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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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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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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%