Lipophilic extracts of *Leucas zeylanica*, a multi-purpose medicinal plant in the tropics, inhibit key enzymes involved in inflammation and gout

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Abstract

Ethnopharmacological relevance: *Leucas zeylanica* (L.) W.T. Aiton is a popular, multi-purpose medicinal plant in Sri Lanka but the pharmacological potential and the chemical profile have not been systematically investigated to understand and rationalize the reported ethnobotanical significance.

Aim of the study: The present study was undertaken to scientifically validate the traditional usage of this plant for the treatment of inflammatory conditions, gout and microbial infections. Inhibition of 5-lipoxygenase (5-LO), microsomal prostaglandin E_2 synthase (mPGES)-1 and xanthine oxidase (XO) by different extracts of *L. zeylanica* was investigated to determine the anti-inflammatory and anti-gout activity, respectively. The antibacterial and antifungal activities were also studied and the relevant constituents in the bioactive extracts were tentatively identified.

Materials and Methods: Cell-free and/or cell-based assays were employed in order to investigate the effects of the extracts against the activity of human 5-LO, mPGES-1 and XO as well as to assess antioxidant properties. The antibacterial activity of the extracts was determined by the broth micro-dilution method against Gram positive and Gram negative bacteria including methicillin-resistant *Staphylococcus aureus* while the agar dilution method was employed to determine the anti-*Candida* activity. Gas chromatography coupled to mass spectrometric (GC-MS) analysis enabled the characterization of secondary metabolites in the extracts.

Results: A dichloromethane extract of *L. zeylanica* efficiently inhibited 5-LO activity in stimulated human neutrophils (IC_{50} = 5.5 µg/mL) and isolated human 5-LO and mPGES-1 (IC_{50} = 2.2 and 0.4 µg/mL). Potent inhibition of XO was observed by the same extract (IC_{50} = 47.5 µg/mL), which is the first report of XO-inhibitory activity of a Sri Lankan medicinal plant. Interestingly, significant radical scavenging activity was not observed by this extract. Only the *n*-hexane extract exhibited antibacterial activity against *Staphylococcus aureus* and *Staphylococcus saprophyticus* with a MIC of 250 µg/mL while the anti-*Candida* activity was moderate. GC-MS analysis revealed the presence of phytosterols, fatty acids, sesquiterpenes, diterpenes and several other types of secondary metabolites.

Conclusions: Potent inhibition of 5-LO, mPGES-1 and XO rationalizes the ethnopharmacological use of *L. zeylanica* as anti-inflammatory and anti-gout remedy. Interestingly, the antimicrobial activities were not prominent, despite its wide utility as an antimicrobial medication.

Keywords: *Leucas zeylanica*, 5-Lipoxygenase, microsomal prostaglandin E_2 synthase, xanthine oxidase, anti-inflammatory

Compounds studied: zileuton, allopurinol, MK886, Ca^{2+}-ionophore A23187, ascorbic acid, prostaglandin H_2.

Introduction

The genus *Leucas* (Lamiaceae) comprises approx. 80 species naturally distributed in East Africa, India, Sri Lanka, Nepal, Myanmar, China and Bangladesh (Chouhan and Singh, 2011). In Sri Lanka, six species exist with *Leucas zeylanica* (L.) W.T.Aiton, locally known as "Gata-thumba", as most widely distributed species. This plant is an annual herb commonly found at roadsides and in waste, sandy places in both wet and dry zone of the country up to about an elevation of 1700 m. Due to the aromatic, stomachic and carminative properties, *L. zeylanica* is used for several applications in traditional medicine. It is applied as an insecticide in agriculture and the leaves are used as vegetable. The plant is extensively utilized in traditional medicine for the treatment of inflammatory conditions, gout, skin diseases and related disorders (Jayaweera, 1982) as well as an anthelminthic drug. Poultice of leaves are used as sedatives and for treatment of wounds, sores, itches, headaches and vertigo. A decoction prepared from the leaves together with *Nigella* seed or the fresh juice of turmeric and rice is used as a lotion for ulcers of the nose (Department of Ayurveda, 1979; Ediriweera and Rajapaksha, 2014).

Despite its therapeutic importance in folk medicine, the bioactivities of *L. zeylanica* are hardly explored to scientifically rationalize its reported ethnobotanical use. Only a few documentary evidences are available that validate the pharmacological potential of this plant. Recently, hepatoprotective effects of *L. zeylanica* against ethanol- and Fenton's reagent-induced oxidative stress *in vitro* were reported (Hossain et al., 2013), and a decoction of the plant was highly effective against *Enterobius vermicularis* infections in adults (Ediriweera and Rajapaksha, 2014). In addition, the aqueous extract of this plant displayed a strong, broad-range photoprotective activity (Napagoda et al, 2016a). However, scientific reports that support the traditional usage of *L. zeylanica* against inflammatory conditions, gout and microbial infections are scarce. Thus, the present study was undertaken to reveal the anti-inflammatory, anti-gout and antimicrobial potential in *L. zeylanica in vitro* and to identify related secondary metabolites that would be responsible for the observed bioactivity.

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most frequently used therapeutics for the treatment of inflammatory disorders, pain, and fever as well as acute gout attack. The anti-inflammatory actions of NSAIDs are mainly conferred by interference with eicosanoid biosynthesis, particularly by blocking the formation of prostaglandin (PG)E_2 from arachidonic acid

(AA) that involves cyclooxygenase (COX) and microsomal PGE_2 synthase (mPGES)-1 (Grosser et al., 2010; Koeberle and Werz, 2009). Besides PGE_2, the major pro-inflammatory PG, the leukotrienes (LTs) play major roles in the pathogenesis and symptoms of inflammation. Like PGE_2, LTs are also formed from AA by enzymatic oxygenation involving 5-lipoxygenase (5-LO) and LT synthases (Radmark et al., 2007; Pergola and Werz, 2010). Current innovative pharmacological strategies for intervention with inflammation focus on dual inhibitors of mPGES-1 and 5-LO with the aim to block both types of pro-inflammatory eicosanoids, i.e. PGE_2 and LTs, which may result in more efficient drugs exerting fewer side effects (Koeberle et al., 2008; Koeberle and Werz, 2018). Accordingly, we tested in this study whether *L. zeylanica* extracts would inhibit mPGES-1 and 5-LO.

The inhibition of xanthine oxidase (XO) was evaluated to rationalize the utility of the plant as a remedy for gout and gouty arthritis. XO catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid in the purine nucleotide degradation pathway. Thus, one of the therapeutic approaches to treat gout involves the use of XO inhibitors (e.g. allopurinol) that block the production of uric acid (Khan et al., 2013). The traditional claim of *L. zeylanica* as an antimicrobial medication was investigated using several Gram negative and Gram positive bacteria including methicillin-resistant *Staphylococcus aureus* and different species of the fungus *Candida*.

Although a number of phytochemicals such as flavanoids, coumarin, ligans, terpenes, fatty acid and aliphatic long chain compounds have been isolated from the genus *Leucas* (Chouhan and Singh, 2011), the phytochemistry of *L. zeylanica* is not established yet and remains to be explored. Early attempts of phytochemical screening of *L. zeylanica* revealed the presence of β-sitosterol, ursolic acid, β-sitosterol glucoside, oleanoic acid, tricin, stigmastan-7,22-di-ene-3-β-ol, 28-epoxy-stimaststan-7,22-di-ene-3-β-O-glucopyranoside, and 2-hydroxy-dicyclo-2,5-di-ene-7,8-imethyl-3-β-D-glucopyranoside (Begum, 1998; Liyanage, 2002), however, the phytochemical profile was not correlated to any bioactivity.

2. Materials and methods

2.1 Plant Material

L. zeylanica **(**L.) W.T. Aiton plants were collected in Nittambuwa (Gampaha district - Western Province of Sri Lanka) in 2012 and 2016. The plant was identified by the author (M.N.), a botanist,

and confirmed based on the books "A Revised Handbook to the Flora of Ceylon: volume-III, M.D. Dassanayake and F.R. Fosberg" and "Medicinal plants (indigenous and exotic) used in Ceylon: Volume 2 by 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 (MN-01) is deposited at the National Herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka. Morphological features such as height of the plant, shape, size and arrangement of leaves as well as floral characteristics were measured and recorded. The plant name has been checked with http://www.theplantlist.org (accessed on 10/12/2017).

2.2 Preparation of crude extracts

The plant material (whole plant) was thoroughly washed with running water and dried in the shade (30 ± 2 °C) for six days. Dried plants were powdered using a domestic grinder. Thirteen grams of powdered material was successively extracted with 600 mL of *n*-hexane, dichloromethane (DCM), ethyl acetate, and methanol (Roth, Karlsruhe, Germany) at room temperature using a linear shaker for 20 minutes. In addition, 4.0 grams of powdered material was extracted in 300 mL of 70% methanol by heating for 2 h at 60 °C. The extracts were evaporated to dryness with the use of a rotary evaporator (BÜCHI, R-114, Germany), and solubilized in DMSO for bioactivity assays.

2.3 Evaluation of bioactivities

2.3.1 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 with consent 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 pre-incubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO) and incubated for 10 min at 37 °C with 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 were added. The samples were subjected to solid phase extraction on RP18-columns (100 mg, UCT, Bristol, PA, USA) and 5-LO products (LTB_4 and its trans-isomers, 5-hydro(pero)xyeicosatetraenoic acid (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

E. coli (BL21) were 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 pre-incubated 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 the 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 analysed 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

Microsomal preparations of A549 cells were prepared as previously described (Koeberle et al., 2008). Briefly, A549 cells were cultured in DMEM medium containing 2% FCS and IL-1β (2 ng/mL) for 72 hrs (37 °C, 5% CO_2). Cells were then harvested and re-suspended in homogenization buffer (potassium phosphate (0.1 M, pH 7.4), phenylmethanesulfonyl fluoride (1 mM), soybean trypsin inhibitor (60 µg/mL), leupeptin (1 µg/mL), glutathione (2.5 mM), and sucrose (250 mM)). After shock-freezing of the cells in liquid nitrogen, sonication (3 × 20 s), differential centrifugation at 10,000×g (10 min, 4 °C) and 174,000×g (60 min, 4 °C), the pellets were re-suspended in homogenization buffer. The microsomes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) with glutathione (2 mM) and pre-incubated with the extracts or vehicle (0.1% DMSO) on ice

for 15 min. After stimulation (1 min, 4 °C) with 20 µM PGH_2 as substrate the reaction was terminated by addition of stop solution containing $FeCl_3$ (40 mM), citric acid (80 mM), and 11β-PGE_2 (10 µM as internal standard) and analyzed for PGE_2 by RP-HPLC as reported before (Koeberle et al., 2008).

2.3.4 XO inhibitory activity

The XO inhibitory activity of the crude extracts was determined by measuring the rate of hydroxylation of the substrate (xanthine) into uric acid, which is a colorless end product of the reaction and shows UV light absorption at 295 nm (Kahn et al., 2013). The reaction mixture contained 10 µL of the extract dissolved in DMSO, 150 µL of phosphate buffer (50 mM, pH 7.4), 0.003 units of XO dissolved in buffer (20 µL), and 20 µL of 0.1 mM xanthine as substrate. After addition of XO, the mixture was incubated for 10 min at room temperature and the initial absorbance was measured at 295 nm (Multiskan Go Microplate spectrometer, Thermo Scientific). Thereafter, the substrate was added to the reaction mixture, and final readings were carried out for 15 min at an interval of 1 min. Allopurinol (Sigma/Aldrich) was used as the reference drug. The percentage inhibitory activity by the samples was determined against a DMSO blank and calculated by using the following formula.

$$\text{Inhibition (\%)} = 100 - [(\text{Optical Density}_{\text{test compound}} / \text{Optical Density}_{\text{control}}) \times 100]$$

2.3.5 Antioxidant activity

The radical scavenging capability of the extracts was assessed by measuring the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by the method of Blois (1958) with slight modifications. The absorbance was recorded at 517 nm after 30 min incubation of the extracts with DPPH solution under gentle shaking in the dark. The percentage antioxidant activity (AAct) was calculated using the following formula and the EC_{50} was determined using Graph Pad Prism version 6.01.

$$\text{AAct (\%)} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{control}}] \times 100$$

Ascorbic acid was used as reference compound and all the measurements were carried out in triplicates.

2.3.6 Antibacterial activity

Standard bacterial cultures were obtained from the Department of Microbiology, Faculty of Medicine, University of Ruhuna, Sri Lanka. Standard isolates include Gram positive and Gram negative bacteria; *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212) and *Escherichia coli* (ATCC 35218). Furthermore, the antibacterial activity of the plant extracts was determined using the clinical isolates including *Staphylococcus saprophyticus*, *Salmonella* Typhi and nine strains of methicillin-resistant *Staphylococcus aureus* (MRSA) available at the Department of Microbiology, Faculty of Medicine, University of Ruhuna, Sri Lanka. Selected cultures were streaked on blood agar and incubated at 37 °C overnight. A loop full from an isolated colony was dissolved in sterile distilled water. The turbidity of the mixture was adjusted to get equal to that of the McFarland 0.5 standard.

The antimicrobial activity as well as the minimum inhibitory concentrations (MIC) of plant extracts was determined by the broth microdilution method in 96-well microtitre plates as described by Eldeen and Van Staden, 2007 with slight modifications. 100 µL of solvent controls and test samples were added to the first wells of the microplate starting with a concentration of 2 mg/mL and then two-fold serially diluted down the wells. 100 µL of the diluted culture and 50 µL of distilled water were added to all wells. The microtitre plates were incubated for 24 h at 37°C. After incubation, the absorbance was measured by a microplate reader (Biotek, ELx800). The MIC was determined as the lowest concentration of test agent which prevents visible growth of a bacterium. The assay was conducted in triplicates. Gentamicin and cefotaxime were used as the standards for the antimicrobial assay.

2.3.7 Anti-candidal activity

Anti-candidal activity of plant extracts was assessed by an agar dilution method as described by Andrews, 2001. Five standard isolates of *Candida* species, i.e., *C. albicans* (ATCC 90028), *C. parapsilosis* (ATCC 22019), *C. glabrata* (ATCC 90030), *C. krusei* (ATCC 6258) and *C. tropicalis* (ATCC 13803) were used. The organisms were maintained on Sabouraud Dextrose Agar (SDA) at 37 °C for 20 - 22 h. Thereafter, a loop full from an isolated colony was dissolved in normal saline. The turbidity of the mixture was adjusted to get equal to that of the McFarland 0.5 standard.

The extract was added to a universal bottle and 19 mL of sterile molten MHA (cooled to 45°C) was added and the contents were mixed thoroughly. Then, the contents in the bottles were added to sterile petri dishes. The plates were allowed to dry at 44 °C to remove any moisture on the surface and 1-2 µL from the prepared broth cultures were placed on agar using a micropipette. Thereafter, the plates were incubated at 37 °C. Presence or absence of any growth of the organisms was observed after 24 h. The assay was carried out in triplicates.

2.4. Gas chromatography coupled mass spectrometric analysis

Dried crude extracts of *n*-hexane and DCM were dissolved in ethyl acetate (1 mg/mL) and analyzed on a gas chromatograph HP6890 (Agilent, USA) connected to a MS02 mass spectrometer from Micromass (Waters, UK) with EI 70 eV using ZB-5ms column (30 m × 0.25 mm, 0.25 µm film thickness; Phenomenex). The carrier gas was helium at a flow rate of 1 mL/min. The injector temperature was kept at 250 °C and the temperature program was set as 100 °C (2 min), 15 °C / min to 200 °C, 5 °C/ min to 305 °C (20 min).

2.5. Statistical analysis

Data are expressed as mean ± S.E.M. The IC_{50} values were calculated from averaged measurements at 4-5 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.

3. Results

3.1. Morphological features of *L. zeylanica*

The observed morphological features of *L. zeylanica* are listed in **Table 1**.

Feature	Description
Habit	An erect, branched herb with a height around 30-45 cm.
Stem	Hairy, quadrangular, light green color.

Leaves	Simple, sessile-sub sessile, linear to lanceolate in shape with blunt tip, both surfaces are hairy, leaf margin-distantly serrate.
Flowers	Arranged as terminal whorls, directly attached to the base without a peduncle, corolla white, five petals consist of short upper-lip and a broadly spathulate lower-lip with a large middle lobe and small lateral lobes.

Table 1: Morphological features of *L. zeylanica.*

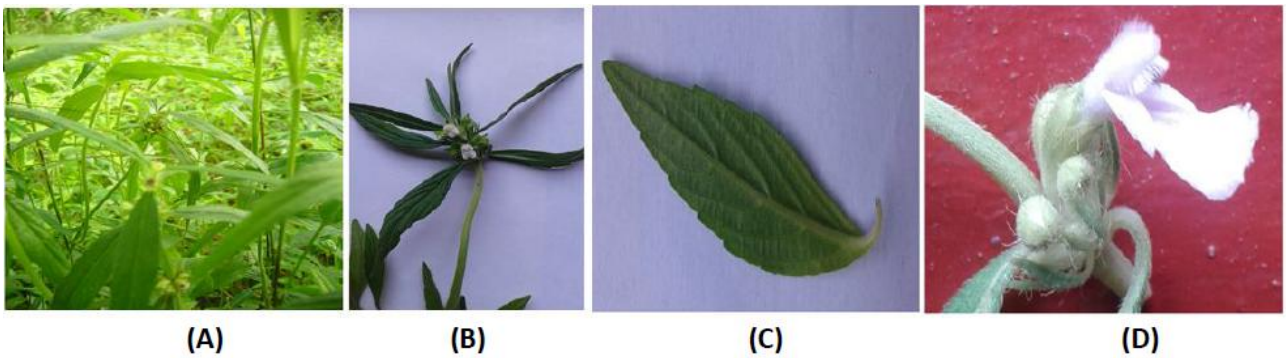

Fig. 1: Morphological features of *L. zeylanica.*

3.2. Evaluation of 5-LO inhibition

In order to study whether or not the extracts of *L. zeylanica* inhibit 5-LO product biosynthesis *in vitro*, isolated human neutrophils stimulated with the Ca^{2+}-ionophore A23187 were used as test system, which is a well-established cell-based model for investigating pharmacological inhibition of 5-LO activity in intact cells (Werz, 2007). Potent suppression of 5-LO activity in neutrophils was observed for the DCM extract and to a somewhat minor extent also for the *n*-hexane extract of *L. zeylanica* (10 and 100 µg/mL), whereas extracts prepared with methanol or water were much less effective (**Figure 2A**). More detailed concentration-response studies using this cell-based assay revealed an IC_{50} value of 5.5 µg/mL for the DCM extract (**Figure 2B**). The IC_{50} value of the synthetic reference control inhibitor zileuton, an approved anti-asthmatic drug (Israel et al., 1990), was 0.13 µg/mL (corresponding to 0.55 µM).

In order to evaluate whether the extracts directly inhibit the activity of the 5-LO enzyme, a cell-free assay using isolated human recombinant 5-LO as enzyme source and 20 µM AA as substrate was used. Again, the DCM and *n*-hexane extracts efficiently blocked 5-LO activity while extracts based on methanol or water were much less efficient (**Figure 2C**). The DCM extract showed concentration-dependent inhibition of 5-LO activity with IC_{50} = 2.2 µg/mL (**Figure 2D**). For zileuton, the IC_{50} value was determined as 0.11 µg/mL.

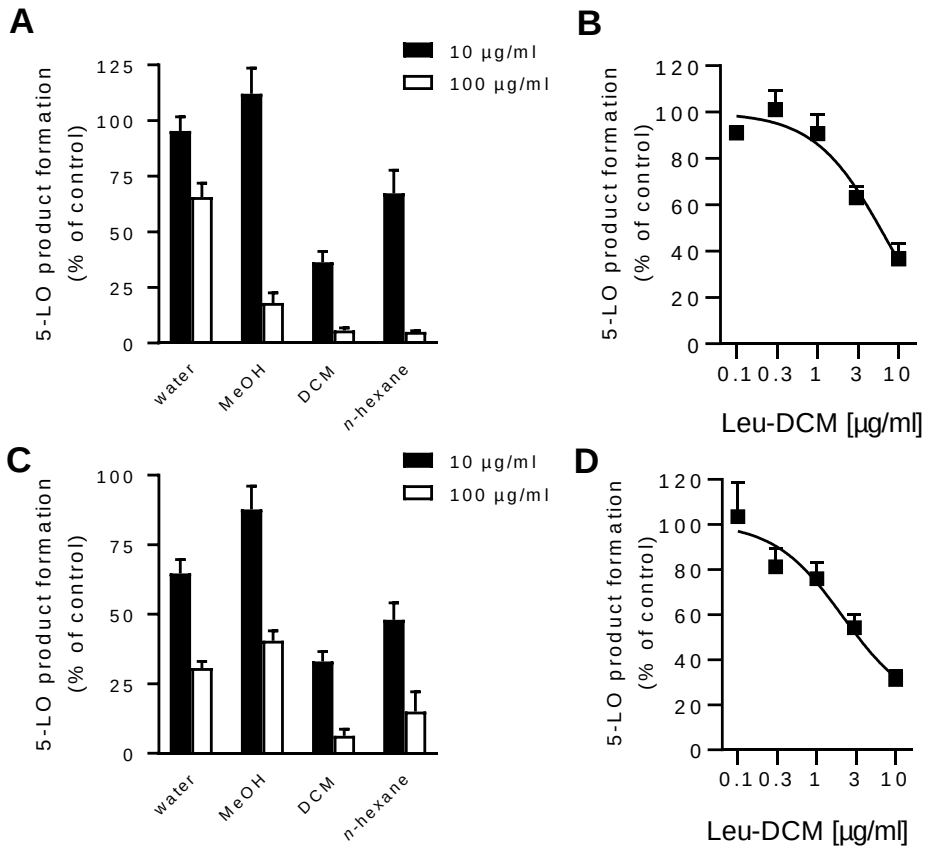

Fig. 2: Inhibition of 5-LO in intact neutrophils (A,B) and in cell-free assays (C,D) by extracts of *L. zeylanica*.

3.3. Inhibition of mPGES-1

Because PGE_2 is the major pro-inflammatory eicosanoid and since several 5-LO inhibitors of natural origin interfere also with PGE_2 formation by blocking mPGES-1 (Koeberle and Werz, 2015), we tested if the DCM extract could inhibit mPGES-1 activity. In fact, the DCM extract caused concentration-dependent inhibition of mPGES-1 activity in a cell-free assay (**Figure 3**),

where mPGES-1 in microsomes derived from human A549 cells was supplied with its substrate PGH_2 (20 µM). The IC_{50} value was determined at 0.4 µg/mL, which is rather low as compared to the IC_{50} = 2.2 µM of reference mPGES-1 inhibitor MK886 (not shown).

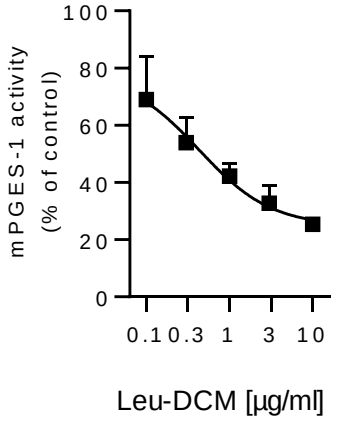

Fig. 3: Inhibition of mPGES-1 in a cell-free assay by the DCM extract.

3.4. XO inhibition

Among the tested extracts, only the DCM extract of *L. zeylanica* exhibited pronounced suppression of human XO activity in a cell-free assay with more than 50% enzyme inhibition at an initial concentration of 50 µg/mL. More detailed analysis revealed concentration-dependent XO inhibition by the extract with an IC_{50} value of 47.5 µg/mL. The reference drug allopurinol inhibited XO activity with an IC_{50} = 16.8 µg/mL (**Figure 4**).

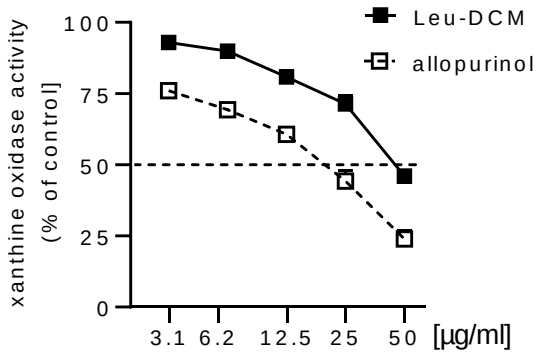

Fig. 4: Concentration-response curve for allopurinol and DCM extract of *L. zeylanica* for the inhibition of XO.

3.5. Antioxidant activity

Inflammatory reactions are characterized by an environment with increased oxidative tone due to elevated levels of reactive oxygen species (ROS). Moreover, 5-LO represents an iron-containing, redox-sensitive dioxygenase that can be effectively inhibited by lipophilic antioxidants/ROS scavengers (Werz, 2007). Thus, the lipophilic extracts of *L. zeylanica* were analyzed for their antioxidant/radical scavenging capacities using the cell-free DPPH assay. Intriguingly, the hydrophilic aqueous and methanolic extracts of *L. zeylanica* were effective as antioxidants in the DPPH assay with comparable potency as ascorbic acid (reference compound), whereas the antioxidant potential of the lipophilic DCM and *n*-hexane extracts was only moderate **(Table 2)**.

Extract	EC_{50} (µg/mL)
n-hexane	311.8
DCM	208.4
Ethyl acetate	35.9
Methanol	32.1
Water	30.5
Ascorbic acid (reference compound)	14.3

Table 2: The antioxidant activity of the different extracts of *L. zeylanica.* The test extracts or ascorbic acid at different concentrations were incubated with DPPH solution for 30 min and the absorbance was recorded at 517 nm. EC_{50} was determined using Graph Pad Prism version 6.01.

3.5 Antibacterial and antifungal activity

Among the tested extracts, only the *n*-hexane extract displayed antibacterial activity against *S. aureus* and *S. saprophyticus* with a MIC of 250 µg/mL, without any activity against other bacterial species used in this study including MRSA strains.

Antifungal activity was observed only with the *n*-hexane extract against *Candida glabrata* with a MIC of 5 mg/mL while no activity was detected against any other fungal strains.

3.6 Phytochemical screening

The GC-MS analysis of the DCM crude extract led to the identification of 16 components, based on the comparison of the obtained experimental mass spectrum with those recorded in the NIST MS Search 2.0 and also by comparison with the respective standards. These components include ubiquitously occurring fatty acids (hexadecanoic acid, 9,12,-octadecadienoic acid, 9,12,15-octadecatrienoic acid, octadecanoic acid), sesquiterpenes (β-caryophyllene, farnesene, caryophyllene oxide), a diterpene alcohol (phytol), an acyclic diterpene (neophytadiene), and α-tocopherol (**Figure 5**). Similarly, ten compounds in the *n*-hexane extract (caryophyllene oxide, neophytadiene, (3,7,11,15)-tetramethyl-2-hexadecen-1-ol, hexadecanoic acid, phytol, octadecanoic acid, 4,8,12,16-tetramethylheptadecan-4-olide, α-tocopherol, stigmasterol and β-sitosterol were tentatively identified (data not shown). However, several compounds present in the DCM extract such as β-caryophyllene, farnesene, α-caryophyllene, β-cubebene and α-selinene were not detected in the *n*-hexane extract while 4,8,12,16-tetramethylheptadecan-4-olide was detected exclusively in the *n*-hexane extract.

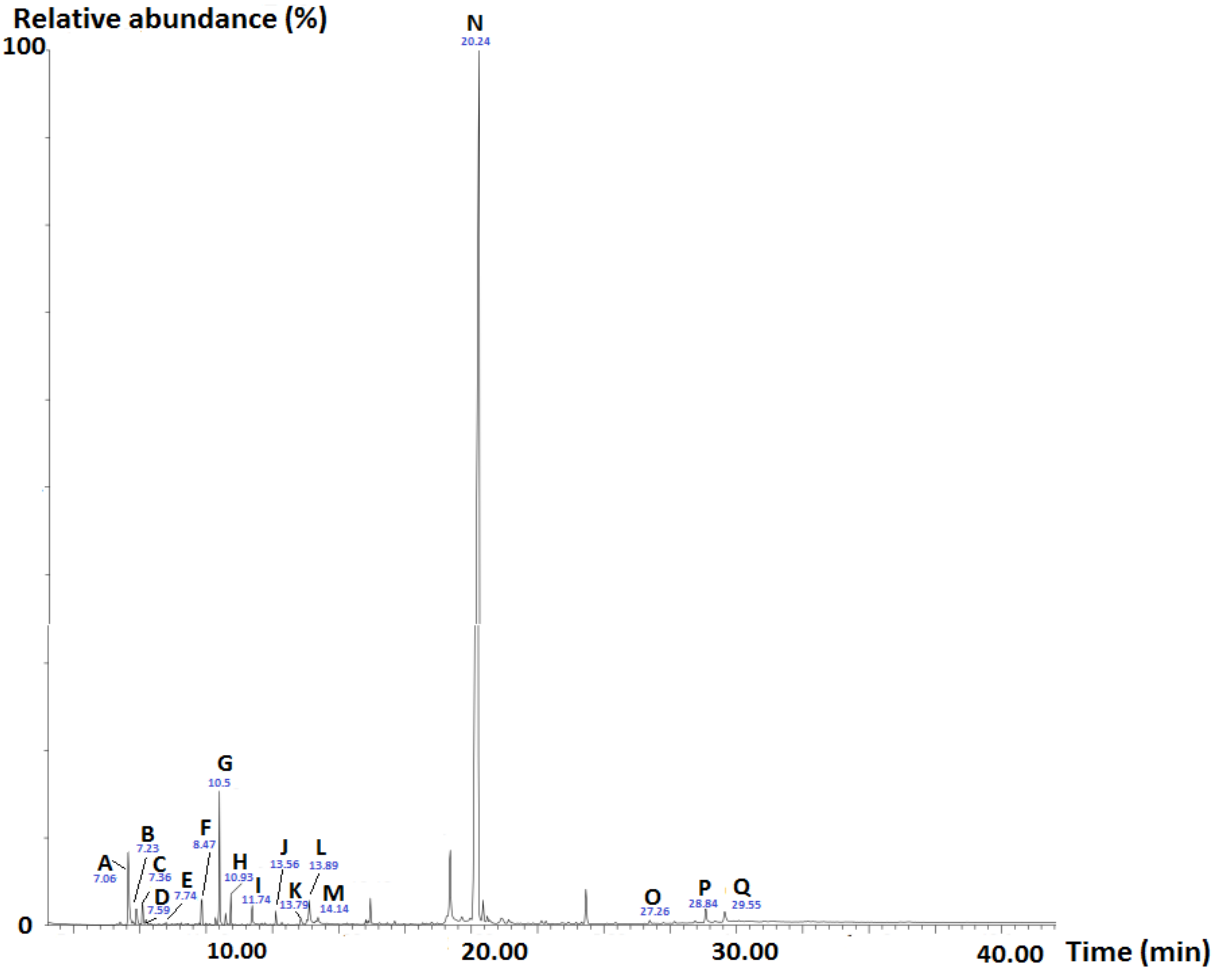

Fig. 5: Total ion chromatogram of the DCM extract of *L. zeylanica* and the tentatively identified compounds.

4. Discussion

Leucas zeylanica is an important medicinal plant in Sri Lanka, but the knowledge on its pharmacological features, phytochemistry, and its bioactive constituents is sparse and insufficient to rationalize its use in the treatment of inflammation-related disorders, gout and infectious diseases. To rationalize and validate its traditional therapeutic use we studied various types of hydrophilic and lipophilic extracts of *L. zeylanica* for their capacities to interfere with key enzymes involved in the biosynthesis of major pro-inflammatory mediators such as PGE_2, LTs, and ROS, as well as with XO, a key enzyme in the pathogenesis of gout. Our data show that a DCM extract of *L. zeylanica* effectively inhibits 5-LO and mPGES-1 as well as XO at comparably low concentrations,

suggesting a potential of this extract as anti-inflammatory remedy and explaining its use for the treatment of inflammation and gout in folk medicine.

The complex mechanisms of chronic inflammation fostered the development of dually or multiple acting anti-inflammatory drugs, in particular in the field of pro-inflammatory eicosanoids (Koeberle and Werz, 2014). NSAIDs inhibit prostanoid formation and represent the prevalent therapeutics for the treatment of inflammatory diseases, fever and pain (Grosser et al., 2010). However, NSAIDs are afflicted with severe side effects, which might be circumvented by more selective suppression of pro-inflammatory eicosanoid biosynthesis. This led to the development of dual inhibitors of mPGES-1 and 5-LO (Koeberle and Werz, 2015). Natural products represent rich sources of privileged structures that share multiple bioactivities, and the interest in exploring their impact on human physiology is still increasing. In particular, plant-derived remedies often exhibit anti-inflammatory properties with multiple modes of action, and they are often preferred by patients with chronic inflammatory disorders over NSAIDs and glucocorticoids due to fewer side effects (Koeberle and Werz, 2014). Diverse popular remedies (e.g., frankincense, cannabis, St. John´s wort) and isolated natural products (e.g., curcumin, EGCG, carnosol and carnosic acid) from plant origin are commonly used in inflammation therapy, where dual inhibition of PGE_2 and LT formation may contribute to the anti-inflammatory features (Koeberle and Werz, 2018). Along these lines, the anti-inflammatory properties of *L. zeylanica* reported in folk medicine may be explained by dual interference with the activities of mPGES-1 and 5-LO, as reported in the present study.

Since ancient times, inflammatory disorders and related diseases have been treated with plants and plant-derived formulations. Many studies showed that plant extracts or plant secondary metabolites can control the levels of various inflammatory cytokines including IL-1β, IL-6, and TNF-α, pro-inflammatory mediators and their key enzymes such as NO and iNOS, PGE_2 and COX-2, or LTs and 5-LO, as well as the activation of transcription factors such as NF-κB and Nrf2 (Calixto et al., 2003). As 5-LO plays a key role in LT biosynthesis (Radmark et al., 2007) and has been intensively explored as a drug target for many inflammatory diseases (Werz et al., 2017), we addressed 5-LO inhibition as anti-inflammatory property of *L. zeylanica*. Although numerous plant species were reported as 5-LO inhibitors, the inhibitory potencies of the investigated extracts are often rather low. Thus, relatively high IC_{50} values in the range of 15-50 µg/mL for 5-LO inhibition were

observed for lipophilic extracts of various medicinal plants (Schneider and Bucar, 2005). In this respect, the comparatively low IC_{50} obtained for the DCM extract of *L. zeylanica* (i.e., 2.2 µg/mL) suggests a high pharmacological potential for intervention with 5-LO under pathological conditions where LT play key roles.

Besides 5-LO and its pro-inflammatory products, PGE_2 is a major lipid mediator promoting inflammation, which under inflammatory conditions is mainly produced by the inducible enzymes COX-2 and mPGES-1 (Koeberle and Werz, 2015). mPGES-1 is currently under intensive investigation as alternative drug target to COX enzymes, and several classes of mPGES-1 inhibitors, synthetic compounds or natural products, have been identified and pre-clinically evaluated (Koeberle et al., 2016; Koeberle and Werz, 2018; Psarra et al., 2017). Our data show that the DCM extract of *L. zeylanica* potently inhibits mPGES-1 activity, supporting interference with mPGES-1 as underlying anti-inflammatory mechanism of *L. zeylanica.*

In addition to the dual 5-LO/mPGES-1 inhibitory action, the DCM extract displayed XO inhibition that could be beneficial in the management of gout, a painful disease with strong inflammatory component. Gout is a metabolic disorder associated with accumulation of uric acid crystals in the joints, tendons and surrounding tissues leading to local inflammation due to invasion of neutrophils that phagocytose these crystals. The subsequent release of lysosomal enzymes from neutrophils together with liberated urate crystals fosters inflammation along with decreasing the pH in the environment. This facilitates further precipitation of urate crystals and promotes worsening of the diseases. Uric acid is the final product of the catabolism of purine nucleotides where XO catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid. NSAIDs such as indomethacin and naproxen are frequently used as first-line therapies to treat acute attacks of gout (Cronstein and Terkeltaub, 2006). Another therapeutic approach involves the use of XO inhibitors that block the production of uric acid. Allopurinol and febuxostat are the only XO inhibitors currently employed under clinical applications with adverse effects such as hepatitis and nephropathy (Khan et al., 2013). Hence, the search for novel XO inhibitors with good therapeutic efficacy and fewer side effects are desired for the treatment of gout. However, the potential of developing effective natural products for the management of XO-related disorders is still largely unexplored and so far, no study has been reported on the XO-inhibitory potential in medicinal plants from Sri Lanka. Therefore, *L.*

zeylanica is the first Sri Lankan plant species that is reported as a potential source of natural XO inhibitor.

Although free radical scavenging properties are frequently observed for natural 5-LO and XO inhibitors, our investigations revealed that the DCM extract exhibits only weak antioxidant activity. Antioxidants such as polyphenols, flavonoids and coumarins are effective 5-LO inhibitors that interfere with the redox cycle of the 5-LO active-site iron or that chelate the active-site iron (Werz, 2007). However, our findings suggest that the DCM extract may contain phytochemicals that interact with 5-LO in a nonredox-dependent manner. XO is capable of generating ROS such as superoxide radicals and hydrogen peroxide that can led to oxidative stress in gout patients. Thus, herbal extracts with antioxidant properties are believed to be effective in abrogating ROS produced by XO. As a result, in previous studies the XO inhbition has been correlated to the presence of antioxidants such as flavonoids and other phenolic compounds (Nile and Park, 2013). In contrast, our data suggest that for *L. zeylanica* extracts, secondary metabolites devoid of antioxidant properties are capable of inhibiting XO.

L. zeylanica is claimed as an effective antimicrobial medication but our results revealed that its antimicrobial potential is negligible as compared to other plant species that are used in traditional medicine in Sri Lanka belonging to the Lamiaceae family, like *Plectranthus zeylanicus* (Napagoda et al., 2016b). Some antibacterial activities in the *n*-hexane extract against *S. aureus* and *S. saprophyticus* was obvious and the Gram-positive bacteria turned out to be were more susceptible. However, the anti-fungal activity of the *n*-hexane extract was very weak.

Among the identified constituents in the bioactive DCM extract by GC-MS, the presence of β-caryophyllene and α-caryophyllene may reasonably correlate to the observed anti-inflammatory activity. Thus, anti-inflammatory properties of β-caryophyllene was demonstrated in models of acute carrageenan-induced inflammation (Gertsch et al., 2008). Similarly, α-caryophyllene ameliorated carrageenan-induced paw oedema in mice and rats accompanied by reduced PGE_2 levels and impaired expression of iNOS and COX-2 (Fernandes et al., 2007). α-Tocopherol, which was identified in the DCM extract, was reported as a moderate inhibitor of 5-LO (Goetzl et al., 1980) and might be responsible for the 5-LO-inhibitory activity. Although α-tocopherol is a classical lipophilic antioxidant (Kontush et al., 1996), we observed only weak radical scavenging

activity by the lipophilic DCM or *n*-hexane extract of *L. zeylanica*, possibly because of a low content of α-tocopherol. In terms of XO supression, β-caryophyllene was reported to inhibit the enzyme with IC_{50} = 65 µM, corresponding to 13 µg/mL (Umamaheswari et al., 2012), supporting that this compound might contribute to XO inhibition by the DCM extract. Bioassay-guided fractionation will be carried out to identify the secondary metabolites that are responsible for the observed bioactivities, and to further evaluate their suitability for the development as phytotherapeutics.

5. Conclusion

Leucas zeylanica is a multi-purpose medicinal plant in Sri Lanka and has been widely employed in traditional medicine to treat inflammatory conditions, gout and microbial infections. Here, we provide insights into the biological activities of *L. zeylanica* that rationalize its ethnobotanical significance, that is, interference with major pro-inflammatory mediators and key enzymes in inflammation and gout, and identification of the chemical profile of constituents in the bioactive extracts of the plant. Dual interference with 5-LO and mPGES-1 along with XO inhibition by the DCM extract imply anti-inflammatory and anti-gout potential and supports its traditional usage. Noteworthy, *L. zeylanica* is the first Sri Lankan medicinal plant for which XO inhibitory activity was reported. To our surprise, analysis of the antimicrobial properties of this plant indicates only moderate potential for treatment of microbial infections. Taken together, the present study identified 5-LO, mPGES-1 and XO as target enzymes of a lipophilic *L. zeylanica* extract with pharmacological relevance, and thus provides a significant scientific basis that rationalizes the ethnobotanical use of *L. zeylanica* as remedy to treat inflammatory disease and gout.

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Author contributions

Conceived and designed the experiments: M.N., J.G., A.N., G.W., A.S., L.J., A.K., O.W.

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Study supervision: O.W., M.N

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Legends to the figures

Fig. 1: Morphological features of *L. zeylanica.*
(A), (B) habit (C) appearance of the abaxial side of the leaves (D) flower.

Fig. 2: Inhibition of 5-LO in intact neutrophils (A,B) and in cell-free assays (C,D) by extracts of *L. zeylanica*. (A, B) Extracts of *L. zeylanica* prepared with water, methanol, DCM or *n*-hexane at the indicated concentrations were added to human neutrophils and after 15 min at 37 °C, cells were stimulated with 2.5 µM A23187 and 20 µM AA. After 10 min, formed 5-LO products were extracted by SPE and analyzed by RP-HPLC. **(C, D)** The extracts of *L. zeylanica* at the indicated concentrations were added to isolated human recombinant 5-LO and after 15 min at 4 °C, samples were pre-warmed for 30 sec at 37 °C, and 20 µM AA was added as substrate. After 10 min, formed 5-LO products were extracted by SPE and analyzed by RP-HPLC. Data are means ± S.E.M, n = 3-4.

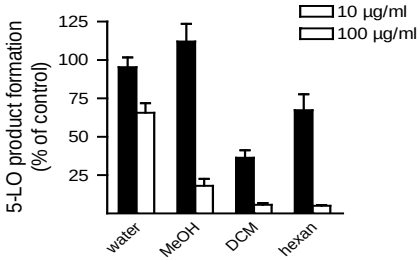
Fig. 3: Inhibition of mPGES-1 in a cell-free assay by the DCM extract. The DCM extract of *L. zeylanica* at the indicated concentrations was added to microsomes derived from A549 cells containing mPGES-1 and after 15 min at 4 °C, 20 µM PGH_2 was added as substrate. After 30 sec at 4°C the reaction was stopped and formed PGE_2 was analyzed by RP-HPLC. Data are means ± S.E.M, n = 3-4.

Fig. 4: Concentration-response curve for allopurinol and DCM extract of *L. zeylanica* for the inhibition of XO. The DCM extract of *L. zeylanica* or allopurinol at the indicated concentrations were added to the reaction mixture consisting of 0.003 units XO and 50 mM phosphate buffer. After incubation of 10 min, the initial absorbance was measured and 0.1 mM xanthine was added as substrate. Thereafter the absorbance at 295 nm was measured for 15 min at an interval of 1 min. Data are means ± S.D, n = 3.

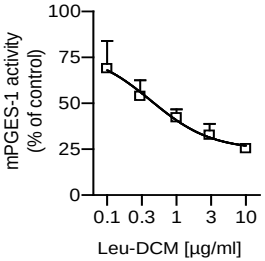
Fig. 5: Total ion chromatogram of the DCM extract of *L. zeylanica* and the tentatively identified compounds. **A**: β-caryophyllene **B**: farnesene, **C**: α-caryophyllene, **D**: β-cubebene, **E**: α-selinene **F:** caryophyllene oxide, **G**: neophytadiene, **H**: (3,7,11,15)-tetramethyl-2-hexadecen-1-ol, **I**: hexadecanoic acid, **J**: phytol, **K**: 9,12-octadecadionoic acid, **L**: 9,12,15-octadecatrienoic acid, **M**: octadecanoic acid, **N:** unidentified terpenoid, **O:** α-tocopherol **P**: stigmasterol, **Q:** β-sitosterol.

EXTRACTS

5-lipoxygenase activity



mPGES-1 activity



xanthine oxidase activity