products was significantly reduced by the novel ω-3 PUFA fraction. The AI effect of the novel ω-3 PUFA in this *in vitro* assay can be explained on the basis of modulation of AA metabolism by 5-LO. We have previously demonstrated strong inhibition of the AA-derived eicosanoids for the CO_2-SFE crude lipid extract of *P. canaliculus*, as well as production of alternate LT and PG metabolites (McPhee et al., 2001, 2007). Leukotriene B_4 is a potent chemoattractant which also induces neutrophil aggregation *in vivo*, and inhibition of LTB_4 is emerging as an important target for RA drug therapy (Jala and Haribabu, 2004).

The AI activity of the CO_2-SFE crude lipid extract and of the mussel FFA class of *P. canaliculus* was established *in vivo* by a standard animal model that demonstrates generalised polyarthritis with systemic inflammation (McColl et al., 1987). The animals used in this study were male Long Evans rats, which is a strain that is quite sensitive to the Freund's adjuvant, but typically 25% will not respond fully and are termed 'non-responders' (NR) (Billingham, 1983). In this study, suspect NR animals were identified and their values removed from group results to avoid skewed data. Parameters used to assess the AI activity of the treatments included changes in rear paw swelling, disease activity scores, body weight changes, somatic index and histopathology. However, measurements of these parameters alone are insufficient for determining the extent of ongoing active inflammation, whereas changes in important immune organs (i.e. spleen), and in serum levels of acute phase proteins (i.e. ceruloplasmin), provide considerably more information about the severity of the adjuvant arthritis (Walz et al., 1971; Rainsford, 1982). Consequently, these additional parameters were also investigated in this study.

The arthritic rats showed soft tissue swelling around the ankle joints during the development of arthritis which is considered as edema of the periarticular tissues. The AI results obtained for the 5 day treatment protocol demonstrate that only the mussel FFA class at 30 mg/kg BW/day has clinically significant AI activity, which was clearly demonstrated by significantly reduced rear paw swelling and less deterioration of total body condition when compared to the arthritic control. Reductions of paw swelling in the *P. canaliculus*-treated arthritic rats are likely to be due to the modulation of AA metabolism rendered by the lipid extracts. Eicosanoids produced from ω-3 PUFA exhibit lower vasodilation and chemotactic activity, and have lower pro-inflammatory activity compared to the ω-6 AA-derived eicosanoids (Gil, 2002). The AI effects of the mussel FFA class were accompanied by a reduced infiltration of inflammatory cells into the spleen, and a significant decrease in the serum activity of ceruloplasmin, indicating a less severe disease state. Notably, the AI response observed for the FFA class at 30 mg/kg BW was similar to the known anti-inflammatory agent, piroxicam (dosed at 2 mg/kg BW). Although this represents a 15-fold greater dosage amount of mussel FFA class for similar AI activity as piroxicam, the novel ω-3 PUFA fraction at 3.4% w/w of the FFA class is present at 1.02 mg/kg BW, clearly demonstrating the potency of the novel ω-3 PUFA compounds.

The 5 day treatment of the CO_2-SFE crude lipid extract exhibited no significant AI activity at either 50 or 100 mg/kg BW/day, apart from a significantly reduced serum ceruloplasmin activity. It was noticed that deposits of the crude material

remained at the injection sites, and may relate to inadequate dispersion from the injection sites. This finding led to a further investigation of the CO_2-SFE lipid extract at 100 mg/kg BW/day for a longer dosage period of 15 days, starting on the day of adjuvant injection. A clinically significant AI activity was subsequently observed whereby the crude lipid extract suppressed both rear paw swelling and deterioration of total body condition. These observations were accompanied not only by a significant decrease in serum ceruloplasmin activity but also by a lowered splenic inflammatory response, when compared to the arthritic control. Previous experimental observations in a similar animal study conducted by Whitehouse et al. (1997) using a different, commercially available lipid extract of *P. canaliculus* (obtained from CO_2-SFE-extracted stabilised dried mussel powder formulated with olive oil, and vitamin E as an antioxidant given orally) indicated that the crude extract is a potent but relatively slow-acting AI agent *in vivo*.

Preliminary toxicology assessment studies were performed to investigate whether the mussel lipids of *P. canaliculus* had the potential for adverse side effects. Investigations were performed in both inflamed (arthritic) and uninflamed (healthy, non-arthritic) rats, as it is important to also test new AI drugs in diseased animals due to impaired drug metabolism in rats with adjuvant-induced arthritis (Whitehouse and Beck, 1973).

As the liver is the major metabolic organ and important in lipid metabolism, it is necessary to investigate the effects of potential therapeutic lipid compounds on this organ. The present results show that administration of the CO_2-SFE crude lipid and mussel FFA extracts did not alter levels of microsomal ECOD and EROD activities when compared to the olive oil-vehicle controls of uninflamed rats. Marked interference with the xenobiotic-metabolising P450s enzymes would have otherwise indicated the possibility of drug interactions during polypharmacy. Because inflammation reduces P450 activity to very low levels (Ivens et al., 1993), EROD and ECOD activity was measured in uninflamed animals only. Sorbitol dehydrogenase is a specific intracellular enzyme of the liver, and leakage of this enzyme into the bloodstream is predictive of hepatocellular injury (Travlos et al., 1996). Administration of the *P. canaliculus* lipid extracts did not elevate serum SDH levels in either inflamed or uninflamed rats indicating that hepatotoxicity had not occurred. The histopathology assessment performed on liver sections from both inflamed and uninflamed groups did not reveal any significant changes in the histology of the mussel extract-treated groups when compared to olive oil-vehicle controls. In addition, histology of kidney and stomach was performed, as these are also target organs for the adverse side effects caused by NSAID treatment (Celotti and Laufer, 2001), particularly as NSAIDs can cause gastric irritation and ulceration of the stomach lining. Preliminary histopathology examination of stomach sections also revealed no adverse changes, which correlate with an earlier study whereby *P. canaliculus* lipids are shown to be gastroprotective (Whitehouse et al., 1997). This is an interesting observation, as it could overcome one of the major adverse effects associated with the prolonged use of NSAIDs, such as piroxicam-induced gastric ulceration (Tagliati et al., 1999). The present study also shows no adverse effects pertaining to the mussel lipid extracts in relation to the general non-specific indicators of changes in body or relative organ weights.

As lipophilic extracts, it is essential to investigate the effects of the mussel treatments on endogenous lipid metabolism. A causal relationship between hyperlipidemia and atherosclerosis is well established (Jain et al., 2007). Triglyceride levels in serum and mitochondrial β-oxidation of fatty acids in the liver were assayed in both inflamed and uninflamed treatment groups, and neither parameter was altered significantly upon treatment with *P. canaliculus* lipid extracts.

There is much evidence for beneficial effects of ω-3 PUFA in human health and disease (Gil, 2002; Stamp et al., 2005). Ingestion of ω-3 PUFA from the diet partially replace the AA of cellular membranes, including those of inflammatory cells. Since ω-3 PUFA modulate the production of AA-derived eicosanoids, there is a potential benefit of *P. canaliculus* lipids in the treatment of not only RA, but of other inflammatory diseases including osteoarthritis, psoriasis, asthma and cardiovascular disease. In conclusion, this study has established that both the CO_2-SFE crude lipid component and the FFA class of tartaric acid-stabilised *P. canaliculus* contain biologically significant AI activity without observable adverse side effects. The AI activity increases with progressive purification of the AI components, as the mussel FFA class exhibited greater AI activity at a lower dosage (30 mg/kg BW) and for a shorter dosage period (5 days) when compared to the significant AI activity obtained for the crude lipid component (100 mg/kg BW for 15 days).

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