Anti-inflammatory natural products from plants used in traditional medicine: A mass spectrometric approach for fast screening and discovery

Napagoda MT

Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka

ABSTRACT

The traditional use of *Plectranthus zeylanicus* and *Munronia pinnata* as anti-inflammatory remedy was rationalized by the current study. Extremely potent 5-LO / mPGES-1 inhibitory activities were observed for the lipophilic extracts of the two plant species and the bioactive constituents were characterised by a rapid and convenient mass spectrometric approach.

Corresponding author: MT Napagoda

<mayurinapagoda@yahoo.com>

Introduction

Natural products (secondary metabolites) are low molecular weight compounds made by living organisms (plants, animals, microorganisms), and have been exploited for treating and preventing diseases since ancient times. Despite the recent interest in other drug discovery approaches such as molecular modeling, combinatorial chemistry etc., natural products still play a major role as new clinical candidates and drugs (1). About half of the drugs currently in clinical use are based on natural product scaffolds (2,3).

Although plants and their products have been systematically used in Sri Lanka for treating illnesses for over thousand years. Sri Lankan flora has not yet been adequately studied phytochemically or pharmacologically.

The isolation and structural elucidation of novel natural products from medicinal plants and investigations on their bioactivities is rewarding, however, the interest in this field is dramatically declining due to the long persisting problems associated with conventional natural products isolation approaches. The requirement for large scale extractions, as well as laborious isolation and purification methods which are highly technically demanding are no longer cost-effective and time-effective, thus hindering the chemical profiling of medicinal plants, and thereby the validation of their uses in traditional medicine.

Over the years, mass spectrometry (MS) has increasingly become an analytical tool of choice in the field of natural products chemistry owing to its high throughput nature, quantitative capability and the facility to integrate with chromatographic separation methods. These advanced hyphenated spectrometric techniques afford for a rapid identification and characterisation of secondary metabolites without the necessity of isolation and purification, and detailed information on their metabolic profiles can be obtained with a minimal amount of material (4). Therefore, the present study was focused on the application of MS-based rapid screening strategies to characterise bioactive metabolites in two popular Sri Lankan medicinal plants; *Plectranthus zeylanicus* (Iruveriya) and *Munronia pinnata* (Binkohomba), that are neither phytochemically nor pharmacologically evaluated yet.

Plectranthus zeylanicus Benth is a perennial herb of the family Lamiaceae with aromatic,

astringent and stomachic properties and is used in folk medicine in decoctions for fevers, dysentery, diarrhoea, vomiting and thirst (5,6). Furthermore, it is used as a constituent of various ayurvedic and traditional medicinal preparations (Kalkaya, Prameha, Kvathaya) and the plant is described to be effective in combating asthma, common cold, varieties of fever, cough, leucoderma, diarrhea, chronic ulcers etc (7). *Munronia pinnata* (Wall) Theob (Family Meliaceae) is a small herb which is considered as a rare species but is also distributed in several other Asian countries (8). It is considered to be one of the most expensive plant materials (US$ 50-110/kg) used in traditional medicine in Sri Lanka (9). 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 (10,11). 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 (7,11). Despite the therapeutic importance, the scientific evidences are insufficient to rationalize the reported ethnopharmacological use of these two plant species.

Prostaglandins (PG) and leukotrienes (LTs) are formed from arachidonic acid (AA) and act as important mediators of inflammation, allergy and pain (12). LTs contribute to various inflammatory and allergic reactions in the pathophysiology of asthma, allergic rhinitis, atherosclerosis, cancer, etc (13). 5-lipoxygenase (5-LO) that catalyzes the first two key steps in LT biosynthesis from AA is considered as a valuable drug target (14,15). 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 (16). 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 (17,18) which rationalizes these pro-inflammatory enzymes as functional targets for anti-inflammatory phytomedicine.

Therefore the present study was undertaken to evaluate the anti-inflammatory mode of action of *P. zeylanicus* and *M. pinnata* and characterise the related secondary metabolites.

Materials and Methods

Plant Material

P.zeylanicus was collected in Nittambuwa (Gampaha district) while *M. pinnata* was collected in Weerasuriyakanda (Gampaha district) and Algama (Kegalle district) in 2011/ 2012. The plants were identified by the author and authenticated by comparing herbarium specimens at the National herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka.

Preparation of crude extracts

The plant materials (whole plants) were dried, powdered (13 g of *P. zeylanicus*, 15 g of *M. pinnata*) and successively extracted with 600 ml of *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol. Besides, 3g of powdered materials were extracted in 300 ml of 70% methanolwater. The solvents were evaporated and the crude extracts were then subjected to the bioactivity studies.

Evaluation of Bioactivity

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

Human neutrophils were isolated from leucocyte concentrates obtained from the University Hospital Jena, Germany. The neutrophils were treated as described (19) and 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. Thereafter the 5-LO products formation (LTB_4 and its trans-isomers, 5-H(P)ETE) were analyzed by HPLC as described (19). Cysteinyl-LTs C_4, D_4 and E_4 and oxidation products of LTB_4 were not determined.

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

E.coli (BL21) was transformed with pT3-5-LO plasmid and recombinant 5-LO protein was expressed and partially purified as described (20). 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) and the 5-LO inhibition was detected as described (20).

Determination of mPGES-1 activity

A549 cells were prepared and the microsomal fraction was obtained (21). 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 (20 μM). After 1 min at 4°C, the reaction was terminated, and PGE_2 was separated by solid-phase extraction and analyzed by RP-HPLC as described (21).

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 (22). The absorbance was recorded at 520 nm after 30 min incubation of the test samples (100 il of 5, 25, 50 μg/ml) with DPPH in ethanol (50 μM , 100 μl) under gentle shaking in the dark. Ascorbic acid and L-cysteine were used as reference compounds. All analyses were performed in triplicates.

Measurement of reactive oxygen species in neutrophils

Neutrophils 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 to the 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) NOVO star microplate reader (BMG Lab technologies GmbH, Offenburg, Germany).

Statistical analysis

Data are expressed as mean ± S.E. IC_{50} values were calculated by nonlinear regression using Graph Pad Prism software one site binding competition. Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni/ Tukey-Kramer post-hoc test for multiple comparisons respectively. A p value < 0.05 (*) was considered significant.

Phytochemical screening

Bioassay-guided fractionation

M. pinnata hexane extract (130 mg) and *P. zeylanicus hexane and DCM extracts (230 mg and 120 mg respectively)* were fractionated over a silica gel column. The samples were eluted with *n*-hexane, different mixtures of EtOAC in *n*-hexane (3%, 5%, 10%, 15%, 25%, 35%, 50%, 75%, 100%) and methanol, successively, yielding 11 fractions. The collected fractions were evaporated and then subjected to bioactivity assays and LC-MS analysis.

Liquid Chromatography coupled Mass Spectrometric (LC-MS) analysis

n-Hexane and DCM extracts of *P. zeylanicus and n*-hexane extract of *M. pinnata* and the fractions obtained thereof were analyzed on LTQ-Orbitrap instrument (Thermo Fisher, San Jose, CA) with electrospray ionization (ESI) /atmospheric pressure chemical ionization (APCI). 15 μl aliquots of the diluted samples (10 μg/ml in EtOAc) were injected and separated by liquid chromatography by DionexAcclaim® RSLC 120 C18 column (2.1 × 150 mm packed with 2.2 μm, 120 Å). Reversed phase UPLC gradient separations were performed using water and methanol as mobile phases. The ESI/APCI conditions were optimized to generate full scan and collision-induced dissociation (CID) mass spectra with 30,000 and 7500 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.

Molecular formula identification

Following a published method (23), the molecular formula were identified by isotope pattern and fragmentation tree analysis. The fragmentation tree that explains the data best is calculated by an optimization algorithm.

Gas Chromatography coupled Mass Spectrometric (GC-MS) analysis

GC-MS analysis of the crude extracts and the fractions of interest 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).

Results

Bioactvity and Phytochemistry of P. zeylanicus

Evaluation of 5-LO inhibitory activities of *P. zeylanicus*

P. zeylanicus extracts (100 µg/ml) that were prepared by using *n*-hexane or DCM as solvent strongly inhibited 5-LO activity in A23187-stimulated neutrophils (inhibition >85%), whereas extracts obtained by the use of hydrophilic solvents, (water or methanol) were comparably ineffective (Figure 1A). More detailed concentration-response studies using this cell-based assay revealed IC_{50} values of 6.6 and 12 µg/ml for *P. zeylanicus* extracts prepared with *n*-hexane and DCM, respectively (Figure 1B). The synthetic reference inhibitor zileuton (approved as anti-asthmatic drug) blocked 5-LO activity with IC_{50}=0.13µg/ml. The 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.

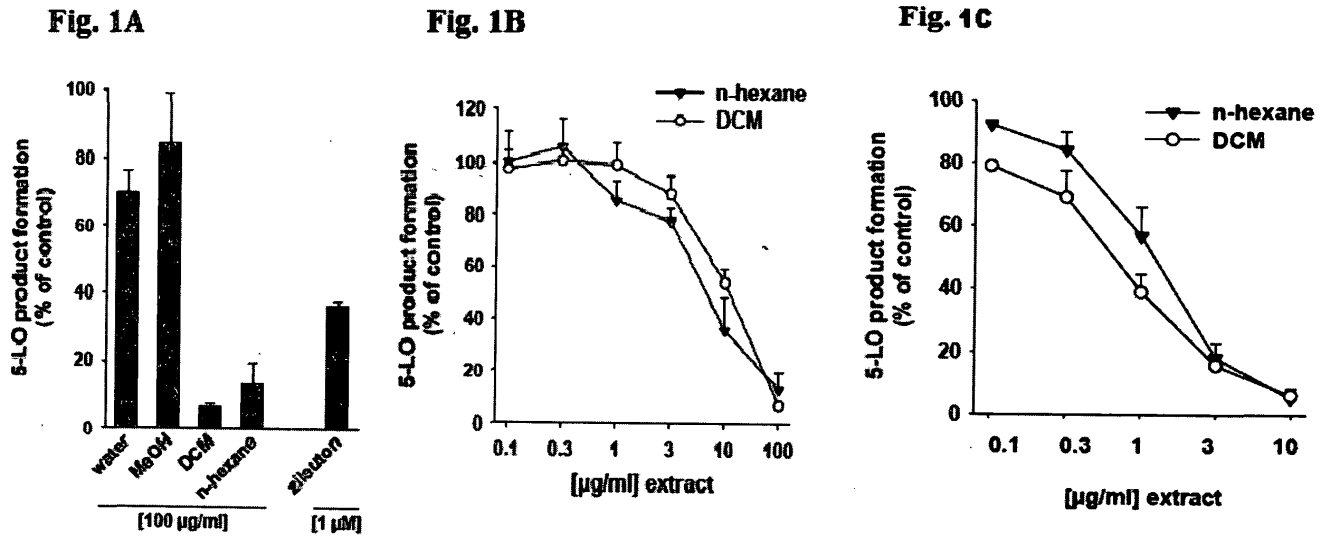

Figure 1: Inhibition of 5-LO activity by *P. zeylanicus* extracts: (A), (B) in intact human neutrophils and (C) in cell free assay. Data are given as mean +/-S.E.M., n = 3-4.

Suppression of 5-LO product synthesis in the cell may be caused by diverse mechanisms, others than interference with the 5-LO enzyme activity, thus to investigate whether or not the extracts directly inhibit 5-LO activity, a cell-free assay was applied. *n*-hexane and DCM extracts of *P. zeylanicus* caused concentration-dependent inhibition of cellular 5-LO with IC_{50} = 1.2 and 0.7 µg/ml, respectively **(Figure 1C)**. For zileuton, the IC_{50} value was determined at 0.11 µg/ml.

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

The majority of natural products from plant origin that suppress 5-LO activity may confer their inhibitory action by unselective antioxidant reactions as they reduce the active-site iron, decompose 5-LO-activating lipid hydroperoxides or scavenge intermediate fatty acid radicals within LT synthesis (18). To investigate whether such unselective antioxidant properties may account also for 5-LO inhibition by *P. zeylanicus* extracts, radical scavenging properties were assessed using the cell-free DPPH assay. In contrast to the reference antioxidants, the *n*-hexane or DCM extracts of *P. zeylanicus* were not able to significantly reduce radical formation, suggesting that 5-LO inhibition is not mediated by a redox-based mechanism. In accordance with the DPPH assay, neither the *n*-hexane nor the DCM extract of *P. zeylanicus* caused significant inhibition of ROS formation in neutrophils stimulated with the bacterial peptide fMLP.

Bioassay-guided separation of the *P. zeylanicus* extracts

To get more insights into the identity of the ingredient and composition of the extracts that might be responsible for the potent inhibition of 5-LO, the *n*-hexane and DCM extracts were separated by liquid column chromatography into 11 fractions that were analyzed for inhibition of isolated 5-LO in the cell-free assay at concentrations of 1 and 10 µg/ml, each. Out of the 11 fractions (F) of the *n*-hexane extract, F-6, F-7, F-8, F-9, F-10 and F-11 at a concentration of 10 µg/ml inhibited 5-LO activity by > 50%, and only F-9 and F-10 were active at 1 µg/mL (> 50% 5-LO inhibition). For the DCM extract, F-6, F-7, F-9, F-10 and F-11 were active at 10 µg/ml concentration in the 5-LO cell free assay, but none of these fractions showed significant 5-LO inhibitory activity at 1 µg/ml.

GC-MS analysis

Highly potent 5-LO inhibitory F-9 and F-10 from the *n*-hexane extract of *P. zeylanicus*, were subjected to a phytochemical screening by GC-MS and UPLC-MS. The GC-MS analysis of F-9 and F-10 revealed 13 and 12 components respectively, identified by comparison of their experimental mass spectrum with those recorded in the NIST MS Search 2.0 and Adams mass spectrum libraries as well as by comparison with the respective standards (Figure 2).

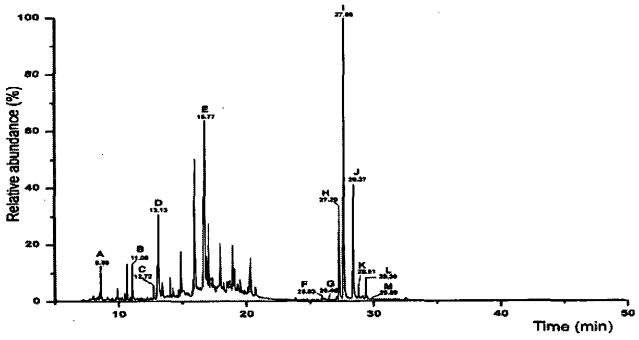

Figure 2: Characterisation of constituents of bioactive fraction F-9 of the *n*-hexane extract of *P. zeylanicus* by GC-MS

Total ion chromatograph of F-9 of the *n*-hexane extract of *P. zeylanicus* and its identified compounds. **A**: Eudesm-7(11)-en-4-ol, **B**: hexadecanoic acid, **C**: phytol, **D**: 9,12,15-octadecatrienoic acid, **E**: callitrisic acid, **F**: Cholest-5-en-3β-ol, **G**: ergosta-5,22-dien-3β-ol, **H**: campesterol, **I**: stigmasterol, **J**: β- sitosterol, **K**: β-amyrin, **L**: α-amyrin, **M**: stigmast-4-en-3-one.

UPLC - MS analysis

Analysis of F-9 by UPLC-MS revealed two uncommon compounds (denoted as compound A and B, Figure 3A) and two common phytosterol derivatives were also detected in the TIC.

Only compound B yielded a substantial peak in the TIC of F-10.

The accurate mass measurements of compound B using the Orbitrap instrument and the search in the METLIN database suggested the presence of coleone P ($C_{22}H_{30}O_6$, Figure 3B) in both F-9 and F-10. The identity of coleone P was further confirmed by its fragmentation pattern, which agrees with literature data[26]. In addition, the peak at *m/z* 383.20648 which corresponds to compound A, fits well with the molecular formula $C_{20}H_{30}O_7$ (mass accuracy of 0.131 ppm) suggesting the presence of cinncassiol A / cinncassiol C3, a diterpenoid which has been isolated from the family Lauraceae.

Fig. 3A

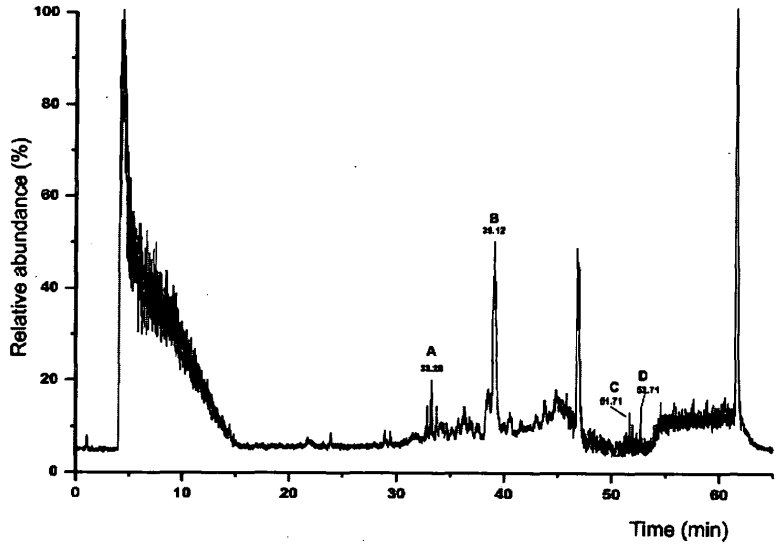

Fig. 3B

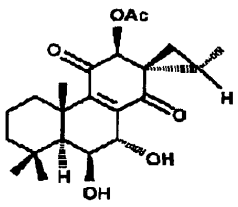

Figure 3: Identification of constituents of bioactive fractions of the *n*-hexane extract of *P. zeylanicus* by UPLC-MS

(A) Total ion chromatogram of F-9 of the hexane extract of *P. zeylanicus* and its identified compounds. **A**: cinncassiol A/ cinncassiol C3, **B**: coleone P, **C**: stigmasterol, **D**: stigmasterol-5, 22, 25-trien-3-β-ol

(B) Chemical structure of coleone P.

Bioactivity and Phytochemistry of *M. pinnata*

Evaluation of 5-LO and mPGES-1 inhibition

A potent inhibition of 5-LO activity in neutrophils was observed for the *n*-hexane and DCM extracts of *M. pinnata* (10 and 100 µg/ml, Figure 4A), whereas methanol and water extracts were almost ineffective and reduced 5-LO activity only by 15 and 16% at 100 µg/ml, respectively (Figure 4A). 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 (Figure 4B).

n-hexane and DCM extracts (at 10 µg/ml), efficiently blocked 5-LO activity in the cell free assay while extracts prepared with water or methanol were much less effective (Figure 5A). As shown in Figure 5B, 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. As *n*-hexane extract appeared to be most interesting, the potential of this extract to interfere also with the formation of the pro-inflammatory PGE_2 produced by mPGES-1 was investigated.

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 (Figure 5C), which is even slightly lower than that the IC_{50} for MK886 (1.3 µg/ml), a well-recognized mPGES-1 inhibitor, used as control (not shown).

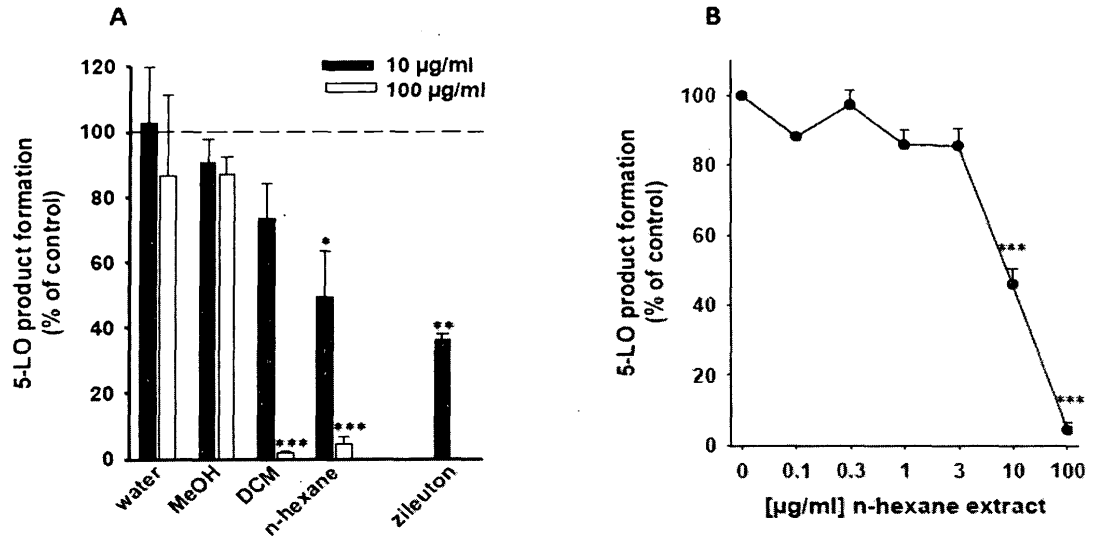

Figure 4: Inhibition of 5-LO activity in intact neutrophils (A) Inhibition of 5-LO activity by various extracts of *M. pinnata*. (B) Concentration-response analysis for the *n*-hexane extract of *M. pinnata*. Data are given as mean +/- S.E.M., n = 3-4.

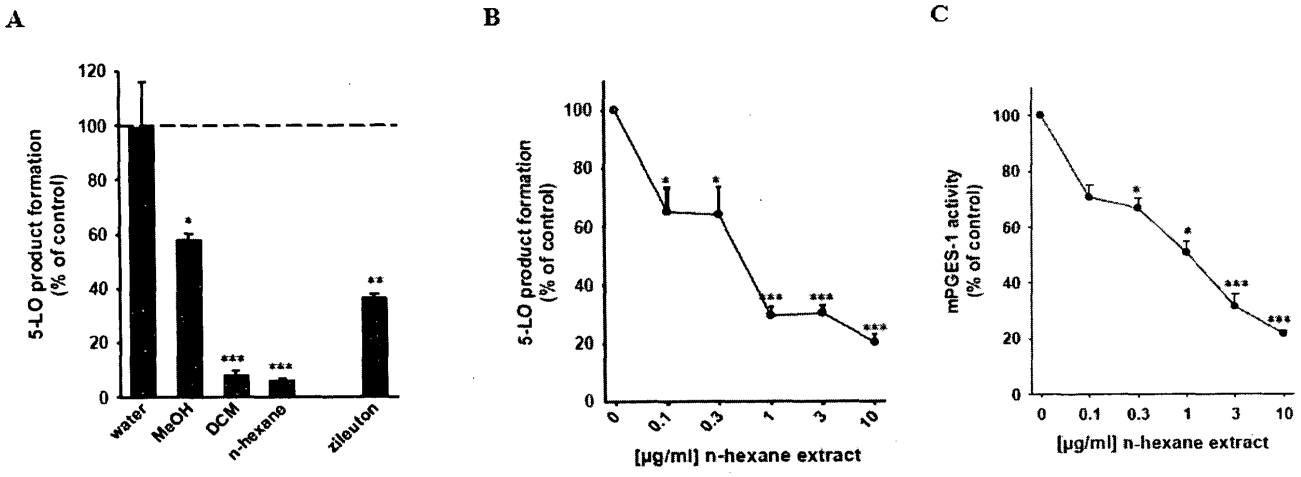

Figure 5: Inhibition of 5-LO activity in a cell-free assay and inhibition of mPGES-1, (A) Inhibition of 5-LO by various extracts (10 µg/ml) of *M. pinnata* or zileuton (3 µM) (B) Concentration-response analysis for the *n*-hexane extract. (C) Inhibition of mPGES-1 by the *n*-hexane extract of *M. pinnata*. Data are given as mean +/- S.E.M., n = 3-4.

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

The *n*-hexane extract of *M. pinnata* up to 50 µg/ml is not able to significantly reduce radical formation while it did not cause significant inhibition of ROS formation. Of interest, extracts based on water, methanol or DCM were able to reduce ROS formation, with the methanol extract being most potent (54.7 +/- 6.9% inhibition).

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

Early attempts of phytochemical screening of *M. pinnata* either failed (12) or provided incomplete information[27]. 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 and the resulted 11 fractions 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, F-6, F-7, F-8, F-9 and F-11 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. These data imply a good correlation of the fractions for dual inhibition of 5-LO and mPGES-1.

Identification of constituents of the *n*-hexane extract and its bioactive fractions 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 (Figure 6). Among the above identified compounds, 12 compounds were detected in F-6 and only 3 compounds in F-8 after analysis.

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 (Figure 7). 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 comprehensive analysis of the fragmentation pattern suggested the most possible structure for the 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 and the analysis of CID spectra proposed compound B to be most likely the triterpenoids, conicasterol C or theonellasterol E according to its characteristic fragmentation pattern. In addition, two common phytosterol derivatives were also detected in the TIC and (compound C and D) and were proposed as stigmastentriol and tigmasterol, respectively.

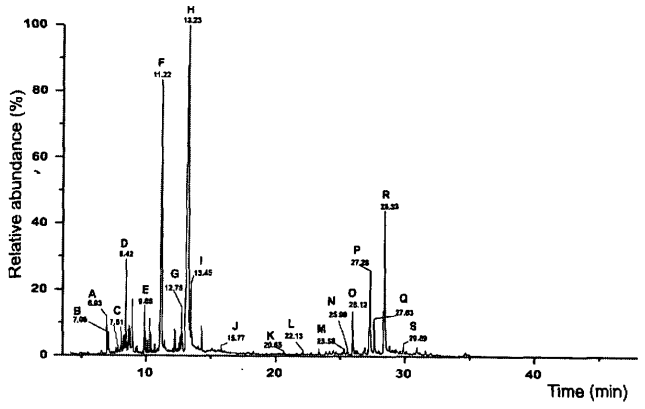

Figure 6: Total ion chromatograph of the *n*-hexane extract of *M. pinnata* and its identified compounds. **A**: β-caryophyllene, **B**: isocaryophyllene, **C**: dodecanoic acid, **D**: caryophyllene oxide, **E**: neophytadiene, **F**: hexadecanoic acid, **G**: phytol, **H**: 9,12 - octadecadienoic acid, **I**: octadecanoic acid, **J**: 4,8,12,16 tetramethylheptadecan-4-olide, **K**: heptacosane, **L**: squalene, **M**: stigmastan-3,

5-diene, **N**: hentriacontane, **O**: á-tocopherol, **P**: campesterol, **Q**: stigmasterol, **R**: β-sitosterol, **S**: stigmast-4-en-3-one.

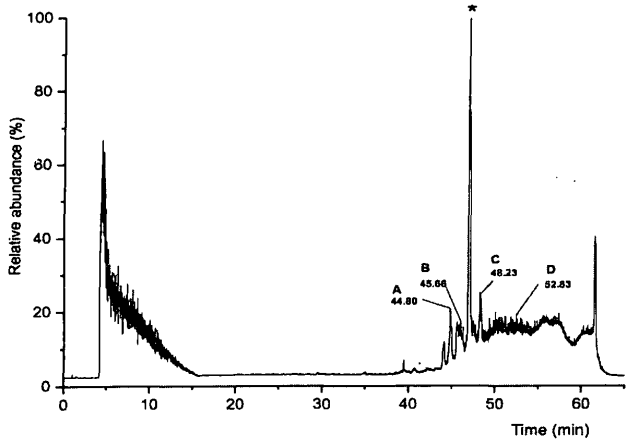

Figure 7: Total ion chromatograph of F-6 of the *n*-hexane extract of *M. pinnata* when analyzed with the ESI source and the presumed compounds. **A:** ganoderiol F, **B:** conicasterol C/ theonellasterol, **C**: stigmastentriol, **D:** stigmasterol (the peak denoted as " *" is due to erucylamide, a contaminant in the LC system).

Discussion

Here the attempts were made to (I) rationalize and validate the traditional use of the medicinal plants *P. zeylanicus* and *M. pinnata* as anti-inflammatory remedies by analysis of their ability to interfere with 5-LO/mPGES-1 activity and (II), to identify relevant constituents of the bioactive fractions. In fact, *n*-hexane or DCM extracts of *P. zeylanicus* and *n*-hexane extract of *M. pinnata* caused direct and potent inhibition of human 5-LO and suppressed the biosynthesis 5-LO products in isolated human neutrophils while the latter inhibited the human mPGES-1 as well. Notably, these extracts exhibited no significant radical scavenging or antioxidant activities in cell-free (DPPH assay) or cell-based (ROS generation in neutrophils) test systems, and failed to reduce neutrophil viability. Hence, the lipophilic *P. zeylanicus* and *M. pinnata* extracts contain nonredox-related principles that specifically interact with 5-LO supporting an anti-inflammatory potential. Instead of performing extensive chromatographic separations and isolation procedures aiming to reveal potential bioactive constituents by applying traditional phytochemical analysis, rapid and convenient chromatographic/MS approaches were employed for compound identification.

Since the *P. zeylanicus* and *M. pinnata* are widely used in traditional medicine in Sri Lanka to alleviate the pathological conditions caused by inflammation (6,7,10) the emphasis was given on inhibition of 5-LO and/or mPGES-1 as potential underlying mode of action. In fact, 5-LO as key enzyme in the biosynthesis of the pro-inflammatory LTs (14) is explored as a drug target for many inflammatory disorders (28). A large number of plants and their extracts and/or specific secondary metabolites thereof have been reported that are capable of suppressing the biosynthesis of 5-LO products. Such interference with 5-LO activity is considered as basis for the anti-inflammatory features of the respective plants (and medicinal preparations thereof) in folk medicine. However, many of these investigations lacked sufficient and detailed experimentation and the 5-LO inhibitory potencies of the extracts often turned out to be comparably low (IC_{50} values of approx. 20-80 µg/ml)(29). Even for extracts of the gum resin of *Boswellia serrata,* which is considered as potent 5-LO inhibiting natural product and thus frequently used as anti-inflammatory remedy (30), IC_{50} values of 8.4-30 µg/ml were determined (31). In direct comparison to these potencies, the results obtained with the *n*-hexane and DCM extracts of *P. zeylanicus* (IC_{50} of 0.7-12 µg/ml) and *n*-hexane extract of *M. pinnata* (IC_{50} of 0.48 -8.7 µg/ml) are remarkable and suggest a high pharmacological potential for intervention with 5-LO-related disorders.

Previous studies showed that plant derived 5-LO inhibitors such as hyperforin, myrtucommulone, boswellic acids etc, also inhibit the activity of mPGES-1 (17,34), 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

(12). 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 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. 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 use in folk medicine.

Efficient chromatographic and MS techniques were utilized in the current study on the putative bioactive constituents in restricted fractions of the *n*-hexane extracts of the two plant species. Tandem mass spectrometry was employed here and the accurate mass measurements data were used to tentatively identify the compound in the active fractions. This approach is novel in the fact that it allows for fast data de-replication and will give the researchers a hint on the class of compounds. The amounts of compounds that are able to assay using MS/MS experiments is typically much smaller and it can work on mixtures.

The current study provides new insights towards phytochemicals of *P. zeylanicus*, and among the identified compounds in the most active fractions, some have been proposed to possess in vitro and in vivo anti-inflammatory properties (35,36). Among the identified constituents in the active fractions of the *n*-hexane extract of *P. zeylanicus,* coleone P is of particular interest. The genus *Plectranthus* is rich in coleone-type diterpenoids which exert several biological activities such as anticancer and antimicrobial activities (37,38,39). Coleone P has been isolated from the *Plectranthus* species *P. caninus* (26), and current spectral analysis proposes the presence of this compound in the active fractions F-9 and F-10 of the *P. zeylanicus* *n*-hexane extract. Besides coleone P, cinncassiol A/ C3 as well as callitrisic acid were identified for which the knowledge regarding bioactivities is rare. Furthermore, the pentacyclic triterpenes α- and β-amyrin which have displayed anti-inflammatory activity in in vitro and in vivo models are present in the active fractions (35).

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, the current study has unveiled the phytochemistry of this medicinal plant for the first time. Among the identified constituents in the *n*-hexane extract by GC-MS, â-caryophyllene and caryophyllene oxide might be of interest. Anti-inflammatory activity of â-caryophyllene was revealed in models of acute (carrageenan-induced) inflammation[40] while caryophyllene oxide exhibited significant cytotoxicity against the human cancer cell lines[41]. Furthermore, the presence of á-tocopherol could be correlated to the 5-LO inhibition as it has displayed a potent inhibition by selective and tight binding to 5-LO (42).

The LC-MS analysis of F-6 and of F-8 of the *n*-hexane extract of *M. pinnata* 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. Particularly, the tentative identification of ganoderiol F is of interest as this triterpenoid was reported to exhibit strong anti-HIV-1 protease activity (43) as well as *in vivo* antitumor effects (44). Further optimization of LC-MS/MS conditions in planned follow-up studies may permit confirmation of the identified structures.

Although it is reasonable to correlate the antiinflammatory activities of *P. zeylanicus* and *M. pinnata* to the presence of the identified compounds supported also on literature reports, there are several unknown compounds that are not in any database which might also contribute 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 (23) will provide better insights into the chemical profiles of the above plants. 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.

Conclusion

By demonstrating potent inhibition of 5-LO/mPGES-1 activity by lipophilic extracts of *P. zeylanicus* and *M. pinnata* in different biological test systems, the traditional use of *the above plants* in Sri Lanka for the treatment of inflammatory conditions could be explained. The phytochemical analysis of the bioactive fractions *n*-hexane extracts of *P. zeylanicus* by MS techniques led to the identification of coleone P for the first time in this plant, along with other thus far unknown or potentially interesting constituents for which anti-inflammatory activity has been proposed. The phytochemistry of *M. pinnata* was revealed for the first time and several secondary metabolites with known anti-inflammatory properties were identified by mass spectrometric techniques. The solid platform laid by our study will be indispensable for further phytochemical and bioactivity research on these popular and valuable medicinal plants in the future. The current data may stimulate for more detailed preclinical analysis of the pharmacological properties of *P. zeylanicus* and *M. pinnata* that may further support its therapeutic potential in the treatment of inflammatory disorders.

Acknowledgements

The author sincerely acknowledge the International Max Planck Research School for the PhD fellowship (2010-2013). The study was conducted under the supervision of Dr. Aleš Svatoš (Research Group Mass Spectrometry/Proteomics, Max Planck Institute for Chemical Ecology, Jena, Germany), Prof. Oliver Werz (Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Germany) and Prof. Sebastian Boecker (Chair for Bioinformatics, Friedrich-Schiller-University Jena, Germany). The author expressed her sincere thanks to Jana Gerstmeier, Andreas Koeberle, Sandra Wesely, Sven Popella (Institute of Pharmacy, Friedrich-Schiller-University), Sybille Lorenz (Max Planck Institute for Chemical Ecology) and Kerstin Scheubert (Department of Bioinformatics, Friedrich-Schiller-University) for their contribution to this study.

References

1. Butler MS. The role of natural product chemistry in drug discovery. *Journal of Natural Products* 2004;**67**:2141-2153.
2. Newman DJ, Cragg GM,.Natural products as sources of new drugs over the last 25 years. *Journal of natural products* 2007;**70**(3):461-477.
3. Harvey AL. Natural products in drug discovery. *Drug Discovery Today* 2008;**13**(19/20): 894-901.
4. Sarker SD, Nahar L. An introduction to natural products isolation. *Methods in molecular biology* 2012; **864**:1-25.
5. Arambewela L, Wijesinghe A. Sri Lankan medicinal plant Monographs and analysis - Vol-11 *Plectranthus zeylanicus*, 2006; National Science Foundation, Sri Lanka.
6. Jayaweera DMA. Medicinal plants (Indigenous and exotic) used in Ceylon, Part **2**. 1982; National Science Council, Sri Lanka 108-109.
7. Ayurveda Pharmacopeia**1**. Part (1). 1979; Department of Ayurveda, Sri Lanka.
8. Dassanayake MD, Fosberg FR. A Revised Handbook to the Flora of Ceylon. Vol. **3**. 1981; Amerind Publ. Co. Ltd. India.
9. Dharmadasa RM, Hettiarachchi PL, Premakumara GAS. Geographical distribution and conservation of a rare medicinal plant *Munronia pinnata* (Wall) Theob (Meliacea) in Sri Lanka. *Bangladesh Journal of Plant Taxonomy* 2011;**18**:39-49.
10. Jayaweera DMA. Medicinal plants (Indigenous and exotic) used in Ceylon, Part **4,** 1982; National Science Council, Sri Lanka.
11. Arambewela L, Wijesinghe A. Sri Lankan medicinal plant Monographs and analysis- Vol-12 *Munronia Pumila*. 2006: National Science Foundation, Sri Lanka.
12. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001;**294**:1871-1875.
13. Werz O, Steinhilber D. Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacology and Therapeutics* 2006;**112**:701-718.

14. Radmark O, Werz O, Steinhilber D, Samuelsson B. 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends in Biochemical Sciences* 2007;**32**:332-341.

15. Pergola C, Werz O. 5-Lipoxygenase inhibitors: a review of recent developments and patents. *Expert Opinion on Therapeutic Patents* 2010;**20**(3):355-375.

16. Samuelsson B, Morgenstern R, Jakobsson PJ. Membrane prostaglandin E synthase-1: a novel therapeutic target. *Pharmacological Reviews* 2007;**59**:207-224.

17. Koeberle A, Werz O. Inhibitors of the microsomal prostaglandin E(2) synthase-1 as alternative to non-steroidal anti-inflammatory drugs (NSAIDs) - a critical review. *Current Medicinal Chemistry* 2009;**16**:4274-4296.

18. Werz O. Inhibition of 5-lipoxygenase product synthesis by natural compounds of plant origin, *Planta Medica* 2007;**73**(13):1331-1357.

19. Pergola C, Dodt G, Rossi A, Neunhoeffer E, Lawrenz B, Hinnak H, Samuelsson B, Rådmark O, Sautebin L, Werz O. ERK-mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for gender differences in inflammation and asthma. Proceedings of the National Academy of Sciences of the United States of America 2008;**105**:19881-19886.

20. Fischer L, Szellas D, Radmark O, Steinhilber D, Werz O. Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors. *FASEB Journal* 2003;**17**:949-951.

21. Koeberle A, Siemoneit U, Buehring U, Northoff H, Laufer S, Albrecht W, Werz O. Licofelone suppresses prostaglandin E2 formation by interference with the inducible microsomal prostaglandin E2 synthase-1. *Journal of Pharmacology and Experimental Therapeutics* 2008;**326**:975-982.

22. Blois MS. Antioxidant Determinations by the Use of a Stable Free Radical, *Nature* 1958; **181**:1199-1200.

23. Rasche F, Scheubert K, Hufsky F, Zichner T, Kai M, Svatoš A., Böcker S. Identifying the unknowns by aligning fragmentation trees. *Analytical Chemistry* 2012;**84**(7):3417-3426.

24. Werz O, Steinhilber D. Development of 5-lipoxygenase inhibitors-lessons from cellular enzyme regulation. *Biochemical Pharmacology* 2005;**70**:327-333.

25. Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, Summers JB, Brooks DW. 5-lipoxygenase inhibitory activity of zileuton. *Journal of Pharmacology and Experimental Therapeutics* 1991;**256**(3):929-937.

26. Arihara S, Ruedi P, Eugster CH. Neue spiro-Cyclopropyl-cyclohexendion-Diterpene: Coleone M, N, P, Q, R sowie Barbatusin aus *Plectranthus caninus* ROTH und Coleon 0 aus *Coleus somaliensis S.* MOOR. *Helvetica Chimica Acta* 1975;**58**(2):343-356.

27. Munasinghe MLAMS. Phytochcmical screening of *Munronia pinnata* (Wall) Theob. *Ayurveda Sameekshava* 2002;**1**:164-171.

28. Peters-Golden M, Henderson WR. Leukotrienes. *The New England Journal of Medicine* 2007;**357**:1841-1854.

29. Schneider I, Bucar F. Lipoxygenase inhibitors from natural plant sources. Part 1: Medicinal plants with inhibitory activity on arachidonate 5-lipoxygenase and 5-lipoxygenase/ cyclooxygenase. *Phytotheraphy Research* 2005;**19**(2):81-102

30. Ammon HP. Boswellic acids in chronic inflammatory diseases. *Planta Medica* 2006; **72**(12):1100-1116.

31. Wildfeuer A, Neu IS, Safayhi H, Metzger G, Wehrmann M, Vogel U, Ammon HP. Effects of boswellic acids extracted from an herbal medicine on the biosynthesis of leukotrienes and the course of experimental autoimmune encephalomyelitis. *Arzneimittelforschung* 1998;**48**(6):668-674.

32. Eugster PJ, Guillarme D, Rudaz S, Veuthey JL, Carrupt PA, Wolfender JL. Ultra high pressure liquid chromatography for crude plant extract profiling, *Journal of AOAC International* 2011;**94**(1):51-69.

33. Perry RH, Cooks RG, Noll RJ, Orbitrap mass spectrometry: instrumentation, ion motion and applications. *Mass Spectrometry Reviews* 2008;**27**(6):661-699.

34. Bauer J, Koeberle A, Dehm F, Pollastro F, Appendino G, Northoff H, Rossi A, Sautebin L, Werz O. Arzanol, a prenylated heterodimeric

phloroglucinyl pyrone, inhibits eicosanoid biosynthesis and exhibits anti-inflammatory efficacy in vivo. *Biochemical Pharmacology* 2011;**81**:259-68.

35. Kweifio-Okai G, Macrides TA. Antilipoxygenase activity of amyrin triterpenes. Research communications in chemical pathology and pharmacology 1992;**78**(3):367-372.
36. Prieto-Garcia JM, Recio MC, Giner RM. Anti-inflammatory activity of beta-sitosterol in a model of oxazolone-induced contact delayed type hypersensitivity. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 2006;**6**(4):57-62.
37. Marques CG, Pedro M, Simões MFA, Nascimento MSJ, Pinto MMM, Rodríguez B. Effect of abietane diterpenes from *Plectranthus grandidentatus* on the growth of human cancer cell lines. *Planta Medica* 2002;**68**(9):839-840.
38. Xing X, Wu H, Wang X, Huang Y, Li Q, Li C, Yang Y, Liu Y, Liu J. Inhibition of tumor cell proliferation by Coleon C. *Journal of Chemotheraphy* 2008;**20**(2):238-245.
39. Wellsow J, Grayer RJ, Veitch NC, Kokubun T, Lelli R, Kite GC, Simmonds MSJ, Insect-antifeedant and antibacterial activity of diterpenoids from species of *Plectranthus*. *Phytochemistry* 2006;**67**:18181825.
40. Gertsch J, Leonti M, Raduner S, Racz I, Chen JZ, Xie XQ, Altmann, KH, Karsak M, Zimmer A. Beta-caryophyllene is a dietary cannabinoid. *Proceedings of the National Academy of Sciences of the United States of America* 2008;**105**:9099-9104.
41. Jun JN, Mosaddik A, Moon JY, Jang KC, Lee DS, Kwang SA, Cho SK. Cytotoxic Activity of caryophyllene oxide isolated from Jeju guava (*Psidium cattleianum* Sabine) leaf. *Records of Natural Products* 2011;**5**:242-246.
42. Reddanna P, Rao MK, Reddy CC. Inhibition of 5-lipoxygenase by vitamin E. *FEBS Letters* 1985;**193**:39-43.
43. el-Mekkawy S, Meselhy MR, Nakamura N, Tezuka Y, Hattori M, Kakiuchi N, Shimotohno K, Kawahata T, Otake T. Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum*. *Phytochemistry* 1998;**49**:1651-1657.
44. Gao JJ, Hirakawa A, Min BS, Nakamura N, Hattori M. *In vivo* antitumor effects of bitter principles from the antlered form of fruiting bodies of *Ganoderma lucidum*. *Journal of Natural Medicines* 2006;**60**:42-48.