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Munronia pinnata (Wall.) Theob.: Unveiling phytochemistry and dual inhibition of 5-lipoxygenase and microsomal prostaglandin E_2 synthase (mPGES)-1

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ABSTRACT

Ethnopharmacological relevance: Preparations from *Munronia pinnata* (Wall.) Theob. are extensively used in traditional medicine in Sri Lanka for the treatment of inflammatory conditions. However, neither the pharmacological features nor the phytochemistry of this plant are explored in order to understand and rationalize the reported ethnobotanical significance. As 5-lipoxygenase (5-LO) and microsomal prostaglandin E_2 synthase (mPGES)-1 are crucial enzymes in inflammatory disorders, we evaluated their inhibition by *M. pinnata* extracts and studied the chemical profile of the plant for the identification of relevant constituents.

Materials and methods: Cell-free and cell-based assays were employed in order to investigate the suppression of 5-LO and mPGES-1 activity. Cell viability, radical scavenger activities, and inhibition of reactive oxygen species formation (ROS) in neutrophils were studied to assess cytotoxic and antioxidant effects. Gas and liquid chromatography coupled to mass spectrometric analysis enabled the characterization of secondary metabolites.

Results: The *n*-hexane extract of *M. pinnata* efficiently suppressed 5-LO activity in stimulated human neutrophils (IC_{50} = 8.7 μg/ml) and potently inhibited isolated human recombinant 5-LO (IC_{50} = 0.48 μg/ml) and mPGES-1 (IC_{50} = 1.0 μg/ml). In contrast, no significant radical scavenging activity or suppression of ROS formation was observed, and neutrophil viability was unaffected. The phytochemistry of the plant was unveiled for the first time and phytosterols, fatty acids, sesquiterpenes and several other types of secondary metabolites were identified.

Conclusions: Together, potent inhibition of 5-LO and mPGES-1 activity, without concomitant antioxidant activity and cytotoxic effects, rationalizes the ethnopharmacological use of *M. pinnata* as anti-inflammatory remedy. Detailed chromatographic/mass spectrometric analysis reveals discrete chemical structures of relevant constituents.

1. Introduction

The genus *Munronia* Wight (Meliaceae) comprises 13–15 species naturally distributed in southern China, Vietnam, Myanmar, Java, Sri Lanka, India, Indonesia and the Philippines (Qi et al., 2003). *Munronia pinnata* (Wall.) Theob. (synonyms – *Munronia pumila* Wight, *Melia pumila* Moon), locally known as "Binkohomba", is a small herb widely used in Ayurvedic and folk medicine in Sri Lanka for hundreds of years (Hapuarachchi et al., 2011a). This plant is a rare species and grows in intermediate and wet zone forests and on rocky places in Sri Lanka but is also distributed in Southern and Northern India, China, Vietnam, Burma, Thailand and Timor (Dassanayake et al., 1995). The whole plant is used for commercial purposes (Hapuarachchi et al., 2011a) and is considered to be one of the most expensive plant materials (US$ 50–110/kg) used in traditional medicine in Sri Lanka (Dharmadasa et al., 2011). Nowadays much attention is drawn on the development of *ex situ* conservation methods *via in vitro* propagation techniques in order to establish commercial cultivations of this plant (Senarath et al., 2007).

In Sri Lankan folk medicine, the plant is a major ingredient of decoctions and powders used for the treatment of fever, dysentery, skin diseases, purification of blood upon snake bites and malaria

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(Jayaweera, 1982), and to prevent hiccups, vomiting and sore throats (Arambewela and Wijesinghe, 2006). According to the pharmacopoeia, it exhibits wound purifying, anthelmintic, carminative and laxative properties, it improves digestive power, reduces dermatitis, promotes lactation, destroys worms and interestingly, it is also used for the treatment of polyuria, cough and edema (Arambewela and Wijesinghe, 2006; Department of Ayurveda, 1979).

Despite its therapeutic importance, the bioactivities of *M. pinnata* are hardly explored in order to rationalize the reported ethnopharmacological use. The whole plant extract exhibits marked cytotoxicity and potent anti-malarial activity as claimed by Sri Lankan traditional practitioners (Dharmadasa et al., 2012). A pilot experimental study with aqueous extracts in healthy Wistar rats revealed statistically significant oral hypoglycemic effects (Hapuarachchi et al., 2011a,b). No acute or chronic toxic effects of water and ethanol extracts of natural plant and callus cultures of *M. pinnata* were observed in healthy rats (Hapuarachchi et al, 2013). Although the plant is extensively used to alleviate the pathological conditions caused by inflammation, pharmacological investigations on its anti-inflammatory properties are rare. A decoction of *M. pinnata* (Hapuarachchi et al., 2012) revealed anti-inflammatory effectiveness in the carrageen-induced paw edema. However, neither the anti-inflammatory principle of the plant extract nor the molecular mechanisms were identified.

Prostaglandins (PG) and leukotrienes (LTs) are formed from arachidonic acid (AA) and act as important mediators of inflammation, allergy and pain (Funk, 2001). LTs contribute to various inflammatory and allergic reactions in the pathophysiology of asthma, allergic rhinitis, atherosclerosis, cancer, etc. (Werz and Steinhilber, 2006). 5-Lipoxygenase (5-LO) that catalyzes the first two key steps in LT biosynthesis from AA is considered as valuable drug target (Radmark et al., 2007; Pergola and Werz, 2010). Among the PGs, the PGE_2 is formed from AA under inflammatory conditions essentially by cyclooxygenase (COX)-2 coupled to microsomal PGE_2 synthase (mPGES)-1 (Samuelsson et al., 2007). Dual pharmacological intervention with both LT and PGE_2 biosynthesis proposes a strong therapeutic benefit in inflammatory diseases. In fact, plant-derived natural products have been reported to dually suppress 5-LO and mPGES-1 activity (Koeberle and Werz, 2009; Werz, 2007), which rationalizes these pro-inflammatory enzymes as functional targets for anti-inflammatory phytomedicine.

The phytochemistry of *M. pinnata* is not established yet and remains to be explored. Conventional natural product isolation methodologies involving tedious chromatographic separations are extremely time consuming, technically demanding and require large quantities of sample, and are thus not feasible for phytochemical studies of rare medicinal plants like *M. pinnata*. Therefore, the development of novel methodologies which could provide detailed structural information about phytochemical constituents directly from the crude extract or less purified fractions of the crude extracts is desirable. Due to the dramatic improvement in instrumental methods in the field of mass spectrometry over the last few years, detection and identification of chemical components without extensive purification protocols is possible. Novel hyphenated techniques providing excellent separation efficiency as well as acquisition of online complementary spectrometric data from complex crude extracts enable effective compound identification in plant extracts (Sarker and Nahar, 2012). Together with the modern analytical techniques, tandem mass spectrometry (MS/MS) fragment libraries provide a potential avenue for the study of secondary metabolites at nanomole-scale. The present study was undertaken to reveal anti-inflammatory mechanisms of *M. pinnata* and to identify related secondary metabolites with novel mass spectrometric techniques.

2. Materials and methods

2.1. Plant material

Plants were collected in Weerasuriyakanda (Gampaha district, Western Province of Sri Lanka) and Algama (Kegalle district, Sabaragamuwa Province of Sri Lanka) in 2011/2012. The plant was identified by the author (MN), a botanist, and confirmed based on the books "A Revised Handbook to the Flora of Ceylon: Volume IX – M.D. Dassanayake, F.R. Fosberg and W.D. Clayton" and "Medicinal Plants (indigenous and exotic) used in Ceylon: Volume IV – D.M.A. Jayaweera" and authenticated by comparison with the herbarium specimens at the National herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka. A voucher specimen (Mun-SP-1-0606) is deposited at Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka.

2.2. Preparation of crude extracts

The plant materials (whole plant) were thoroughly washed, chopped into small pieces and dried in shade (30 ± 2 °C) for four days. Dried plants were powdered using an electrical grinder (Singer, model: KA-MIXEE) and the powdered material (15 g) successively extracted with 600 ml of *n*-hexane, dichloromethane, ethyl acetate (EtOAc) and methanol (Roth, Karlsruhe, Germany) at room temperature using a linear shaker for 20 minutes. Besides, 3.3 g of powdered material was extracted in 300 ml of 70% methanol–water in the presence of 0.05% acetic acid by heating for 2 hours at 60 °C. Evaporation of each solvent under reduced pressure (BÜCHI- Rotary evaporator, *R*-114, Germany) yielded dried crude extracts which were then subjected to the bioactivity studies.

2.3. Evaluation of bioactivity

2.3.1. 5-Lipoxygenase (5-LO) activity in intact neutrophils

Human neutrophils were isolated from leukocyte concentrates obtained from the University Hospital Jena, Germany. In brief, peripheral blood was withdrawn from fasted (12 h) healthy donors that had not taken any anti-inflammatory drugs during the last 10 days by venipuncture in heparinized tubes (16 IE heparin/ml blood). The blood was centrifuged at 4000*g* for 20 min at 20 °C. Leukocyte concentrates were subjected to dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria). Contaminating erythrocytes of pelleted neutrophils were lysed by hypotonic lysis. Neutrophils were washed twice in ice-cold PBS and finally resuspended in PBS pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) (purity $>$ 96–97%). The cells were preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO), and incubated for 10 min at 37 °C with the Ca^{2+}-ionophore A23187 (2.5 μM) plus 20 μM AA. Then, the reaction was stopped on ice by addition of 1 ml of methanol, 30 μl 1 N HCl and 500 μl PBS, and 200 ng prostaglandin B_1 was added. The samples were subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA) and 5-LO products (LTB_4 and its trans-isomers, 5-H(P)ETE) were analyzed by HPLC on the basis of the internal standard PGB_1. Cysteinyl-LTs C_4, D_4 and E_4 were not detected (amounts were below detection limit), and oxidation products of LTB_4 were not determined.

2.3.2. 5-LO activity in cell-free assays (purified 5-LO)

Escherichia coli (BL21) was transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed at 30 °C as described (Fischer et al., 2003). Cells were lysed in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/ml),

1 mM phenylmethanesulphonyl fluoride, and lysozyme (1 mg/ml), homogenized by sonication (3 × 15 s), and centrifuged at 40,000*g* for 20 min at 4 °C. The 40,000*g* supernatant was applied to an ATP-agarose column to partially purify 5-LO as described previously (Fischer et al., 2003). Aliquots of semi-purified 5-LO were immediately diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added. Samples were preincubated with the test compounds or vehicle (0.1% DMSO) as indicated. After 10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 μM AA was added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol, and the formed metabolites were analyzed by RP-HPLC as described (Fischer et al., 2003). 5-LO products include the all-trans isomers of LTB_4 as well as 5-HPETE and its corresponding alcohol 5-HETE.

2.3.3. Determination of mPGES-1 activity

Preparation of A549 cells and determination of the activity of mPGES-1 was performed as described previously (Koeberle et al., 2008). In brief, IL-1β-treated A549 cells overexpressing mPGES-1 were sonicated and the microsomal fraction was prepared by differential centrifugation at 10,000*g* for 10 min and at 174,000*g*. The resuspended microsomal membranes were preincubated with the test compounds or vehicle (DMSO). After 15 min, PGE_2 formation was initiated by addition of PGH_2 (final concentration, 20 μM). After 1 min at 4 °C, the reaction was terminated, and PGE_2 was separated by solid-phase extraction (RP-18 material) and analyzed by RP-HPLC as described (Koeberle et al., 2008).

2.3.4. DPPH assay

The radical scavenger capability was assessed by measuring the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described (Blois, 1958). Briefly, 100 μl of extracts (20 and 100 μg/ml) were added to 100 μl of a solution of the stable free radical DPPH in ethanol (50 μM, corresponding to 5 nmol), buffered with acetate to pH 5.5, in a 96-well plate. The absorbance was recorded at 520 nm (Multiskan Spectrum Reader, Thermo Fisher Scientific Oy, Vantaa, Finland) after 30 min incubation under gentle shaking in the dark. Ascorbic acid and L-cysteine were used as reference compounds. All analyses were performed in triplicates.

2.3.5. Measurement of reactive oxygen species in neutrophils

Neutrophils (10^7/ml PG buffer) were preincubated with test compounds (or 0.1% DMSO as vehicle) for 15 min. Then, the peroxide-sensitive fluorescence dye 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, 1 μg/ml) and $CaCl_2$ (1 mM) were added 2 min prior addition of phorbol myristate acetate (PMA, 0.1 μM). The fluorescence emission at 530 nm was measured after excitation at 485 nm in a thermally controlled (37 °C) NOVOstar microplate reader (BMG Labtechnologies GmbH, Offenburg, Germany).

2.3.6. Statistical analysis

Data are expressed as mean ± S.E.M. IC_{50} values were calculated from averaged measurements at 3–5 different concentrations of the compounds by nonlinear regression using GraphPad Prism software (San Diego, CA) one site binding competition. Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni or Tukey–Kramer post hoc test for multiple comparisons respectively. A p value < 0.05 (*) was considered significant.

2.4. Phytochemical screening

2.4.1. Bioassay-guided fractionation

M. pinnata hexane extract (130 mg) was dissolved in dichloromethane (about 100 μl) and was absorbed into silica gel (15 mg) (silica gel 60, 0.04–0.063 mm, 230–400 mesh, Roth, Germany), the solvents were completely removed by rotary evaporation and fractionated over a silica gel (12 g) column. The sample was eluted with *n*-hexane, 3% EtOAc in *n*-hexane, 5% EtOAc in *n*-hexane, 10% EtOAc in *n*-hexane, 15% EtOAc in *n*-hexane, 25% EtOAc in *n*-hexane, 35% EtOAc in *n*-hexane, 50% EtOAc in *n*-hexane, 75% EtOAc in *n*-hexane, EtOAc and methanol, successively, yielding 11 fractions. The collected fractions were evaporated and the dry weight was measured and subjected to bioactivity assays and GC/LC–MS analysis.

2.4.2. Liquid chromatography coupled mass spectrometric (LC–MS) analysis

The *n*-hexane extract and the fractions obtained thereof were analyzed on a LTQ-Orbit rap instrument (Thermo Fisher, San Jose, CA) with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The samples were dissolved in ethyl acetate (LC–MS grade; 1 mg/ml) and diluted to 10 μg/ml. Fifteen μl aliquots of the diluted samples were injected and separated by liquid chromatography using a Dionex–Acclaim® RSLC 120 C18 column (2.1 mm × 150 mm packed with 2.2 μm, 120 Å). Reversed phase UPLC gradient separations were performed using (A) water (LC–MS grade), with 0.1% formic acid (LC–MS grade), and (B) methanol (LC–MS grade), with 0.1% formic acid as mobile phases. The gradient program was set as 0 min – 100% A, 0.3 ml/min, 5 min – 100% A, 0.3 ml/min, 48 min – 100% D, 0.3 ml/min, 60 min – 100% D, 0.3 ml/min, 60.1 min – 100% A, 0.3 ml/min, 65.1 min – 100% A, 0.3 ml/min. All LC–MS grade solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

In the ESI source, the heated capillary temperature was 275 °C and the capillary voltage and tube lens voltage were set to 48 V and 95 V, respectively. The APCI source was operated at 400 °C, the heated capillary temperature was 220 °C and the corona discharge current was set to 4.5 μA. The full scan and collision-induced dissociation (CID) mass spectra were generated using 30,000 and 7500 full width at half maximum (fwhm) resolutions, respectively. The full scan mass spectra were recorded in the m/z range 100–2000. CID mass spectra were obtained at different collision energies between 1 and 55 eV. The activation time was set at 30 ms with the activation parameter $q = 0.25$. An isolation window of 1.0 mass unit was used.

2.4.3. Molecular formula identification

Following a published method (Rasche et al., 2012), the molecular formula were identified by isotope pattern and fragmentation tree analysis. Fragmentation trees annotate fragment peaks with molecular formulas and model fragmentation reactions through dependencies between fragment ions. The fragmentation tree that explains the data best is calculated by an optimization algorithm. The score of a tree takes into account mass deviation between peak masses and assigned molecular formulas, plausibility of molecular formulas, intensity of explained peaks, and whether losses are common.

2.4.4. Gas chromatography coupled mass spectrometric (GC–MS) analysis

GC–MS analysis of the *n*-hexane extract and the fractions of interest (F-6 and F-8) was carried out on a gas chromatograph HP6890 (Agilent, CA, USA) connected to a MS02 mass spectrometer from Micromass (Waters, Manchester, UK) with EI 70 eV

equipped with ZB5ms column (30 m × 0.25 mm, 0.25 μm film thickness; Phenomenex, CA, USA). Helium was used as the carrier gas at the flow rate of 1 ml/min. The injector temperature was kept at 250 °C and the oven temperature was programmed as 100 °C (2 min), 15 °C/min to 200 °C, 5 °C/min to 305 °C (20 min).

3. Results

3.1. Evaluation of 5-LO inhibition

Isolated human neutrophils that are stimulated with the Ca^{2+}-ionophore A23187 are a well-established cell-based model for investigating the suppression of 5-LO activity by small molecule inhibitors, and was thus applied as test system in the present study. A potent inhibition of 5-LO activity in neutrophils was observed for the *n*-hexane and dichloromethane (DCM) extracts of *M. pinnata* (10 and 100 μg/ml, Fig. 1A), whereas methanol and water extracts were almost ineffective and reduced 5-LO activity only by 15 and 16% at 100 μg/ml, respectively (Fig. 1A). More detailed concentration–response studies using this cell-based assay revealed an IC_{50} value of 8.7 μg/ml for the *n*-hexane extract (Fig. 1B). The synthetic reference control inhibitor zileuton (approved as anti-asthmatic drug, Israel et al., 1990) blocked 5-LO activity with IC_{50} = 0.13 μg/ml (corresponding to 0.55 μM). Note that unspecific detrimental effects of the extracts on the viability of neutrophils can be excluded based on the ability of the cells to prevent trypan blue uptake in the presence of 10 or 100 μg/ml extract (not shown).

The pharmacological intervention with 5-LO product synthesis in the cell may be caused by diverse mechanisms, others than interference with the 5-LO enzyme activity, such as inhibition of AA supply, blockade of 5-LO-activating protein (FLAP), or loss of cell viability (Werz and Steinhilber, 2005). Therefore, the evaluation of

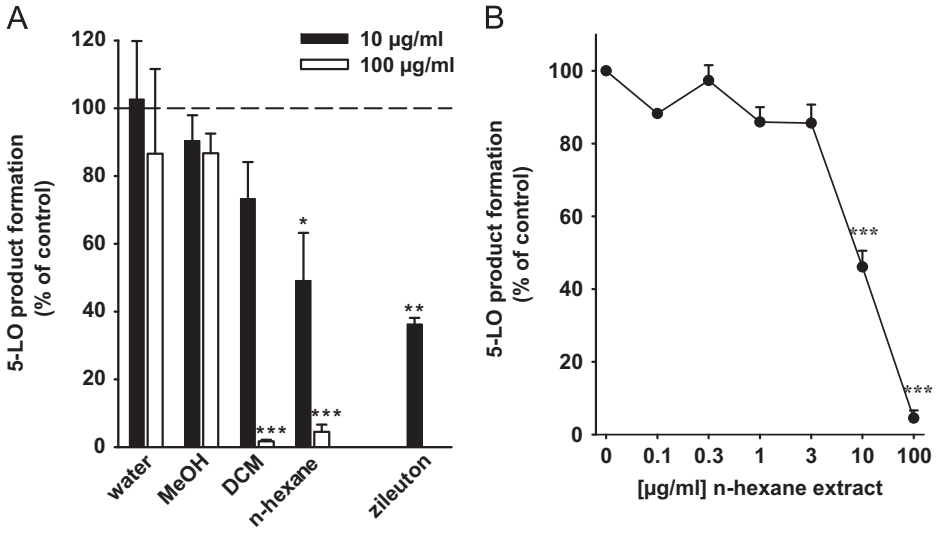

Fig. 1. Inhibition of 5-LO activity in intact neutrophils stimulated with 2.5 μM A23187 plus 20 μM AA. (A) Inhibition of 5-LO activity by various extracts of *M. pinnata*. (B) Concentration–response analysis for the *n*-hexane extract of *M. pinnata*. Neutrophils were preincubated with the extracts, zileuton (3 μM) or vehicle (0.1% DMSO) for 10 min at 37 °C prior to stimulation for another 10 min. Data are given as mean ± S.E.M., n=3–4. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* 100% control.

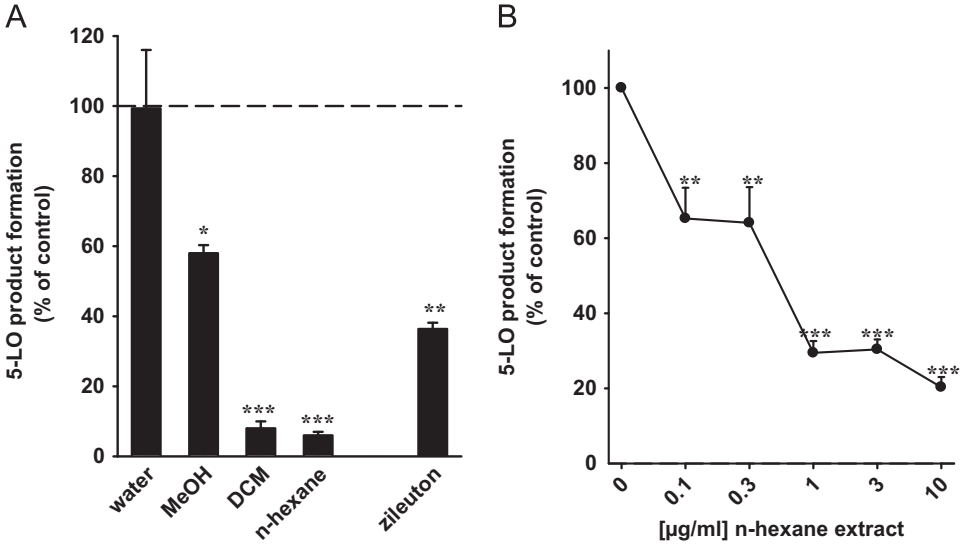

Fig. 2. Inhibition of 5-LO activity in a cell-free assay, where isolated human recombinant 5-LO was used as enzyme source and 20 μM AA as substrate. (A) Inhibition of 5-LO by various extracts (10 μg/ml) or zileuton (3 μM) of *M. pinnata*. (B) Concentration–response analysis for the *n*-hexane extract. Data are given as mean ± S.E.M., n=3–4. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* 100% control.

direct interaction of the extracts with 5-LO itself is important. In order to evaluate whether the extracts directly inhibited 5-LO activity, a cell-free assay using isolated human recombinant 5-LO as enzyme source and 20 μM AA as substrate was employed. Again, extracts (at 10 μg/ml) based on *n*-hexane or DCM as solvent efficiently blocked 5-LO activity while extracts prepared with water or methanol were much less effective (Fig. 2A). As shown in Fig. 2B, the *n*-hexane extract of *M. pinnata* caused potent and concentration-dependent inhibition of 5-LO activity with IC_{50} =0.48 μg/ml. For zileuton, the IC_{50} value was determined at 0.11 μg/ml (not shown), implying comparably marked 5-LO inhibitory potencies of the *M. pinnata* extract.

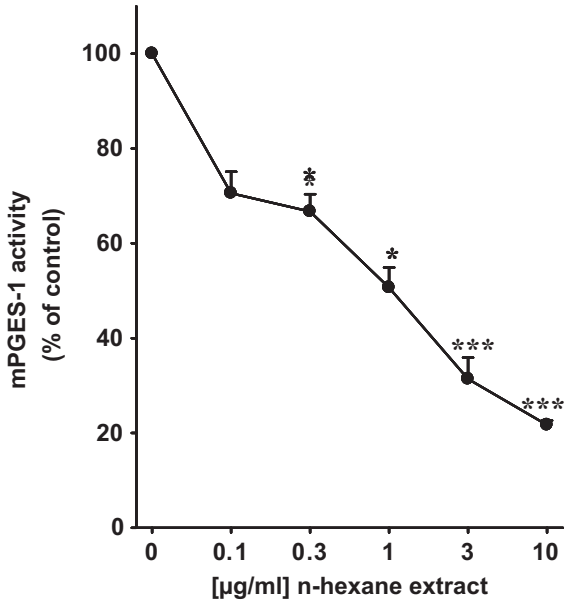

Fig. 3. Inhibition of mPGES-1 by the *n*-hexane extract of *M. pinnata*. The microsomal fraction of IL-1β-stimulated A549 cells was preincubated (10 min) with the extract and then stimulated by addition of 20 μM PGH_2. After 1 min at 4 °C, the formation of PGE_2 was assessed by RP-HPLC. Data are given as mean ± S.E.M., n=3–4. *$p <$ 0.05, ***$p <$ 0.001 *versus* 100% control.

3.2. Evaluation of mPGES-1 inhibition

Because the *n*-hexane extract appeared to be most interesting, we investigated the potential of this extract to interfere also with the formation of the pro-inflammatory PGE_2 produced by mPGES-1. Using the microsomal fraction of IL-1β stimulated A549 cells that strongly express mPGES-1 under these conditions, the extract potently and concentration-dependently inhibited the enzymatic transformation of PGH_2 to PGE_2, catalyzed by mPGES-1. The IC_{50} value was determined at 1.0 μg/ml (Fig. 3), which is even slightly lower than for MK886 (1.3 μg/ml = 2.4 μM), a well-recognized mPGES-1 inhibitor, used as control (not shown).

3.3. Evaluation of radical scavenging properties and suppression of ROS formation in neutrophils

Many natural products from plant origin are proposed to interfere with their molecular targets by unselective antioxidant reactions. In the case of 5-LO, they may reduce the active-site iron, decompose 5-LO-activating lipid hydroperoxides, or scavenge intermediate fatty acid radicals within LT synthesis (Werz, 2007). Therefore, radical scavenging properties of the *M. pinnata* extracts were assessed using the cell-free DPPH assay, in order to investigate whether such unselective antioxidant properties may account for 5-LO inhibition. We find that the *n*-hexane extract of *M. pinnata* up to 50 μg/ml is not able to significantly reduce radical formation, in contrast to the reference antioxidant compounds ascorbic acid or *L*-cysteine (Fig. 4A). This suggests that 5-LO inhibition by *M. pinnata* extract is not mediated by a redox-based mechanism.

In addition, the ability of the extracts (10 μg/ml) to prevent cellular ROS formation in neutrophils stimulated with the bacterial peptide fMLP was assessed. The *n*-hexane extract of *M. pinnata* did not cause significant inhibition of ROS formation, whereas DPI used as reference inhibitor completely prevented ROS generation (Fig. 4B). Of interest, extracts based on water, methanol or DCM

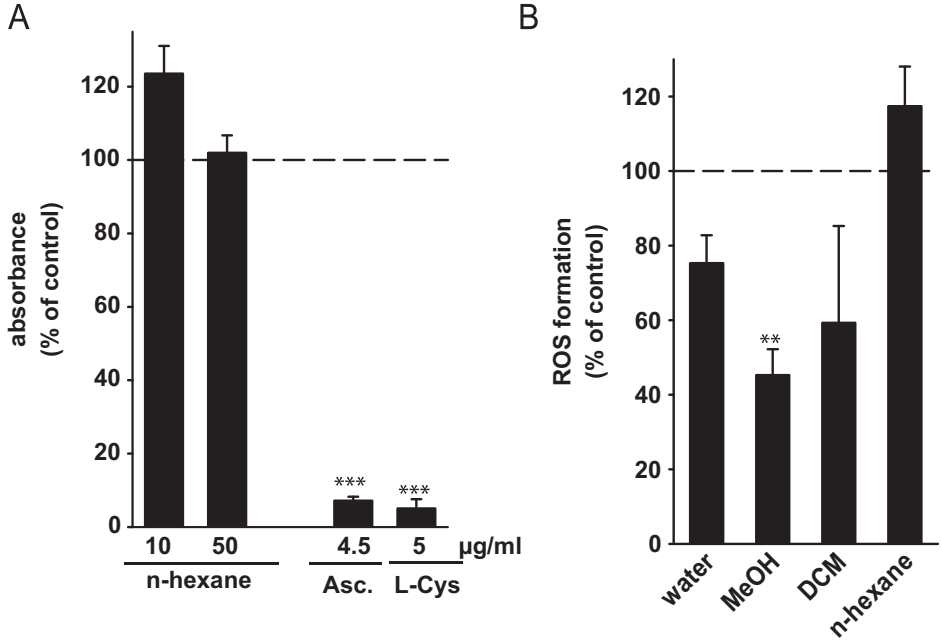

Fig. 4. (A) Radical scavenging activity of the *n*-hexane extract of *M. pinnata*. (B) Effects of the extracts of *M. pinnata* (10 μg/ml) on cellular ROS formation in neutrophils stimulated with fMLP. Neutrophils were preincubated with the extracts (or 0.1% DMSO as vehicle) for 15 min prior to stimulation with fMLP. Data are given as mean ± S.E.M., n=3–4. **$p <$ 0.01, ***$p <$ 0.001 *versus* 100% control.

Table 1
Inhibition of 5-LO and mPGES-1 activity in cell-free assays by fractions of the *n*-hexane extract. Data are given as mean ± S.E.M., $n=3$–4.

Fraction (no.)	Total amount of n-hexane extract (mg)	mPGES-1 residual activity (in %)		5-LO (cell-free) residual activity (in %)	
		10 μg/ml	1 μg/ml	10 μg/ml	1 μg/ml
F-1	1	n.d.	n.d.	n.d.	n.d.
F-2	4	80 ± 5	125 ± 17	60 ± 10	n.d.
F-3	1	n.d.	n.d.	n.d.	n.d.
F-4	24	n.d.	n.d.	n.d.	n.d.
F-5	8	58 ± 6	101 ± 13	57 ± 12	n.d.
F-6	22	29 ± 4	65 ± 6	29 ± 4	78 ± 8
F-7	17	15 ± 5	66 ± 6	40 ± 10	64 ± 4
F-8	1	11 ± 5	42 ± 0	30 ± 9	64 ± 5
F-9	6	49 ± 2	103 ± 1	27 ± 7	72 ± 6
F-10	6	53 ± 2	91 ± 3	64 ± 11	n.d.
F-11	9	55 ± 2	96 ± 3	50 ± 9	n.d.

were able to reduce ROS formation, with the methanol extract being most potent (54.7 ± 6.9% inhibition).

3.4. Bioassay-guided separation of the M. pinnata n-hexane extract

Early attempts of phytochemical screening of *M. pinnata* either failed (Arambewela and Wijesinghe, 2006) or provided incomplete information on an acid with 15 carbon atoms and a triterpenoid with 34 carbon atoms (Munasinghe, 2002) in a lipophilic fraction. In order to get more insights into the identity of secondary metabolites that are responsible for the potent inhibition of 5-LO and mPGES-1, the *n*-hexane extract was fractionated by liquid column chromatography using ethyl acetate, *n*-hexane and methanol. This yielded 11 fractions that were analyzed for inhibition of isolated 5-LO and mPGES-1 in the cell-free assays at 1 and 10 μg/ml, each. Out of the 11 fractions (F) of the *n*-hexane extract, F-5 (15% EtOAc in *n*-hexane), F-6 (25% EtOAc in *n*-hexane), F-7 (35% EtOAc in

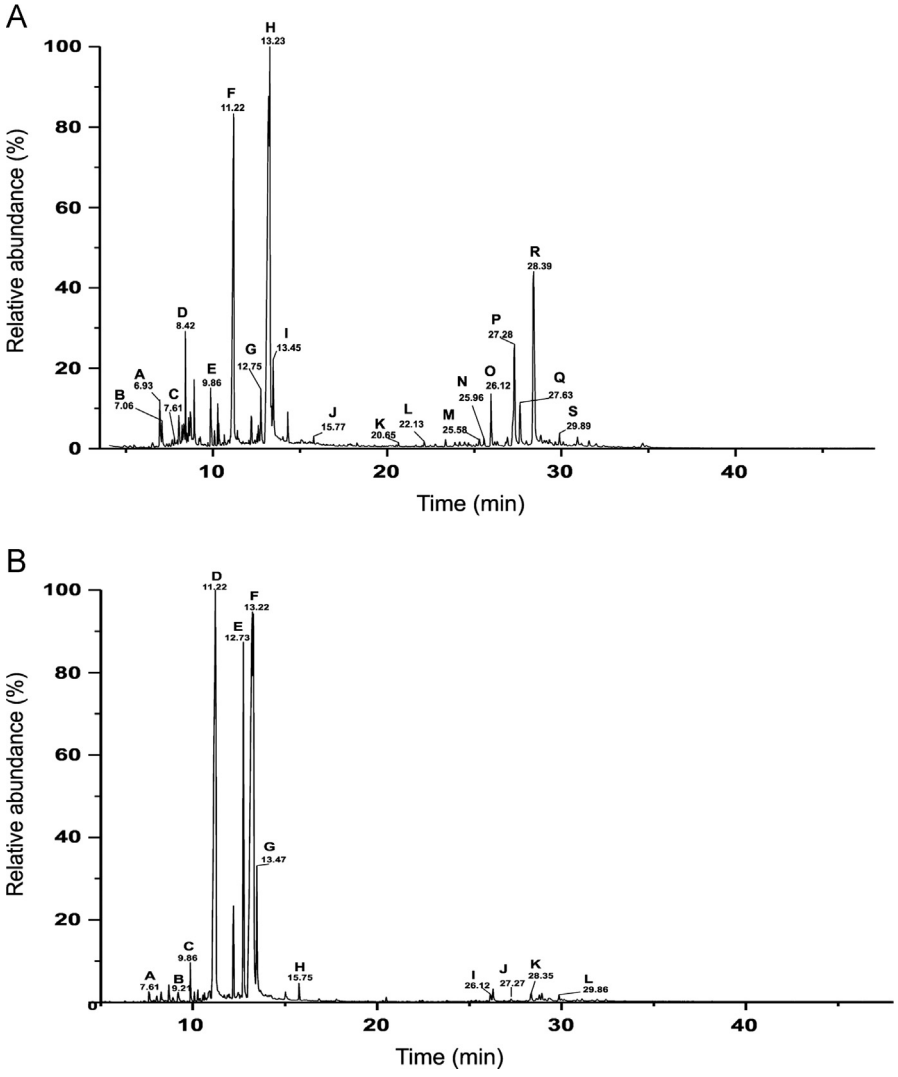

Fig. 5. (A) Total ion chromatograph of the *n*-hexane extract of *M. pinnata* and its identified compounds. **A**: β-Caryophyllene, **B**: isocaryophyllene, **C**: dodecanoic acid, **D**: caryophyllene oxide, **E**: neophytadiene, **F**: hexadecanoic acid, **G**: phytol, **H**: 9,12-octadecadienoic acid, **I**: octadecanoic acid, **J**: 4,8,12,16-tetramethylheptadecan-4-olide, **K**: heptacosane, **L**: squalene, **M**: stigmastan-3,5-diene, **N**: hentriacontane, **O**: α-tocopherol, **P**: campesterol, **Q**: stigmasterol, **R**: β-sitosterol, and **S**: stigmast-4-en-3-one. (B) Total ion chromatograph of F-6 of the *n*-hexane extract of *M. pinnata* and its identified compounds. **A**: dodecanoic acid, **B**: tetradecanoic acid, **C**: neophytadiene, **D**: hexadecanoic acid, **E**: phytol, **F**: 9,12-octadecadienoic acid, **G**: octadecanoic acid, **H**: 4,8,12,16-tetramethylheptadecan-4-olide, **I**: α-tocopherol, **J**: campesterol, **K**: β-sitosterol, and **L**: stigmast-4-en-3-one.

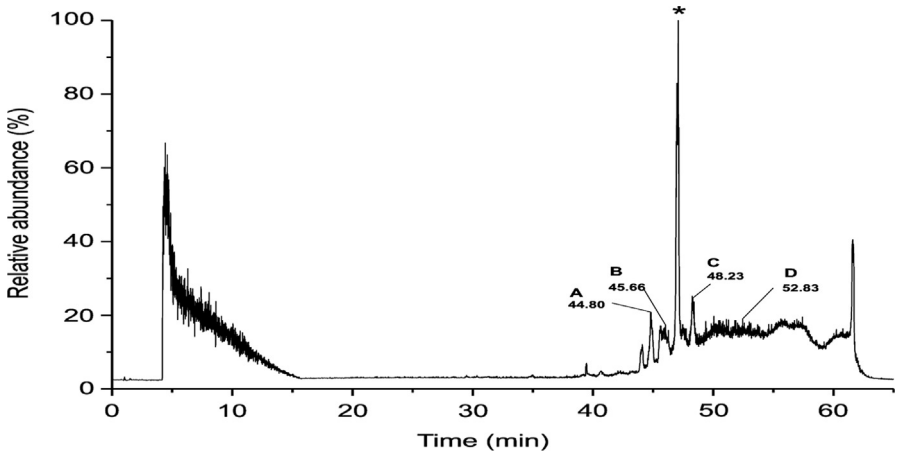

Fig. 6. Total ion chromatograph of F-6 of the *n*-hexane extract of *M. pinnata* when analyzed with the ESI source and the presumed compounds. **A**: Ganoderiol *F*, **B**: conicasterol C/theonellasterol, **C**: stigmastentriol, **D**: stigmasterol (the peak denoted as "*" is due to erucylamide, a contaminant in the LC system).

n-hexane), F-8 (50% EtOAc in *n*-hexane), F-9 (75% EtOAc in *n*-hexane) and F-11 (MeOH) at a concentration of 10 μg/ml inhibited 5-LO activity as well as mPGES-1 by >50%. Among these fractions, F-6, F-7, F-8, and F-9 were significantly active at 1 μg/ml for 5-LO whereas F-6, F-7, and F-8 inhibited mPGES-1 significantly at 1 μg/ml (Table 1). These data imply a good correlation of the fractions for dual inhibition of 5-LO and mPGES-1.

3.5. Identification of constituents of the n-hexane extract and its bioactive fractions

3.5.1. GC–MS analysis

The *n*-hexane extract and the fractions F-6 and F-8, which displayed high 5-LO and mPGES-1 inhibitory activities, were subjected to a phytochemical screening by GC–MS and UPLC–MS. The GC–MS analysis of the *n*-hexane crude extract led to the identification of 19 components, revealed by comparison of their experimental mass spectrum with those recorded in the NIST MS Search 2.0, Adams and Wiley mass spectrum libraries and also by the comparison with the respective standards. These components include ubiquitously occurring fatty acids (dodecanoic acid, hexadecanoic acid, 9,12,-octadecadienoic acid, octadecanoic acid), sesquiterpenes (*β*-caryophyllene, isocaryophyllene, caryophyllene oxide), a diterpene alcohol (phytol), an acyclic diterpene (neophytadiene), higher alkanes (heptacosane, hentriacontane), a triterpene (squalene) as well as the isoprenoid 4,8,12,16-tetramethylheptadecan-4-olide and α-tocopherol (Fig. 5A). Among the above identified compounds, 12 compounds were detected in F-6 (absolute amount 22 mg) and only 3 compounds in F-8 (absolute amount 1 mg, see below) after analysis. The total ion chromatogram (TIC) of F-6 is shown in Fig. 5B.

3.5.2. LC–MS analysis

The accurate mass measurements and the subsequent database search in METLIN suggested several compounds for the peaks denoted as compounds **A**, **B**, **C** and **D** present in F-6 (Fig. 6). Relatively poor fragmentation in the MS/MS experiments obstructed the exact compound identification, nevertheless the analysis of available CID spectra and the fragmentation trees constructed from the CID spectra eliminated many of the possibilities for the given *m*/*z* value and proposed the best fitting compound.

Compound **A**, with a *m*/*z* value of 477.33337, fits the molecular formula of $C_{30}H_{46}O_3Na$ with a mass accuracy of −1.145 ppm. The APCI-MS measurements (not shown) also supported this molecular formula where the protonated adduct ($C_{30}H_{47}O_3$) with a *m*/*z* value of 455.35220 was observed in the spectra with a mass accuracy of −0.703 ppm. The comprehensive analysis of the fragmentation pattern (Figs. S1 and S2) suggested the most possible structure for compound **A** as ganoderiol *F*.

The accurate mass measurements of compound **B** from the Orbitrap instrument suggested the molecular composition of $C_{30}H_{50}O_3Na$ (*m*/*z* 481.36490) with a mass accuracy of −0.658 ppm. The database search suggested several compounds that fit with this molecular formula and mass accuracy, however, the analysis of CID spectra excluded many possibilities and proposed compound **B** to be most likely the triterpenoids, conicasterol C or theonellasterol E according to its characteristic fragmentation pattern (Fig. S3).

In addition, compound **C** represents the molecular composition of $C_{29}H_{50}O_3Na$ (*m*/*z* 469.36456) with a mass accuracy of −0.802 ppm and compound **D** as $C_{29}H_{49}O$ (*m*/*z* 413.37775) with a mass accuracy of −0.043 ppm. Hence, these two compounds are proposed as stigmastentriol and stigmasterol, respectively.

In F8, three compounds were found, two of them (*i.e.*, ganoderiol F with *m*/*z* 477.33337 and conicasterol C or theonellasterol E with *m*/*z* 481.36490) are the same as in F-6 and the third compound with *m*/*z* 479.34921 represents the molecular formula of $C_{30}H_{48}O_3Na$ with a mass accuracy of −0.744 ppm. However, it could not be identified due to the poor fragmentation.

4. Discussion

M. pinnata belongs to the most important medicinal plants in Sri Lanka. However, the knowledge on its pharmacological features, phytochemistry, and its bioactive constituents is insufficient to explain the therapeutic use in the treatment of inflammation-related disorders. Therefore, the present study was conducted in order to rationalize and validate its traditional use as anti-inflammatory remedy by analysis of its ability to interfere with typical pro-inflammatory drug targets (*i.e.*, 5-LO and mPGES-1) and by chemical profiling of the bioactive fractions. In fact, the *n*-hexane extract of *M. pinnata* caused direct and potent inhibition of human mPGES-1 and 5-LO and suppressed the biosynthesis of 5-LO products also in intact human neutrophils. The structural characterization of potential bioactive secondary metabolites was achieved by rapid and convenient mass spectrometric approaches.

In our study, focus was placed on mPGES-1 and 5-LO as potential targets, and interference with these enzymes might help to explain the anti-inflammatory properties of *M. pinnata*. 5-LO

plays a key role in the biosynthesis of the pro-inflammatory LTs (Radmark et al., 2007) and has therefore been intensively explored as drug target for the intervention with asthma, allergic rhinitis, various autoimmune diseases, cardiovascular disease, cancers, and many other inflammatory disorders (Peters-Golden and Henderson, 2007; Werz and Steinhilber, 2006). According to the molecular mode of action, direct 5-LO inhibitors (of synthetic or natural origin) are categorized as (I) redox-type inhibitors that interfere with the redox cycle of the 5-LO active site iron, (II) iron ligand-type inhibitors that chelate the active site iron, and (III) nonredox-type inhibitors that compete with AA as substrate and/or activating fatty acid hydroperoxides or act on 5-LO by so far unrecognized mechanisms (Ford-Hutchinson et al., 1994; Werz and Steinhilber, 2005). Several hundred plants and their extracts and/or specific secondary metabolites have been investigated as inhibitors of the biosynthesis of 5-LO products (Yoshimoto et al., 1983; Laughton et al., 1991; Werz, 2007; Schneider and Bucar, 2005). In fact, the plant kingdom appeared as potential source for 5-LO inhibitors and the interference with 5-LO activity is considered as basis for the anti-inflammatory features of the respective plants (and medical preparations thereof) in folk medicine (Schneider and Bucar, 2005). However, the 5-LO inhibitory potencies of the investigated extracts often turned out to be comparably low and relatively high IC_{50} values in the rough range of 15–50 μg/ml for 5-LO inhibition were observed for lipophilic extracts of various medicinal plants (Schneider and Bucar, 2005). Thus, the IC_{50} of 8.7 μg/ml in neutrophils and of 0.48 μg/ml in the cell-free 5-LO activity assay obtained for the *n*-hexane extract of *M. pinnata* is remarkable and suggests a high pharmacological potential for intervention with 5-LO-related disorders.

Since most of the plants are rich in anti-oxidants such as polyphenols, flavonoids and coumarins, the iron-cheating and antioxidant features of these compounds are often responsible for uncoupling of the 5-LO catalytic cycle and eventually for 5-LO inhibition. Interestingly, the 5-LO inhibitory *M. pinnata* extract neither exhibits significant radical scavenging nor antioxidant activities in cell-free (DPPH assay) or cell-based (ROS generation in neutrophils) test systems. This suggests that the *n*-hexane extract of *M. pinnata* may contain nonredox-related principles that interact with 5-LO. It was observed that the potency of the *n*-hexane extract was more pronounced for inhibition of isolated 5-LO, compared to cellular 5-LO. A possible explanation could be that the active constituents fail to reach equivalent concentrations inside the cell (due to poor permeation) or cellular factors may impair 5-LO inhibition by competition, unspecific protein binding or degradation of the constituents.

Previous studies showed that plant derived 5-LO inhibitors such as hyperforin, myrtucommulone, garcinol, arzanol, curcumin, boswellic acids and embelin also inhibit the activity of mPGES-1 (Koeberle and Werz, 2009; Bauer et al., 2011; Schaible et al., 2013), and such dual suppression of two major pro-inflammatory pathways might be beneficial for effective and safe therapy. PGE_2 is considered as major mediator of inflammation and pain, and non-steroidal anti-inflammatory drugs are assumed to confer their anti-inflammatory effect essentially *via* suppression of PGE_2 biosynthesis (Funk, 2001). In fact, the *n*-hexane extract of *M. pinnata* and also the fractions F-6 to F-9 effectively repressed the activity of mPGES-1 with $IC_{50} = 1$ μg/ml. To the best of our knowledge, no other medicinal plant-derived extract has been reported thus far with such high potency against mPGES-1, and also the well-recognized synthetic mPGES-1 inhibitor MK886 ($IC_{50} = 1.3$ μg/ml) was not superior. Note that a strong correlation regarding the potencies to inhibit 5-LO and to inhibit mPGES-1 for the single fractions was evident, suggesting that both targets are affected by the same ingredient(s). Because mPGES-1 is regarded as a valuable drug target for the treatment of various common disorders including pain, inflammation, fever, and cancer (Samuelsson et al., 2007), the potent suppression of mPGES-1 activity by *M. pinnata* is encouraging and supports its medical and therapeutic use. Moreover, the low IC_{50} values of 0.48–8.7 μg/ml for the *n*-hexane extract against 5-LO and mPGES-1 would roughly correspond to an estimated dose of 36–650 mg per 75 kg body weight (assuming 100% bioavailability and equal distribution) and is thus clearly in the range of the dosage of well-recognized anti-inflammatory phytomedicine used in Western countries such as extracts of nettle, willow bark, or devil's claw that are applied at about 145–600 mg/single dose. Therefore, the high efficiency of *M. pinnata* against 5-LO and mPGES-1 *in vitro* might be of pharmacological relevance and provides a rationale for its use as anti-inflammatory remedy in folk medicine.

The chemical profiling of *M. pinnata* has been hindered for many years mainly due to the dearth of plant materials for large scale extraction and isolation procedures. However, our approach for compound identification was devoid of any extensive purification, thus, required only a few grams of plant materials. Yet, the modern hyphenated techniques enabled us to unveil the phytochemistry of this medicinal plant for the first time. An UPLC system coupled to the Orbitrap instrument and GC coupled to MS were employed as the sole techniques in this study for the characterization of secondary metabolites. While the UPLC provided excellent separation efficiency, the Oribtrap mass accuracy made the determination of molecular composition of precursor and fragment ions straightforward. In addition to the MS database search for the molecular formula of the parent mass, the tandem mass spectra were further analyzed by computer assisted algorithms to yield hypothetical fragmentation trees, which provided better structural insight into the possible compounds.

Among the identified constituents in the *n*-hexane extract by GC–MS, *β*-caryophyllene and caryophyllene oxide might be of interest. Anti-inflammatory activity of *β*-caryophyllene was revealed in models of acute (carrageenan-induced) inflammation (Gertsch et al., 2008) while caryophyllene oxide exhibited significant cytotoxicity against the human cancer cell lines HepG2, AGS, HeLa, SNU-1, and SNU-16 in the MTT assay (Jun et al., 2011). Since the above-mentioned compounds were only sparsely present in the most active fractions of the *n*-hexane extract (F-6/F-8), we conclude that those are not exclusively responsible for the observed bioactivity. However, the presence of α-tocopherol in the F-6 could be correlated to the 5-LO inhibition as it has displayed a potent inhibition by selective and tight binding to 5-LO (Reddanna et al., 1985).

The LC–MS analysis of F-6 and of F-8 of the *n*-hexane extract suggests the presence of some interesting compounds, however, the available tandem mass spectral data which resulted from poor fragmentation of precursor ions, are insufficient for a conclusion. Nevertheless, we could putatively assign the structures after considering the accurate mass, mass accuracy and database search. Particularly, the tentative identification of ganoderiol F is of interest as this triterpenoid was reported to exhibit strong anti-HIV-1 protease activity (El-Mekkawy et al., 1998) as well as *in vivo* antitumor effects (Gao et al., 2006). Further optimization of LC–MS/MS conditions in planned follow-up studies may permit confirmation of the identified structures. Furthermore, the LC–MS analysis has indicated the existence of several unknown compounds that are not in any database which might have also contributed to the bioactivity. Therefore, the planned expansion of this study towards the identification of unknown compounds in the active fraction with the use of fragmentation tree alignments (Rasche et al., 2012) will provide better insights into the chemical profile of the plant. Thereafter, further experiments with synthesized compounds will be worthwhile for a better understanding of their bioactivities, in particular with respect to the inhibition of 5-LO and mPGES-1.

5. Conclusion

The potent inhibition of the activity of the drug targets 5-LO and mPGES-1 in well-established biological test systems provides scientific evidences for *M. pinnata*'s traditional usage in Sri Lanka for the treatment of various inflammatory conditions. It has been revealed that the constituents in the *n*-hexane extract of *M. pinnata* exhibit potent interference with 5-LO in a nonredox-dependent manner rather than *via* an unselective antioxidant mechanism. The phytochemistry of *M. pinnata* was revealed for the first time where phytosterols, fatty acids, sesquiterpenes and several other types of secondary metabolites with known anti-inflammatory properties were identified by mass spectrometric techniques. We believe that the solid platform laid by our study will be indispensable for further phytochemical and bioactivity research on this popular and valuable medicinal plant in the future. Our data may stimulate for more detailed preclinical analysis of the pharmacological properties of *M. pinnata* that may further support its therapeutic potential in the treatment of inflammatory disorders.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.11.052.

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