Journal of Ethnopharmacology 151 (2014) 800–809

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Inhibition of 5-lipoxygenase as anti-inflammatory mode of action of *Plectranthus zeylanicus* Benth and chemical characterization of ingredients by a mass spectrometric approach

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ARTICLE INFO

Article history:
Received 21 May 2013
Received in revised form
1 November 2013
Accepted 2 November 2013
Available online 27 November 2013

Keywords:
Plectranthus zeylanicus
Inflammation
5-Lipoxygenase
Radical scavenger
Neutrophils
Mass spectrometry

ABSTRACT

Ethnopharmacological relevance: The perennial herb *Plectranthus zeylanicus* Benth is extensively used in traditional medicine in Sri Lanka and South India for treating inflammatory conditions, but pharmacological features of *Plectranthus zeylanicus* are hardly explored in order to understand and rationalize its use in ethnomedicine. As 5-lipoxygenase (5-LO) is a key enzyme in inflammatory disorders such as asthma or atherosclerosis, we investigated 5-LO inhibition by *Plectranthus zeylanicus* extracts and analyzed relevant constituents.

Materials and methods: We applied cell-free and cell-based assays to investigate suppression of 5-LO activity. Cell viability, radical scavenger activities, and inhibition of reactive oxygen species formation (ROS) in neutrophils were analysed to exclude unspecific cytotoxic or antioxidant effects. Constituents of the extracts were characterized by bioassay-guided fractionation and by analysis using gas or liquid chromatography coupled to mass spectrometric (Orbitrap) analysis.

Results: Extracts of *Plectranthus zeylanicus* prepared with *n*-hexane or dichloromethane potently suppressed 5-LO activity in stimulated human neutrophils (IC_{50}=6.6 and 12 μg/ml, respectively) and inhibited isolated human recombinant 5-LO (IC_{50}=0.7 and 1.2 μg/ml, respectively). In contrast, no significant radical scavenging activity or suppression of ROS formation was observed, and neutrophil viability was unaffected. Besides ubiquitously occurring ingredients, coleone P, cinncassiol A and C, and callistric acid were identified as constituents in the most active fraction.

Conclusions: Together, potent inhibition of 5-LO activity, without concomitant anti-oxidant activity and cytotoxic effects, rationalizes the ethnopharmacological use of *Plectranthus zeylanicus* as anti-inflammatory remedy. Modern chromatographic/mass spectrometric analysis reveals discrete chemical structures of relevant constituents.

1. Introduction

Plants and their products have been systematically used in Sri Lanka for treating illnesses for over thousand years. Even though modern health care facilities are readily available in most part of the country, many people still rely on indigenous medicines for certain illnesses such as common cold, body aches, minor fractures, etc. Among the native flora of Sri Lanka, more than 1400 plants are used in indigenous medicine (Wijesundera, 2004).

Plectranthus zeylanicus Benth (synonym *Coleus zeylanicus* (Benth) Cramer), locally known as Iruveriya, is a perennial herb of the family Lamiaceae, which is extensively used in traditional medicine (Dassanayake and Fosberg, 1981). Although it is claimed to be an endemic species to Sri Lanka, it can be easily grown in prevailing climatic and soil conditions in the country. The plant is also introduced to South India where it is widely cultivated as a pot herb in home gardens (Sivarajan and Balachandran, 1986). The plant has aromatic, astringent and stomachic properties and is used in folk medicine in decoctions for fevers, dysentery, diarrhea, vomiting and thirst. It acts as a cholagogue but has been also used also as a diuretic and diaphoretic and as an antidote for tarantula bites (Jayaweera, 1982). According to the pharmacopoeia, *Plectranthus zeylanicus* is used as a constituent of various ayurvedic and traditional medicinal preparations (as powders, called Kalkaya, and as oils, called Prameha

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http://dx.doi.org/10.1016/j.jep.2013.11.004

or Kvathaya) and the plant is described to be effective in combating various conditions like asthma, common cold, varieties of fever, cough, leucoderma, diarrhea, chronic liver diseases, chronic ulcers, burning sensation of head, dental diseases, eye diseases and others (Ayurveda Pharmacopeia, 1979).

Despite the wide array of usages in traditional and folk medicine, the bioactivities of *Plectranthus zeylanicus* are hardly explored in order to understand and rationalize the reported ethnopharmacological use of the plant. The in vitro microbicidal activity of essential oils of *Plectranthus zeylanicus* was studied using several bacteria and fungi revealing an inhibitory activity against *Proteus vulgaris, Aspergillus parasiticus, Aspergillus niger, Rhizopus oryzae and Colletotrichum musae* (Deena et al., 2002). The aqueous extract of the plant demonstrated a high inhibitory activity of the terminal route of the complement cascade, thus suggesting the possible use of the extract and/or its active principle(s) in the therapy of septic shock (Beukelman et al., 1994). Extracts of the stem and leaves exhibit antioxidant activities (Rasineni et al., 2008) while the essential oil of the plant showed insecticidal activity against the stored grain pest *Callosobruchus maculatus*, suggesting the plant as an alternative pest control agent with low toxicity to warm blooded mammals (Balachandra et al., 2012).

Although diterpenoids, essential oils, phenolics, and few triterpenoids were isolated from different species of the genus *Plectranthus*, the phytochemistry of this genus is far from being established. In the case of *Plectranthus zeylanicus*, the ethanolic extract afforded abietane-type diterpenoides characterized as 7β-acetoxy-6β-hyroxyroyleanone, 7β-, 6β-dihyroxyroyleanone, and 7α-acetoxy-6β-hydroxyroyleanone (Mehrotra et al., 1989). Geraniol, geranyl acetate, caryophyllene, eudesm-7(11)-en-4-ol, p-cymene, fenchyl acetate, fenchyl formate, and bornyl acetate were identified in the essential oils of aerial parts and roots of *Plectranthus zeylanicus* grown in Sri Lanka (Arambewela and Wijesinghe, 2006), while α-terpeneol was identified as the major component of the essential oil of an Indian variety (Arambewela and Wijesinghe, 2006). Furthermore, peaks corresponding to caffeic acid and coumaric acid were identified by the RP-HPLC-UV spectral analysis of a water/methanol extract of the leaves (Rasineni et al., 2008). However, the current knowledge about the phytochemistry and in particular about the bioactive metabolites in *Plectranthus zeylanicus* is insufficient to rationalize its use in traditional medicine, thus the present study was undertaken to address this aspect.

The leukotrienes (LTs) are crucial mediators of inflammatory and allergic reactions involved in the pathophysiology of for example asthma, allergic rhinitis, atherosclerosis, and cancer (Werz and Steinhilber, 2006). 5-Lipoxygenase (5-LO) catalyzes the first two key steps in LT biosynthesis from arachidonic acid and is considered as attractive drug target (Radmark et al., 2007; Pergola and Werz, 2010). In fact, several natural products from plants used as anti-inflammatory remedies were shown to suppress the formation of LTs, most of them by inhibiting 5-LO activity (Werz, 2007). Mechanistically, many natural products of plant origin (flavonoids, polyphenols, coumarins) interfere with 5-LO activity due anti-oxidant activities, that is, by uncoupling of the redox cycle of the 5-LO active-site iron (Werz, 2007).

Isolation and identification of secondary metabolites, which involves tedious and time consuming purification steps, is the main bottle-neck in natural products chemistry. Therefore, development of new methodologies that facilitate rapid identification of secondary metabolites from natural product mixtures has become a crucial requirement. The advances made in separation technologies and mass spectrometric methods over the past few years have largely revolutionized and tremendously accelerated the compound identification process. Mass spectrometry (MS), coupled to HPLC or UPLC combined with MS/MS data bases have become indispensable tools in structural characterization of small molecules. The present investigation was carried out in order to evaluate 5-LO inhibition as anti-inflammatory mode of action of *Plectranthus zeylanicus* and novel MS methodologies were applied as means to identify related constituents.

2. Materials and methods

2.1. Plant material

Plectranthus zeylanicus plants were collected in Nittambuwa (Gampaha district—Western 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—III, M.D. Dassanayake & 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 (Plec-WP-1-1206) is deposited at the 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 and dried in shade (30 ± 2 °C) for six days. Dried plants were powdered using an electrical grinder (Singer, model KA-MIXEE). Thirteen gram of powdered material was successively extracted with 600 ml of *n*-hexane, dichloromethane (DCM), ethyl acetate, or methanol (Roth, Karlsruhe, Germany) at room temperature using a linear shaker for 20 min. In addition, 3.0 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 h at 60 °C. Though this solvent mixture may cause unstable or degraded products due to hydrolysis, it might extract most of the phenolic compounds potentially responsible for bioactivity. The extracts were evaporated into dryness with the use of rotary evaporator (BÜCHI, R-114, Germany). For bioactivity studies, extracts were freshly solubilized with DMSO (30 mg/ml), except the aqueous extract that was solubilized in water, further diluted by solvent, and immediately used for experiments.

2.3. Evaluation of bioactivity

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

Human neutrophils were freshly isolated from leukocyte concentrates obtained at the University Hospital Jena, Germany, as described (Pergola et al., 2008). In brief, peripheral blood was withdrawn from adult 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 $4000 \times g$ 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 phosphate-buffered saline (PBS) pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) (purity $>$ 96–97%).

For analysis of 5-LO product synthesis in whole blood as described by Pergola et al. (2008), freshly withdrawn blood from healthy adult donors was obtained by venipuncture and collected in monovettes containing 16 I.E. heparin/ml. Aliquots of 2 ml were pre-incubated with the test compounds or with vehicle (0.1%

DMSO) for 15 min at 37 °C, as indicated, and formation of 5-LO products was induced by addition of 1 μg/ml lipopolysaccharide (LPS) for 30 min at 37 °C and then 1 μM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was added. After 15 min at 37 °C, the reaction was stopped on ice and the samples were centrifuged (600 × *g*, 10 min, 4 °C). Aliquots of the resulting plasma (500 μl) were then mixed with 2 ml of methanol and 200 ng prostaglandin (PG)B_1 were added as internal standard. The samples were placed at −20 °C for 2 h and centrifuged again (600 × *g*, 15 min, 4 °C). The supernatants were collected and diluted with 2.5 ml PBS and 75 μl of 1 N HCl. Formed 5-LO metabolites were extracted and analyzed by HPLC as described for intact neutrophils below.

For analysis of 5-LO product synthesis in human neutrophils as described by Pergola et al. (2008), freshly isolated neutrophils were resuspended in 1 ml PGC buffer, preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO or water), and incubated for 10 min at 37 °C with the Ca^{2+}-ionophore A23187 (2.5 μM) plus 20 μM arachidonic acid (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 PGB_1 were 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-$LTsC_4$, 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 using semi-purified 5-LO

E.coli (BL21) was transformed with pT3-5-LO plasmid, and human 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 ethylen-diamine-tetracetic acid (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 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. DPPH assay

The radical scavenging 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 solubilized test compounds (0.9 μg ascorbic acid, 1.2 μg L-cysteine, or 2 and 10 μg of extracts) 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.

2.3.4. 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 5-(and 6-)chloromethyl-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).

2.3.5. Statistical analysis

Data are expressed as mean ± S.E. 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

Plectranthus zeylanicus *n*-hexane extract (230 mg) was subjected to silica gel column chromatography (Roth Kieselgel 60, 0.04–0.063 mm (230–400 mesh)). The sample was 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. Similarly, the DCM extract (120 mg) was subjected to silica gel column chromatography. The sample was successively eluted with the same solvent mixtures mentioned above which also yielded 11 fractions. Both fractionations were repeated, the respective fractions from the two separations were combined, evaporated and the combined dry weight was measured (Table 1) and then subjected to the bioassay and LC–MS analysis.

2.4.2. Liquid chromatography coupled mass spectrometric analysis

n-Hexane and DCM extracts as well as the fractions obtained thereof were analysed on a LTQ-Orbitrap XL instrument (Thermo Fisher, San Jose, CA) with electrospray ionization. The samples were dissolved in ethyl acetate (LC–MS grade, Sigma-Aldrich, St. Louis, MO) at 1 mg/ml and diluted to 10 μg/ml. 15 μl aliquot

Table 1
Bioassay-guided separation of DCM and *n*-hexane extracts and 5-LO inhibitory activity of the fractions 1-11. Fractions F-1 to F-11 were obtained after chromatographic separations (see Methods) and the total amounts from two separate fractionations are given in mg. Isolated 5-LO was preincubated with the dissolved fractions (in DMSO) at 10 or 1 μg/ml and 5-LO activity was determined. Data are given as mean, $n=3$. n.d.=not determined.

Fraction (no.)	Total amount of *n*-hexane extract (mg)	5-LO residual activity (in %) *n*-hexane		Total amount of DCM extract (mg)	5-LO residual activity (in %) DCM	
		@ 10 μg/ml	@ 1 μg/ml		@ 10 μg/ml	@ 1 μg/ml
F-1	200	n.d.	n.d.	>1	n.d.	n.d.
F-2	1	n.d.	n.d.	48	n.d.	n.d.
F-3	1	n.d.	n.d.	>1	n.d.	n.d.
F-4	17	n.d.	n.d.	>1	n.d.	n.d.
F-5	16	n.d.	n.d.	6	n.d.	n.d.
F-6	46	53.0	n.d.	2	28.2	85.7
F-7	24	27.1	76.1	23	32.7	81.3
F-8	20	45.7	n.d.	7	n.d.	n.d.
F-9	11	**19.2**	**44.7**	5	20.2	57.5
F-10	5	**26.1**	**46.5**	7	30.2	79.2
F-11	3	46	n.d.	20	25.5	75.3

of the diluted samples were injected and separated by UPLC using a Dionex–Acclaim® RSLC 120C18 column (2.1 × 150 mm packed with 2.2 μm particles, 120 Å). Reversed phase UPLC gradient separations were performed using channel A: water (LC–MS grade, Sigma-Aldrich, St. Louis, MO, USA) with 0.1% formic acid (LC–MS, Sigma-Aldrich, St. Louis, MO) and channel D: methanol (LC–MS grade, Sigma-Aldrich, St. Louis, MO) with 0.1% formic acid as mobile phases. The gradient program was set as 0 min—100% A, 5 min—100% A, 48 min—100% D, 60 min—100% D, 60.1 min—100% A, 65.1 min—100% A and the flow rate was optimized to 0.3 ml/min.

In the Electro Spray Ionization (ESI) source, the heated capillary temperature was 275 °C and the capillary voltage and tube lens voltage were set to 48.00 V and 95.00 V respectively. 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 formulas 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 analysis

The fractions of interest (1 mg/ml in ethyl acetate) were analyzed on a gas chromatograph HP6890 (Agilent, CA) connected to a MS02 mass spectrometer from Micromass (Waters, Manchester, UK) with EI 70 eV using ZB5ms column (30 m × 0.25 mm, 0.25 μm film thickness; Phenomenex, CA). The carrier gas was helium at the 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). The available standards [stigmasterol (Fluka), β-sitosterol (Aldrich) and phytol (Aldrich)] were also analysed under the same GC–MS conditions.

3. Results

3.1. Evaluation of 5-LO inhibitory activities

To investigate suppression of 5-LO activity in the cell, A23187-stimulated human neutrophils (Werz and Steinhilber, 2005) were used. In the course of a screening approach of extracts of diverse native plants from Sri Lanka used as anti-inflammatory remedies to interfere with 5-LO activity, we identified lipophilic extracts of *Plectranthus zeylanicus* as potential hits. Thus, *Plectranthus 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, that is, water or methanol were comparably ineffective and reduced 5-LO activity only by < 25% at a concentration of 100 μg/ml (Fig. 1A). More detailed concentration-response studies using this cell-based assay revealed IC_{50} values of 6.6 and 12 μg/ml for *Plectranthus zeylanicus* extracts prepared with *n*-hexane and DCM, respectively (Fig. 1B). The synthetic reference 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 (not shown).

Suppression of 5-LO product synthesis in the cell may be caused by diverse mechanisms, others than interference with the 5-LO enzyme activity, including inhibiting of substrate supply,

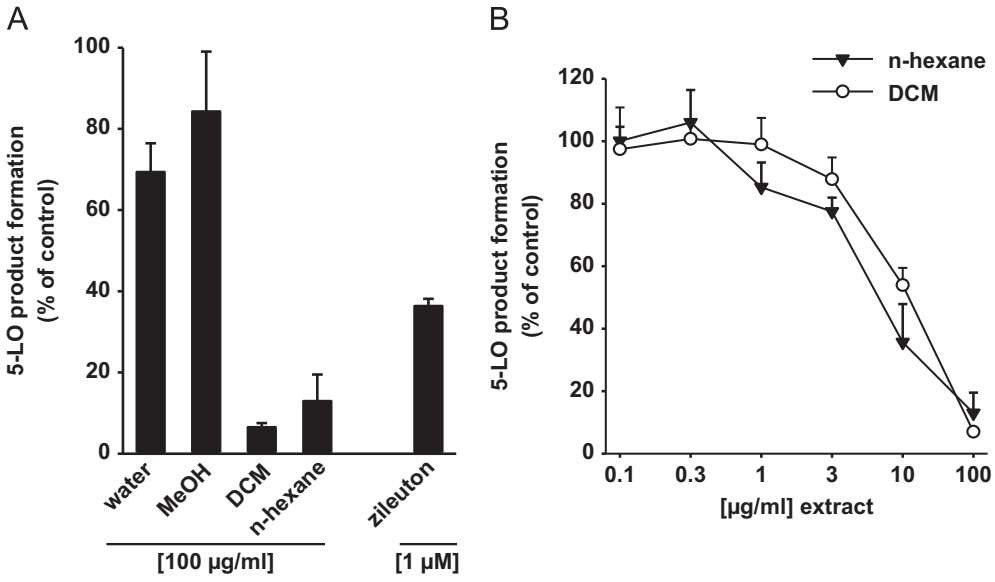

Fig. 1. Inhibition of 5-LO activity in intact human neutrophils. (A) Comparison of the 5-LO inhibitory activity of *Plectranthus zeylanicus* extracts (100 μg/ml, each) prepared by the use of water, methanol (MeOH), dichloromethane (DCM) or *n*-hexane as solvent; zileuton (1 μM) was used as reference compound. (B) Concentration-response curves for DCM and *n*-hexane extracts. Freshly isolated human neutrophils (5×10^6/ml) PGC buffer were preincubated with the indicated concentrations of *Plectranthus zeylanicus* extracts or vehicle (0.1% DMSO) for 15 min at 37 °C. 5-LO product formation was induced by addition of 2.5 μM A23187 plus 20 μM AA, and after 10 min, the reaction was stopped by addition of 1 ml methanol and 5-LO products were analyzed by HPLC. Data are expressed as percentage of vehicle control (100%), means ± S.E., $n=3$.

blockade of FLAP, or loss of cell viability. In order to investigate whether or not the extracts directly inhibit 5-LO activity, we applied a cell-free assay using isolated human recombinant 5-LO. As shown in Fig. 2, the *n*-hexane and DCM extracts of *Plectranthus zeylanicus* caused concentration-dependent inhibition of 5-LO with IC_{50} = 1.2 and 0.7 μg/ml, respectively. For zileuton, the IC_{50} value was determined at 0.11 μg/ml (not shown), implying comparably marked 5-LO inhibitory potencies of the *Plectranthus zeylanicus* extracts.

Finally, 5-LO inhibition by *Plectranthus zeylanicus* extracts was investigated in LPS/fMLP-stimulated human whole blood, a robust and biological relevant test system that includes several variables (e.g., plasma protein binding) which may impede the efficiency and thus, allows for judging the pharmacological potential of a given 5-LO inhibitor. Both, the *n*-hexane and the DCM extract suppressed 5-LO product formation in human whole blood by approx. 40% at 100 μg/ml (Fig. 2B). Higher extract concentrations could not be tested due to solubility issues. For zileuton, an IC_{50} of

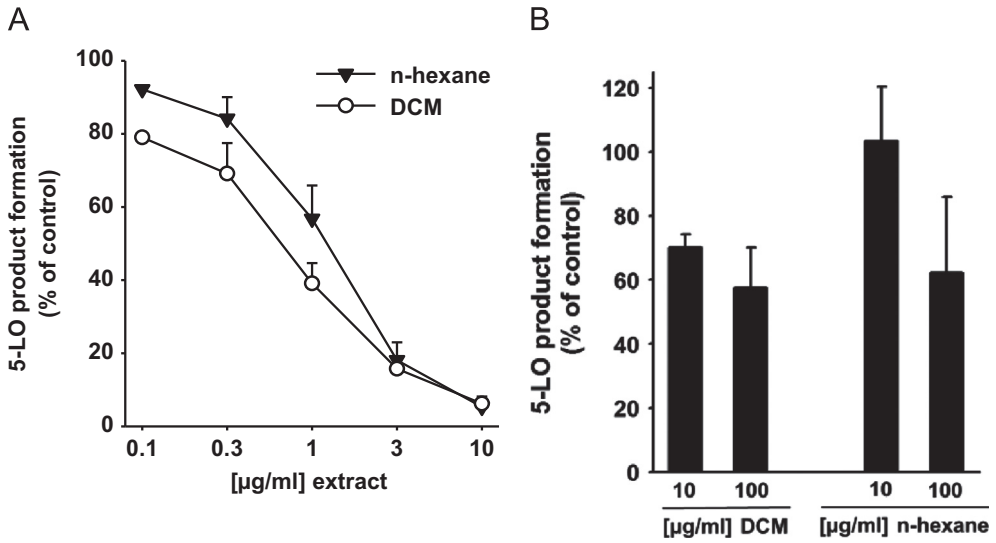

Fig. 2. Inhibition of isolated 5-LO in a cell-free assay and suppression of 5-LO activity in human whole blood. (A) Inhibition of 5-LO activity in a cell-free assay. Partially purified recombinant 5-LO (0.5 μg/ml) was incubated with dichloromethane (DCM) and *n*-hexane extracts of *Plectranthus zeylanicus* or vehicle (0.1% DMSO) at 4 °C for 15 min. Samples were prewarmed for 30 s at 37 °C, 2 mM $CaCl_2$ and 20 μM AA were added, and 5-LO product formation was determined after 10 min. Data are expressed as percentage of control (100%), means ± S.E., n=3. (B) Inhibition of 5-LO activity in whole blood. Freshly withdrawn human whole blood was preincubated with the test compounds or with vehicle (0.1% DMSO) for 15 min at 37 °C, as indicated, and 1 μg/ml LPS was added. After 30 min at 37 °C, 1 μM fMLP was added and after another 15 min the reaction was stopped and 5-LO product synthesis was analysed. Data are expressed as percentage of vehicle control (100%), means ± S.E., n=3.

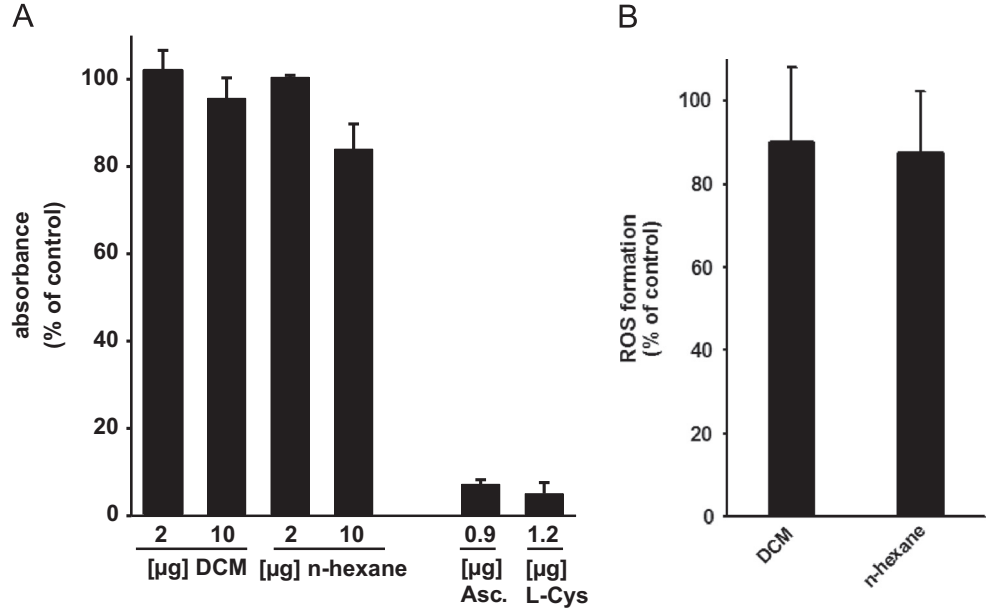

Fig. 3. Analysis of radical scavenging activities and cellular formation of reactive oxygen species. (A) Radical scavenging activities. *Plectranthus zeylanicus* extracts were incubated with 5 nmol DPPH for 30 min at RT and the absorbance was measured at 520 nm. Ascorbic acid and L-cysteine were used as controls. Values are given as percentage of control (100%) mean ± S.E., n=3. (B) Analysis of cellular ROS formation. Neutrophils were pre-incubated with *Plectranthus zeylanicus* extracts for 15 min, loaded with the fluorescent dye DCF-DA and stimulated with 0.1 μM PMA. The relative increase in fluorescence was determined after at 37 °C after 360 s. Data (mean ± S.E., n=3) are expressed as—fold increase (t=0 and t=360 s).

0.9 μM was obtained that agrees with literature data (Carter et al., 1991).

3.2. 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 (Werz, 2007). To investigate whether such unselective antioxidant properties may account also for 5-LO inhibition by *Plectranthus zeylanicus* extracts, we assessed radical scavenging properties using the cell-free DPPH assay. In contrast to the antioxidants ascorbic acid or L-cysteine (used a reference compounds) the *n*-hexane or DCM extracts of *Plectranthus zeylanicus* were not able to significantly reduce radical formation up to a final concentration of 50 μg/ml (corresponding to 10 μg/200 ml in the assay, Fig. 3A), suggesting that 5-LO inhibition is not mediated by a redox-based mechanism.

In addition, we studied the ability of the extracts to prevent cellular ROS formation in neutrophils stimulated with fMLP. Diphenyleneiodonium chloride (DPI, 10 μM) was used as reference inhibitor that completely abolished ROS formation (not shown), as

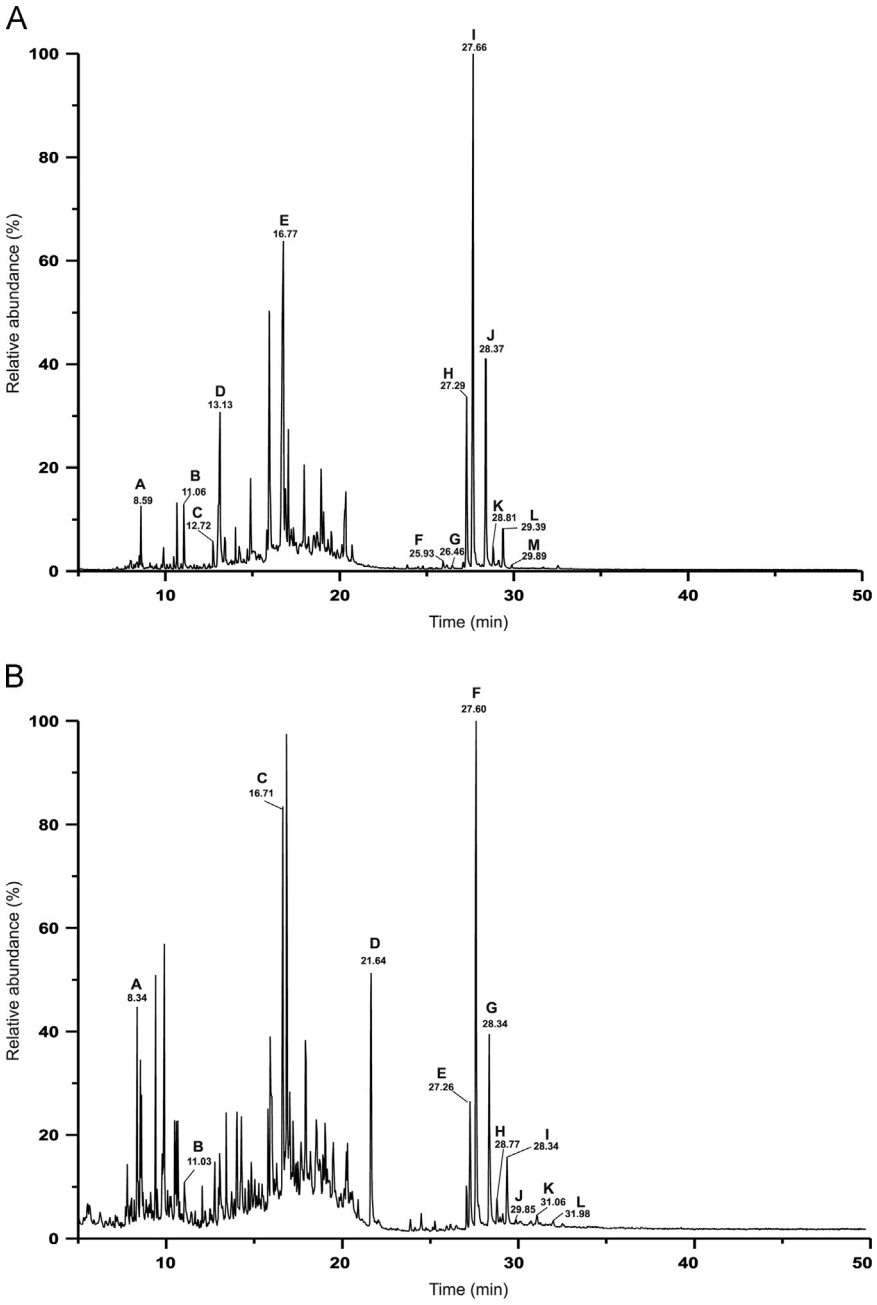

Fig. 4. Identification of constituents of bioactive fractions of the *n*-hexane extract of *Plectranthus zeylanicus* by GC–MS. (A) Total ion chromatograph of F-9 of the *n*-hexane extract of *Plectranthus zeylanicus* and its identified compounds. A: Eudesm-7(11)-en-4-ol, B: hexadecanoic acid, C: phytol, 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. (B) Total ion chromatograph of F-10 of the hexane extract of *Plectranthus zeylanicus* and its identified compounds. A: 4-[(1E)-3-hydroxy-1-butenyl]-3,5,5-trimethyl-2-cyclohexen-1-one, B: hexadecanoic acid, C: callitrisic acid, D: 13-docosenamide, E: campesterol, F: stigmasterol, G: β-sitosterol, H: β-amyrin, I: α-amyrin, J: stigmast-4-en-3-one, K: 4-methylstigmast-22-en-3-one, L: stigmastane-3,6-dione.

expected. In accordance with the DPPH assay, neither the *n*-hexane nor the DCM extract of *Plectranthus zeylanicus* caused significant inhibition of ROS formation (Fig. 3B).

3.3. Bioassay-guided separation of the Plectranthus zeylanicus extracts

We attempted to get more insights into the identity of the ingredients and composition of the extracts that might be responsible for the potent inhibition of 5-LO. Hence, we separated the *n*-hexane and DCM extracts by liquid column chromatography using ethyl acetate, *n*-hexane, and methanol as solvents into 11 fractions that were analyzed for inhibition of isolated 5-LO in the cell-free assay at 1 and 10 μg/ml, each. Out of the 11 fractions (F) of the *n*-hexane extract, F-6 (25% EtOAc in *n*-hexane), F-7 (35% EtOAc in *n*-hexane), F-8 (50% EtOAc in *n*-hexane), F-9 (75% EtOAc in *n*-hexane), F-10 (EtOAc) and F-11 (MeOH) 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) (Table 1). For the DCM extract, F-6 (25% EtOAc in *n*-hexane), F-7(35% EtOAc in *n*-hexane), F-9 (75% EtOAc in *n*-hexane), F-10 (EtOAc) and F-11 (MeOH) 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.

3.4. Characterization of constituents of bioactive fractions of the n-hexane extract of Plectranthus zeylanicus

An UPLC system coupled to LTQ Orbitrap XL instrument and GC–MS have been employed as sole techniques for structural characterization of secondary metabolites. The accurate mass measurements of adduct ions by the Orbitrap instrument enabled the determination of molecular composition within 1–5 ppm mass errors and database searching of exact masses for possible relevant secondary metabolites. MS/MS data has provided a powerful tool to de-replicate possible structures. To obtain better overview, the fragments obtained from the MS/MS experiments were further analyzed by computer-assisted algorithms to yield hypothetical fragmentation trees which allow the assignment of specific relevant fragments and fragmentation pathways.

3.4.1. GC–MS analysis

Due to the high 5-LO inhibitory activity of F-9 and F-10 from the *n*-hexane extract of *Plectranthus zeylanicus*, these fractions were subjected to a phytochemical screening by (I) GC–MS and (II) UPLC–MS. The GC–MS analysis of F-9 revealed 13 components, identified by comparison of their experimental mass spectrum with those recorded in the NIST MS Search 2.0 and Adams mass

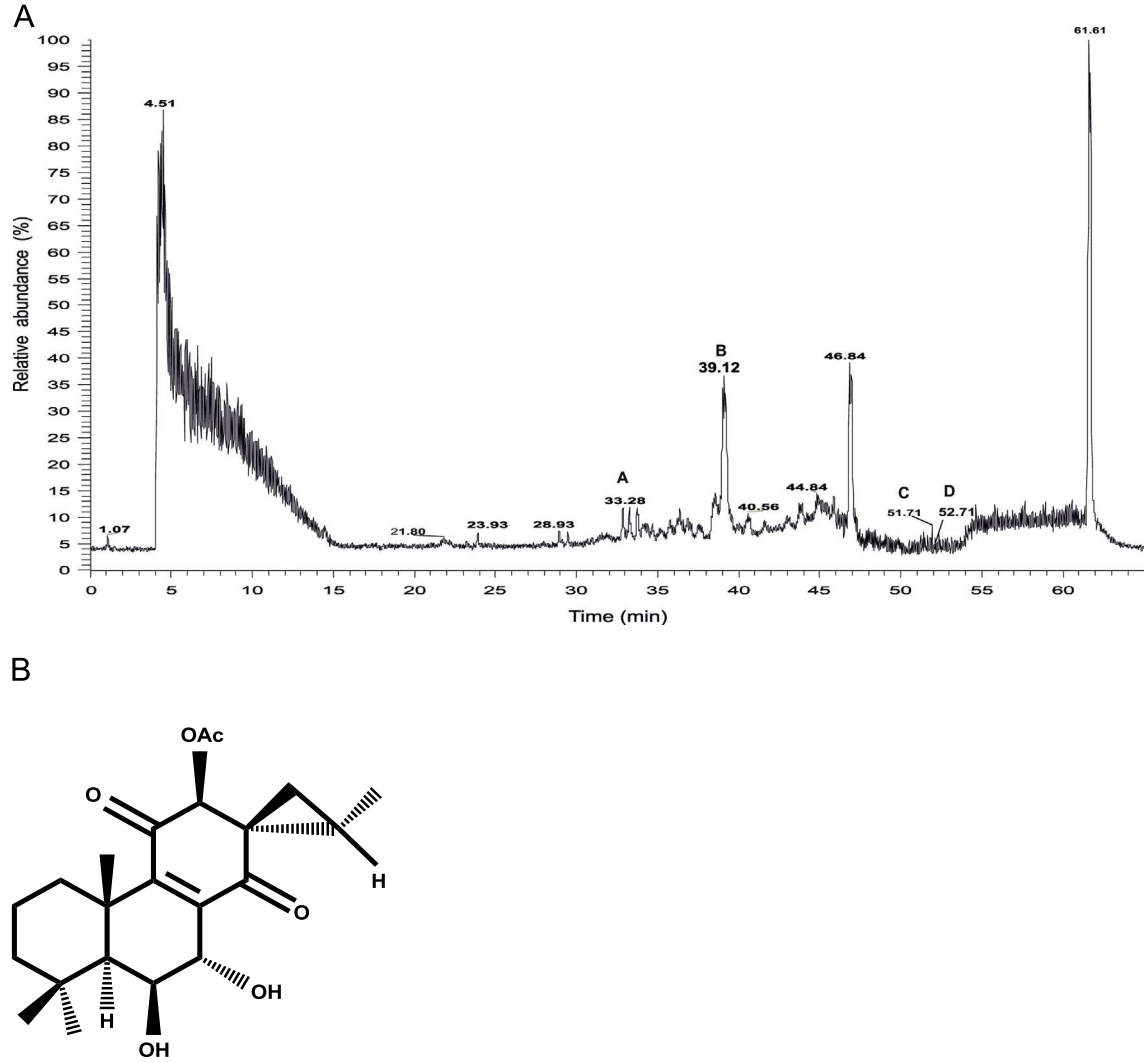

Fig. 5. Identification of constituents of bioactive fractions of the *n*-hexane extract of *Plectranthus zeylanicus* by UPLC–MS. (A) Total ion chromatogram of F-9 of the hexane extract of *Plectranthus zeylanicus* and its identified compounds. A: cinncassiol A/cinncassiol C3, B: coleone P, C: stigmasterol, D: stigmasterol-5,22,25-trien-3-β-ol. (B) Chemical structure of coleone P.

spectrum libraries as well as by comparison with the respective standards. Among these components, ubiquitously occurring fatty acids (hexadecanoic acid, 9,12,15-octadecatrienoic acid), phytol and eudesm-7(11)-en-4-ol, as well as several frequent phytosterols and derivatives (i.e., cholest-5-en-3β-ol, ergosta-5,22-dien-3β-ol, campesterol, stigmasterol, β-sitosterol, stigmast-4-en-3-one) were detected. However, also α- and β-amyrin and callitrisic acid, a quite uncommon diterpenoic phenanthrenecarboxylic acid, were present (Fig. 4A).

GC–MS analysis of F-10 revealed 12 compounds; many of them were the same as in F-9, including phytosterol derivatives, hexadecanoic acid, amyrins, and callitrisic acid (Fig. 4B). In addition, 4-[(1E)-3-hydroxy-1-butenyl]-3,5,5-trimethyl-2-cyclohexen-1-one and 13-docosenamide were detected in F-10.

3.4.2. UPLC–MS analysis

Analysis of F-9 by UPLC–MS revealed two uncommon compounds (denoted as compound A and B, Fig. 5A) and two common phytosterol derivatives that were detected in the TIC. In F-10, only compound B yielded a substantial peak reflecting significant amounts present in this fraction (not shown). In addition, a small peak corresponding to compound A was apparent 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 ($C_{22}H_{30}O_6$, Fig. 5B) and its sodium salt at *m*/*z* 391.21255 and 413.19431, respectively, in both F-9 and F-10. The identity of coleone P was further deduced by its fragmentation pattern, which agrees with literature data (Arihara et al., 1975). The collision-induced dissociation spectra of the protonated adduct at different collision energies are given in Fig. S1. The computed hypothetical fragmentation trees for both protonated and sodium adducts, which annotate fragment masses with molecular formulas and show the dependencies between the fragments through neutral losses, are given in Figs. S2 and S3.

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. The computed hypothetical fragmentation tree for the protonated adduct is given in Fig. S4. Unfortunately, the unavailability of authentic standards of compound A and B has hindered the UPLC–MS confirmation of the respective structures.

4. Discussion

Here we attempted to (I) rationalize and validate the traditional use of the medicinal plant *Plectranthus zeylanicus* as anti-inflammatory remedy by analysis of its ability to interfere with 5-LO activity and (II), to identify relevant constituents of the bioactive fractions. In fact, lipophilic extracts of *Plectranthus zeylanicus* using *n*-hexane or DCM as solvents caused direct and potent inhibition of human 5-LO and suppressed the biosynthesis 5-LO products in isolated human neutrophils and even in human whole blood. 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, we conclude that lipophilic *Plectranthus zeylanicus* 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. Provision of these so far unavailable compounds (i.e., coleone P) in large scale will allow for future comprehensive bioactivity studies and reveal whether or not they inhibit 5-LO.

Since the *Plectranthus zeylanicus* is widely used in traditional medicine in Sri Lanka and South India to alleviate the pathological conditions caused by inflammation (Ayurveda Pharmacopeia, 1979; Jayaweera, 1982), we focused on inhibition of 5-LO as potential underlying mode of action. In fact, 5-LO as key enzyme in the biosynthesis of the pro-inflammatory LTs (Radmark et al., 2007) is explored as drug target for the intervention with asthma, allergic rhinitis, various autoimmune diseases, cardiovascular disease, and many other inflammatory disorders (Peters-Golden and Henderson, 2007). Direct 5-LO inhibitors (of synthetic or natural origin) are essentially 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 fatty acid substrate and/or activating fatty acid hydroperoxides or undefined modes of action (Werz and Steinhilber, 2005; Pergola and Werz, 2010). Starting from early 1980s until today, several hundred plants and their extracts and/or specific secondary metabolites thereof have been reported that are capable of suppressing the biosynthesis of 5-LO products (for review see: Schneider and Bucar, 2005). Such 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. 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. Thus, lipophilic extracts of well-recognized anti-inflammatory medicinal plants such as *Tripterygium wilfordii*, *Urtica dioica*, *Zingiber officinale* or *Harpagophytum procumbens* blocked cellular 5-LO activity with relatively high IC_{50} values of approx. 20–80 μg/ml (Schneider and Bucar, 2005). Also, the reviewed literature (Schneider and Bucar, 2005) reveals 5-LO inhibitory activity of lipophilic extracts of various other medicinal plants with IC_{50} values in the rough average range of 15–50 μg/ml. For example, 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 (Ammon, 2006), IC_{50} values for 5-LO in neutrophils of 8.4–30 μg/ml were determined (Ammon et al., 1993; Wildfeuer et al., 1998). In direct comparison to these potencies, the results obtained with the *n*-hexane and DCM extracts of *Plectranthus zeylanicus* with IC_{50} of 0.7–12 μg/ml are remarkable and suggest a high pharmacological potential of *Plectranthus zeylanicus* for intervention with 5-LO-related disorders.

The active constituents of various plant preparations responsible for 5-LO inhibition were often proposed to be flavonoids, polyphenols, coumarins, and hydroquinones that all possess antioxidant activities, and this was correlated to enzyme inhibition (Werz, 2007). In contrast, for the 5-LO inhibitory *Plectranthus zeylanicus* extracts, we could not observe significant antioxidant properties, suggesting that the respective bioactive constituents mediate 5-LO inhibition by specific interference in a nonredox-dependent manner. Note that the potencies of the *Plectranthus zeylanicus* extracts were more pronounced (about 8-fold) when inhibition of isolated 5-LO was studied as compared to cellular 5-LO suppression. Possibly, the active constituents fail to reach equivalent concentrations inside the cell (poor permeation) or cellular factors may impair 5-LO inhibition by competition, unspecific protein binding or degradation of the constituents.

MS in combination with the hyphenated chromatographic techniques is emerging as a powerful tool in small molecule identification. The resolving power of the chromatography has tremendously enhanced by the introduction of UPLC (Eugster et al., 2011), while the Orbitrap mass accuracy permits a straightforward determination of the molecular composition of parent and

fragment ions (Perry et al., 2008). Although in its infancy, computational methods for the automated analysis of tandem MS data are now developing as potential utensils in characterization of small molecules. Moreover, the GC–MS analysis followed by the comparison of a measured spectrum to reference spectra in databases has been well established as an effective identification method of volatile natural products since many years.

These outstanding developments in the areas of separation methods and spectrometric techniques afford a rapid identification and characterization of secondary metabolites without the necessity of isolation and purification, while the detailed information about their metabolic profiles can be obtained with a minimal amount of material. Utilizing efficient chromatographic and MS techniques, we have performed an initial study on the putative bioactive constituents in a restricted fraction of the *n*-hexane extract of *Plectranthus zeylanicus* after chromatographic separation. We have used tandem mass spectrometry and the accurate mass measurements data to tentatively identify the compound in the active fractions. Our approach is novel in the fact that allows for fast data de-replication and will give the researchers a hint on the class of compounds. The amounts of compounds we are able to assay using MS/MS experiments is typically much smaller and it can be successfully applied to mixtures. For NMR measurements more sample will be needed, therefore large scale extraction and purification procedures are required, thus the pure compound isolation was beyond the scope of this initial phytochemical study.

None of the studies conducted so far on this plant has correlated the occurrence of a specific secondary metabolite to bioactivity, apart from a study which assigned caffeic acid and coumaric acid to antioxidant activity of the plant extract (Rasineni et al., 2008). Therefore, our study provides new insights towards phytochemicals of *Plectranthus zeylanicus*, paving the way for more detailed analysis in future. Among the identified compounds in the most active fractions, some have been proposed to possess in vitro and in vivo anti-inflammatory properties (Kweifio-Okai and Macrides, 1992; Prieto-Garcia et al., 2006).

Among the identified constituent in the active fractions of the *n*-hexane extract of *Plectranthus zeylanicus*, coleone P appears to be of particular interest. The genus *Plectranthus* is rich in coleone-type diterpenoids which exert several biological activities. For example, coleone U and C showed strong antiproliferative activities against human cancer cell lines, respectively (Marques et al., 2002; Xing et al., 2008), and for coleone U, antimicrobial activities were observed (Wellsow et al., 2006). Coleone P has been isolated from the *Plectranthus* species *Plectranthus caninus*, and our spectral analysis proposes the presence of this compound in the active fractions F-9 and F-10 of the *Plectranthus zeylanicus n*-hexane extract. However, the absence of authentic standards of this compound impedes the detailed studies on its anti-inflammatory properties. 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 (Kweifio-Okai and Macrides, 1992).

Although it is reasonable to correlate the 5-LO inhibitory activity of *Plectranthus zeylanicus* 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, we expect to expand our research towards the identification of these compounds in the active fractions in future studies. The recently introduced fragmentation trees for the automated comparison of fragmentation patterns of small molecules (Rasche et al., 2012) will be used for this purpose. The computed fragmentation trees (employed in this study) for compound identification has provided a good explanation of our observed data, and hence, could be a valuable tool for the identification of the unknown constituents. Once the identification is completed, we hope either to synthesize or conduct a large scale extraction (depending on the availability of plant material) to isolate such compounds in sufficient amounts in order to perform discrete analysis of their bioactivities, in particular with respect to inhibition of 5-LO.

5. Conclusion

By demonstrating potent inhibition of 5-LO activity by lipophilic extracts of *Plectranthus zeylanicus* in different biological test systems, our results may explain the traditional use of *Plectranthus zeylanicus* in Sri Lanka and South India for the treatment of inflammatory conditions. We conclude that even though 5-LO is a redox-sensitive oxygenase, the potent inhibition of 5-LO activity is not related to unselective antioxidant properties but rather due to more discrete and direct interference of the respective constituents with the enzyme. The phytochemical analysis of the bioactive fractions 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. Provision of these candidate compounds in large scale may afford to analyze in detail whether or not they interfere with 5-LO and thus, constitute active principles rationalizing the anti-inflammatory use of *Plectranthus zeylanicus* in folk medicine.

Acknowledgements

M.N is supported by the International Max Planck Research School, Jena. We thank Dr. Marco Kai (MPI, Jena) for his assistance in LC–MS measurements.

Appendix A. Supplementrary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.11.004.

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