Fitoterapia 81 (2010) 289–295

Contents lists available at ScienceDirect

Fitoterapia

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

Ocotea quixos Lam. essential oil: In vitro and in vivo investigation on its anti-inflammatory properties

Vigilio Ballabeni [a], Massimiliano Tognolini [a], Carmine Giorgio [a], Simona Bertoni [a], Renato Bruni [b], Elisabetta Barocelli [a,*]

[a] Department of Pharmacological, Biological and Chemical Applied Sciences, University of Parma, Via GP Usberti 27/a, 43100 Parma, Italy
[b] Department of Evolutive and Functional Biology, Sec. Vegetal Biology and Botanic Garden, University of Parma, Via GP Usberti 11/a, 43100 Parma, Italy

ARTICLE INFO

Article history:
Received 31 July 2009
Accepted in revised form 16 September 2009
Available online 13 October 2009

Keywords:
Ocotea quixos
Trans-cinnamaldehyde
Inflammation
NO production

ABSTRACT

Here we investigated the anti-inflammatory properties of *Ocotea quixos* essential oil and of its main components, trans-cinnamaldehyde and methyl cinnamate, in in vitro and in vivo models. Ocotea essential oil and trans-cinnamaldehyde but not methyl cinnamate significantly reduced LPS-induced NO release from J774 macrophages at non-toxic concentrations, inhibited LPS-induced COX-2 expression and increased forskolin-induced cAMP production. The essential oil (30–100 mg/kg os) and trans-cinnamaldehyde (10 mg/kg os) in carrageenan-induced rat paw edema showed anti-inflammatory effect without damaging gastric mucosa. In conclusion we provide the first evidence of a significant anti-inflammatory gastro-sparing activity of *O. quixos* essential oil.

1. Introduction

In the context of the development of novel therapeutics from natural sources we focused our attention on the pharmacological profiling of some essential oils used as herbal remedy in the traditional medicine [1–4]. The chemical characterization of the essential oils performed in these works allowed us to identify the active principles responsible for the pharmacological properties of the overall phytocomplex studied. Among the botanicals examined particular attention was addressed to the essential oil extracted from wild *Ocotea quixos* (Lam.) Kosterm. (Lauraceae) calices whose main components were trans-cinnamaldehyde (27.9%) and methyl cinnamate (21.6%) [5,6]. In a previous investigation the antithrombotic activity evoked in mice by subacute treatment with *O. quixos* essential oil was related to its ability to inhibit platelet aggregation, clot retraction and vasoconstriction [6]. It was speculated that trans-cinnamaldehyde could be the primary oil's constituent responsible for these effects since it shared a similar activity profile. Actually a number of different biological effects are attributed to trans-cinnamaldehyde from antifungal to antimicrobic and anti-inflammatory activities and they are related to its antioxidant properties and its ability to inhibit NF-κB transcriptional activity [7,8]. Through a number of in vitro studies it has been demonstrated that cinnamaldehyde is able to suppress iNOS expression and NO production in LPS-stimulated RAW264.7 cells, IL-1 induced cyclooxygenase 2 activity and PGE_2 production from rat microvascular endothelial cells and ROS release as well as pro-inflammatory cytokines expression in cultured LPS-stimulated monocytes/macrophages [8–11]. All these activities strongly suggest that this agent might possess immuno-modulating properties and lead us to investigate the anti-inflammatory potential of *O. quixos* essential oil containing trans-cinnamaldehyde as one of its principal constituents.

Accordingly, in the current work we assess both the in vivo and in vitro anti-inflammatory properties of *O. quixos* oil and of its main components, trans-cinnamaldehyde and methyl cinnamate, evaluating also their gastric tolerability.

* Corresponding author. Dipartimento di Scienze Farmacologiche, Biologiche e Chimiche Applicate, Università di Parma, Via Usberti 27/a, 43100 Parma, Italy. Tel.: +39 0521 905093; fax: +39 0521 905091.
E-mail address: elisabetta.barocelli@unipr.it (E. Barocelli).

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doi:10.1016/j.fitote.2009.10.002

2. Experimental

2.1. Chemicals and drugs

Thiazolyl blue tetrazolium bromide (MTT), isopropanol, ethanol, trans-cinnamaldehyde, methyl cinnamate, dexamethasone, lipopolysaccharide (LPS, Escherichia Coli serotype 0111: B4), methocel, 3-isobutyl-l-methylxanthine (IBMX), forskolin, dimethylsulfoxide (DMSO), Griess reagents and carrageenan were obtained from Sigma (St. Louis, USA), indomethacin megluminate from Chiesi Farmaceutici (Parma, Italy), goat anti-mouse COX-2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and β-mercaptoethanol and chlorophenol red β-D-galactopyranoside from Roche Molecular Biochemicals, (Indianapolis, IN, USA). Murine macrophage cell line J774 and human neuroblastoma cell line SK-N-MC were from ATCC collection.

2.2. Animals

Male Wistar rats (250–300 g), purchased from Charles River, Italy, were housed with a 12:12 h of light:darkness and received food and water ad libitum. The animals, fasted 12 h before the experiment but with free access to water, were used applying experimental procedures supervised and approved by the "Ministero della Salute" (DL116/92).

2.3. Isolation and analysis of essential oil

O. quixos (Lam.) Kosterm. (Lauraceae) calices (vernacular Shuar and Achuar name: Ishpink) were collected by agronomists of Fundacion Chankuap' (Macas, Ecuador) in January 2002 from wild trees on the outskirts of the Wasak'entsa reserve in eastern Ecuador (77° 15″ W/2° 35″ S) and positively identified by the National Herbary of Pontificia Universidad Catolica del Ecuador (J. Jaramillo). The fresh plant material was dried on a ventilated dryer at room temperature. A voucher specimen of the herbal drug was deposited in the Dipartimento delle Risorse Naturali e Culturali, University of Ferrara, Italy with code ISH01. Essential oil was extracted by steam distillation of the entire dried flower calices with a commercial Clevenger apparatus. After 8 h of steam distillation, 3.74 g of essential oil were obtained from 200 g of crude drug (yield $1.9 \pm 0.25\%$). The essential oil content was determined on a volume to dry weight basis. Detailed composition and analysis of the essential oil was reported elsewhere [3,5,6].

2.4. In vitro assays

2.4.1. LPS-stimulated J774 macrophages

Nitric oxide was produced by incubating J774 cell culture with 10 μg/ml lipopolysaccharide (LPS) for 24 h. Nitrite accumulation in culture supernatant was measured using Griess' method as described previously [12]. Briefly, 50 μl of 1% sulphanilamide in 5% phosphoric acid, followed by 50 μl of 0.1% N-(1-naphtyl)-ethylene diamine in 5% phosphoric acid were added to 100 μl of culture medium. After 10 min of incubation at room temperature the absorbance was read at 550 nm with a microplate reader (Biorad microplate reader 550, Segrate, MI, Italy). Concentrations of nitrite were calculated from a standard curve constructed with sodium nitrite as reference compound. Ocotea oil, trans-cinnamaldehyde, methyl cinnamate or vehicle (DMSO, final concentration 0.5%) was incubated at different concentrations 1 h before LPS addition. Dexamethasone was used as reference drug. The inhibition of NO release was indicated as percentage of inhibition calculated considering the maximal release of NO from control LPS-stimulated macrophages.

2.4.2. Cell viability

Cell viability was determined through colorimetric quantification of formazan derived from thiazolyl blue tetrazolium bromide (MTT) metabolic reduction [13]. J774 cells, cultured in RPMI 1640 medium with addition of 10% fetal calf serum, were suspended at the final concentration of 10^5 cells/ml and plated in 96 well plates. The following day cells were incubated with the compounds under study or the vehicle for 24 h at proper concentrations. At the end of the incubation, 10 μl of 5 mg/ml MTT solution was added to each well and the plate returned in the incubator for 3 h. Afterwards, the culture medium was removed, the cells washed with phosphate buffer solution (PBS) and 200 μl of formazan solubilization solution (0.1 N HCl in anhydrous isopropanol) added. Culture medium absorbance was spectrophotometrically read at 570 nm (Biorad microplate reader 550, Segrate, MI, Italy). Cells viability was expressed as relative viability compared to control.

2.4.3. Western blot analysis

J774 macrophages, previously incubated with 10 μg/ml LPS for 24 h and with appropriate concentrations of vehicle or of the compounds under study 1 h before LPS addition, were lysed in RIPA buffer (Triton X-100 1%, deoxycholate 1%, SDS 0.1%, 158 mM NaCl, Tris 10 mM pH 7.2, NaEDTA 5 mM) in the presence of protease inhibitors. Insoluble materials were removed by centrifugation at 16,000*g* for 15 min at 4 °C. The resulting supernatants were boiled in sample buffer (2% SDS, 1% β-mercaptoethanol, 0.008% bromophenol blue, 80 mM Tris pH 6.8, 1 mM EDTA). The samples were then analyzed by SDS-PAGE and transferred to nitrocellulose membranes. After the transfer, the membranes were blocked in 5% milk, incubated for 1 h with primary antibody (R-α-M-α-COX-2, 1:1000), washed and incubated for 1 h with secondary antibody (α-R-HRP conjugated, 1:2000).

2.4.4. Forskolin-stimulated SK-N-MC cells

SK-N-MC cells were grown in 75 cm^2 culture flasks at 37 °C in a humidified atmosphere with 5% CO_2 in Eagle's minimal essential medium (EMEM), supplemented with 10% (v/v) fetal calf serum, 1% non-essential amino acids, 1% penicillin–streptomycin, 1% L-glutamine and 1% disodium–pyruvate in the presence of 500 μg/ml hygromicine. Cells were used for experiments when they reached about 70–80% confluence and transferred in 96-well plates before the assay. The cells expressed the reporter gene β-galactosidase under the control of multiple cAMP responsive element. To investigate the variation of cAMP levels, the compounds or the vehicle were added at proper concentrations to the cell media, followed 5 min later by addition of forskolin (1 μM) or saline (3 ml). After a 6 h incubation at 37 °C, media was aspirated and the cells were lysed with 25 μl of 0.1× assay buffer (composition mM: 10 NaH_2PO_4, 10 Na_2HPO_4, pH 8, 0.2 $MgSO_4$, 0.01 $MnCl_2$)