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

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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.

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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 antimicrobial and anti-inflammatory activities and they are related to its antioxidant properties and its ability to inhibit NF- κ B 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 PGE₂ 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.

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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-1-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 Herbarium 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 μ g/ml lipopolysaccharide (LPS) for 24 h. Nitrite accumulation in culture supernatant was measured using Griess' method as described previously [12]. Briefly, 50 μ l of 1% sulphanylamide in 5% phosphoric acid, followed by 50 μ l of 0.1% N-(1-naphthyl)-ethylene diamine in 5% phosphoric acid were added to 100 μ l 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 μ l 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 μ l 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 μ g/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 16,000g 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 cm² culture flasks at 37 °C in a humidified atmosphere with 5% CO₂ 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 μ g/ml hygromycin. 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 μ M) or saline (3 ml). After a 6 h incubation at 37 °C, media was aspirated and the cells were lysed with 25 μ l of 0.1 \times assay buffer (composition mM: 10 NaH₂PO₄, 10 Na₂HPO₄, pH 8, 0.2 MgSO₄, 0.01 MnCl₂)

and after 10 min with 100 μ l of 1 \times assay buffer (composition mM: 100 NaH₂PO₄, 100 Na₂HPO₄, pH 8, 2 MgSO₄, 0.1 MnCl₂) containing 0.5% Triton and 40 mM β -mercaptoethanol. Color was developed using 25 μ l of 1 mg/ml substrate solution of chlorophenol red β -D-galactopyranoside; and quantitated on a microplate reader at absorbance 550 nm after overnight incubation at 20 °C [14]. Data were expressed as % of the maximal response produced by IBMX 3 μ M.

2.5. In vivo assays

2.5.1. Rat paw edema

In vivo anti-inflammatory activity was studied in rat paw edema according to Winter's method [15]. A small volume of 1% carrageenan (0.1 ml) was injected into the plantar surface of the rat hind paw 30 min after oral administration of the test compounds or vehicle. Indomethacin (10 mg/kg), was used as reference drug. Paw volume was determined immediately after the injection of the phlogogen agent and again 3, 4, 5 h later by means of a plethysmometer (Basile Mod. 7150, Comerio, VA, Italy). The pharmacological activities of the compounds were expressed as the percentage of inhibition calculated from the difference between the responses of the treated and the control groups.

2.5.2. Gastrolesivity

The acute gastrolesivity of the test compounds was evaluated by examining the stomach excised 5 h after oral administration of the compounds (300 mg/kg) in rats. The stomach, fixed in 4% formalin, was opened and examined with a stereomicroscope by an observer unaware of the treatment the rats received [16]. Acute gastrolesivity was expressed as the number of animals with gastric damage over the number of treated animals.

2.5.3. Ethanol-induced gastric lesions

Gastroprotective effects of the essential oil or of its components were evaluated in rats orally treated with different doses of the test substances 1 h before the administration of the necrotizing agent ethanol (1 ml for rat 90%, v/v). 1 h later the stomach was removed, fixed in 4% formalin, opened along the great curvature and examined: the area of gastric lesions was measured for each stomach by means of a digital scanner and an image analyzer software (ImageJ 1.35, from NIH, USA) by an operator unaware of the treatment applied [17]. Results were expressed as mean damaged area (mm²) for stomach.

2.6. Statistical analysis

All the results were expressed as means \pm standard error of the mean (SEM) or with indication of confidence limits. Differences between groups were analyzed by unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post-test where indicated. **P*<0.05 and ***P*<0.01 were indicative of significant and highly significant difference, respectively.

3. Results and discussion

The essential oil of *O. quixos* flower calices is mainly composed of oxygenated monoterpenes and aldehydes (32.2%) and compounds involved in the biosynthetic phenylpropanoids pathway accounted for a global 54.4%. The most abundant chemicals, mainly responsible for the cinnamon-like taste of the spice, were trans-cinnamaldehyde and methyl cinnamate, which accounted for 27.9% and 21.6% respectively, as previously reported [6]. *O. quixos* essential oil, trans-cinnamaldehyde and methyl cinnamate were evaluated in vitro and in vivo for their anti-inflammatory properties.

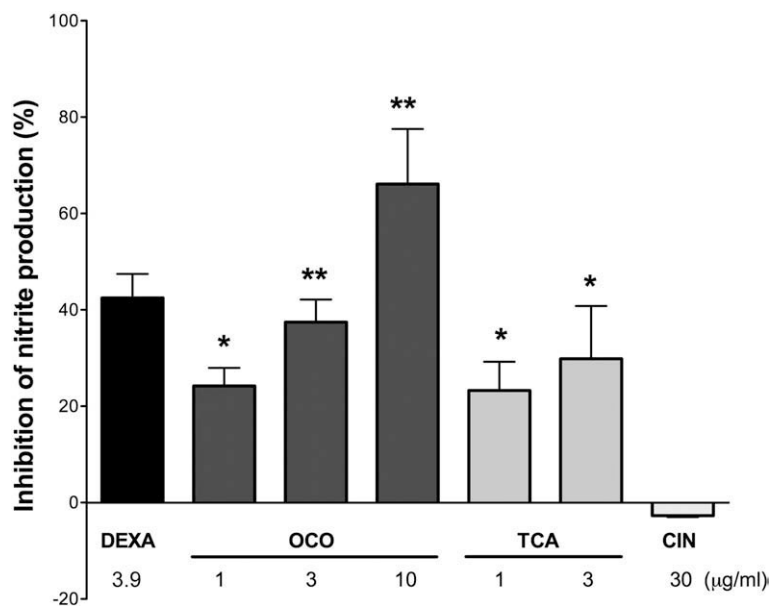


Fig. 1. Percent inhibition of nitrite production from murine J774 macrophages stimulated with 10 mg/ml LPS and treated with dexamethasone (Dexa), *Ocotea quixos* essential oil (OCO), trans-cinnamaldehyde (TCA) or methyl cinnamate (CIN). Nitrite concentrations were measured after 24 h incubation. Mean \pm S.E.M. from 3 independent experiments. ***P*<0.01, **P*<0.05, unpaired Student's *t*-test vs. control cells.

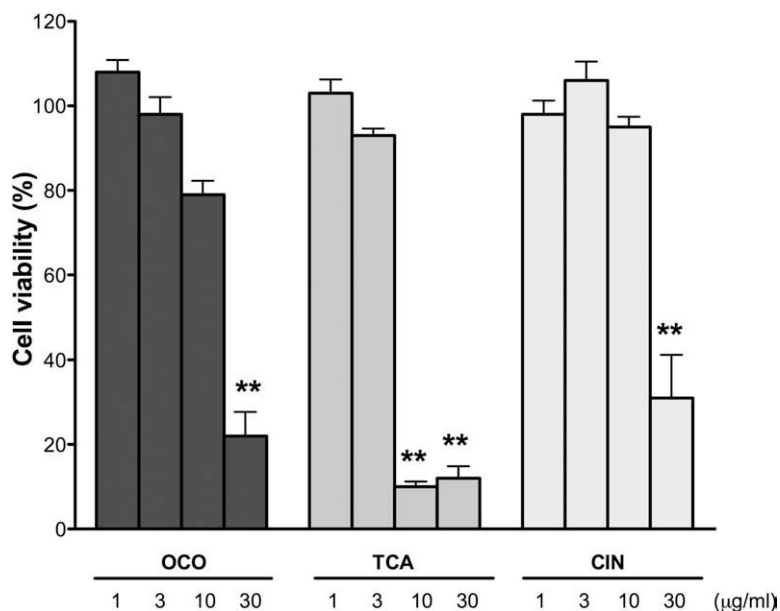


Fig. 2. Murine J774 macrophage viability after 24 h incubation with *Ocotea quixos* essential oil (OCO), trans-cinnamaldehyde (TCA) or methyl cinnamate (CIN) at concentrations ranging from 1 to 30 µg/ml. Data are expressed as percent viability compared to control in MTT test. ** $P < 0.01$, unpaired Student's *t*-test vs. control cells.

The inhibition of nitrite production can be considered an index of potential anti-inflammatory activity since tissue iNOS expression and nitrite levels increase during inflammatory processes. For this reason we tested in vitro the inhibitory effect of the compounds on macrophage LPS-induced NO production

and we measured the nitrite levels in the supernatant of cultured J774 cells incubated for 24 h with the compounds under study (Fig. 1). Like the classic glucocorticosteroid dexamethasone, chosen as positive control (3.9 µg/ml), both *O. quixos* essential oil and its main component trans-cinnamal-

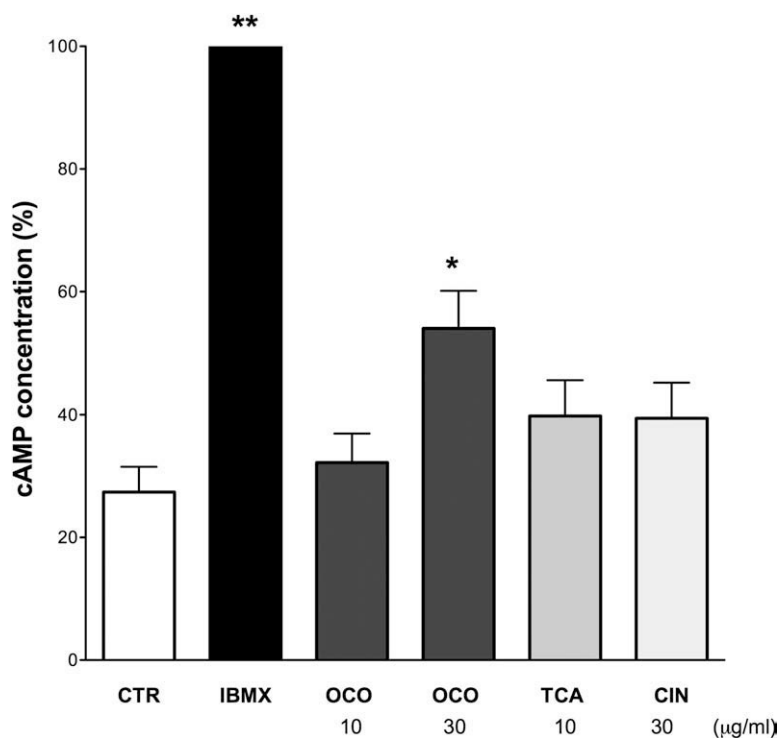


Fig. 3. cAMP concentrations in SK-N-MC cells incubated 6 h with forskolin 1 µM and *Ocotea* (OCO), trans-cinnamaldehyde (TCA), methyl cinnamate (CIN) or IBMX. Data are expressed as percentage assuming as 0% the absorbance of unstimulated cells and as 100% the absorbance of forskolin-stimulated cells treated with IBMX 3 µM. Control cells (CTR) are stimulated with forskolin 1 µM. Mean \pm S.E.M. from 3 independent experiments. ** $P < 0.01$, * $P < 0.05$, unpaired *t*-test vs. control cells.

dehyde dose-dependently and significantly inhibited NO release from J774 macrophages at non-toxic concentrations (1–10 $\mu\text{g/ml}$). At the highest concentration tested (10 $\mu\text{g/ml}$), *Ocotea* essential oil exhibited a stronger activity (66% inhibition of nitrite production) than that demonstrated by trans-cinnamaldehyde (30% inhibition) at the concentration of 3 $\mu\text{g/ml}$ which is comparable to that present in the natural phytocomplex. Methyl cinnamate, the second main component of the plant extract, failed to affect nitrite levels up to the highest non-toxic concentration of 10 $\mu\text{g/ml}$.

Data based on MTT assay confirmed the substantial absence of cytotoxic effect of *Ocotea* oil when incubated with J774 cells for 24 h up to 10 $\mu\text{g/ml}$ at variance with trans-cinnamaldehyde which exhibited, at the same concentration, toxic effects with only 10% of the cells surviving (Fig. 2).

Ocotea essential oil significantly increased the levels of cAMP in forskolin-stimulated SK-N-MC cells after 6 h incubation at variance with trans-cinnamaldehyde and methyl cinnamate when tested at the maximal tolerated concentrations of 10 and 30 $\mu\text{g/ml}$ (Fig. 3), respectively. In the same concentration range (10–30 $\mu\text{g/ml}$) the compounds did not modify significantly the cAMP content in unstimulated cells (data not shown). These findings suggest that the phytocomplex impairs cAMP degradation behaving as the phosphodiesterase inhibitor IBMX although with a lower efficacy and potency. Trans-cinnamaldehyde and methyl cinnamate do not seem to account for this inhibitory activity that could be rather attributable to the synergic effects of the multiple constituents composing the phytocomplex. Furthermore, LPS-induced increase of COX-2 expression in murine J774 macrophages was

dose-dependently (1–10 $\mu\text{g/ml}$) reduced by *Ocotea* essential oil when analyzed with SDS-PAGE (Fig. 4). This finding is in accordance with the inhibition exhibited by cinnamaldehyde on COX-2 expression induced by LPS in RAW 264.7 cells [18].

As concerns *in vivo* experiments, in rat paw edema test, *Ocotea* essential oil as well as trans-cinnamaldehyde significantly prevented carrageenan-induced swelling in a dose-dependent manner after oral administration (Fig. 5). The maximal inhibition (about 20% vs. vehicle-treated rats) was exhibited by *Ocotea* at 30 mg/kg and by trans-cinnamaldehyde at 10 mg/kg. On the other hand, methyl cinnamate exerted negligible anti-inflammatory effects when tested at 30 mg/kg, while the reference drug indomethacin, at 10 mg/kg, afforded approximately 60% significant protection against acute inflammation. It is worth noting that indomethacin at this dosage is gastrolesive in rats (8 ulcerated/8 treated rats) whereas *Ocotea* essential oil and trans-cinnamaldehyde did not damage rat gastric mucosa (0 ulcerated/8 treated rats) even if orally administered at a 30-fold higher dose (300 mg/kg) than the anti-inflammatory ones. It is noteworthy that even if administered at these high doses the compounds were well tolerated and no alterations in behaviour or body functions were detected. The overall phytocomplex (100 mg/kg) as well as trans-cinnamaldehyde (10 mg/kg), but not methyl cinnamate (30 mg/kg), dose-dependently prevented gastric mucosa erosions caused by oral administration of 90% ethanol in rats (Fig. 6). The maintenance of an adequate blood supply to gastric mucosa could account for this antiulcer activity. Indeed, it is worth mentioning that ethanol gastrolesive action is due to the vasocongestion characterized by vascular stasis of gastric

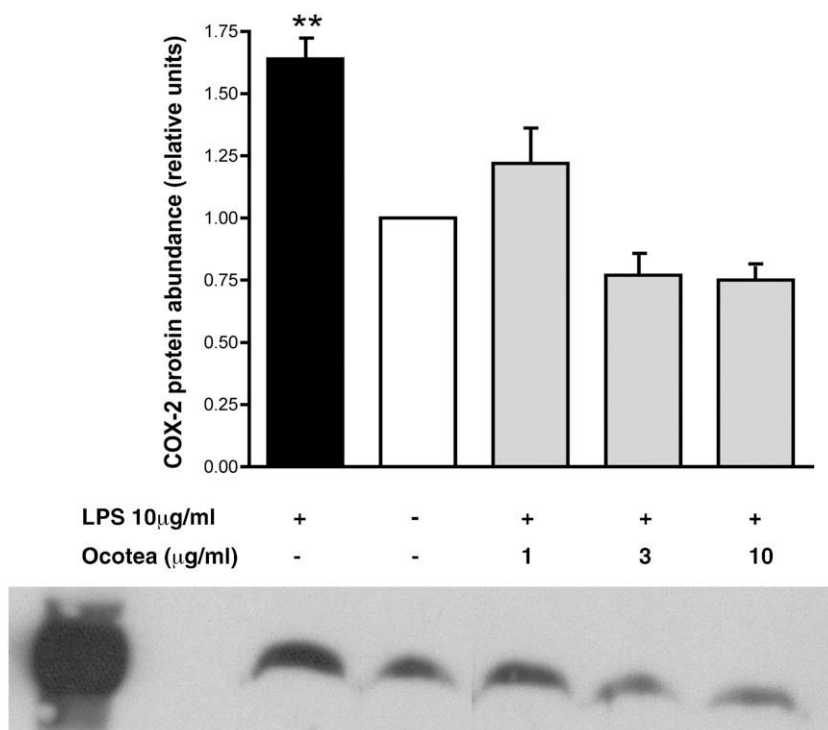


Fig. 4. COX-2 expression in J774 macrophages stimulated by 10 $\mu\text{g/ml}$ LPS for 24 h in the absence and in the presence of *Ocotea quixos* essential oil (1–10 $\mu\text{g/ml}$). Equal amounts of total proteins (20 mg/ml) were subjected to 10% SDS-PAGE and blotted with specific antibodies as specified in Section 2. Graph represents the mean \pm SEM of three different experiments; ** $p < 0.01$ unpaired Student's *t*-test vs. control cells. Lower panel depicts a representative Western blot.

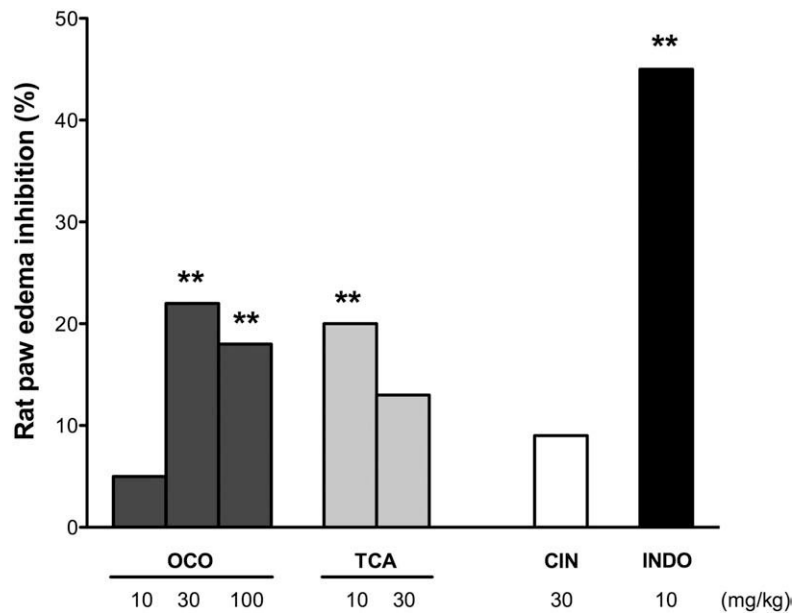


Fig. 5. Anti-inflammatory activity in carrageenan-induced rat paw edema. Groups of 8 rats were orally treated with *Ocotea quixos* essential oil (OCO), trans-cinnamaldehyde (TCA) or methyl cinnamate (CIN) 30 min before plantar subcutaneous injection of 0.1 ml carrageenan 1%. Indomethacin (INDO) was used as reference drug. Inhibition of paw edema registered 5 h after phlogogen injection is reported. ** $P < 0.01$, * $P < 0.05$, unpaired Student's *t*-test vs. vehicle-treated rats.

microcirculation. Consequently both *Ocotea* essential oil and trans-cinnamaldehyde might protect gastric mucosa thanks to their antiplatelet and vasorelaxant activities already described [6]. It has been recently recognized that trans-cinnamaldehyde is an agonist of the TRPA1 excitatory ion channel expressed by a subpopulation of primary afferent somatosensory neurons that contain substance P and calcitonin gene-related peptide [19].

The ability of trans-cinnamaldehyde to activate this target and to produce nociceptive response and neurogenic inflammation has been documented in vivo after its topic/regional administration while in the present study the systemic application through the oral route was adopted. In the experimental model of rat paw inflammation caused by subcutaneous injection of carrageenan, used in this study, the systemic application of trans-cinnamal-

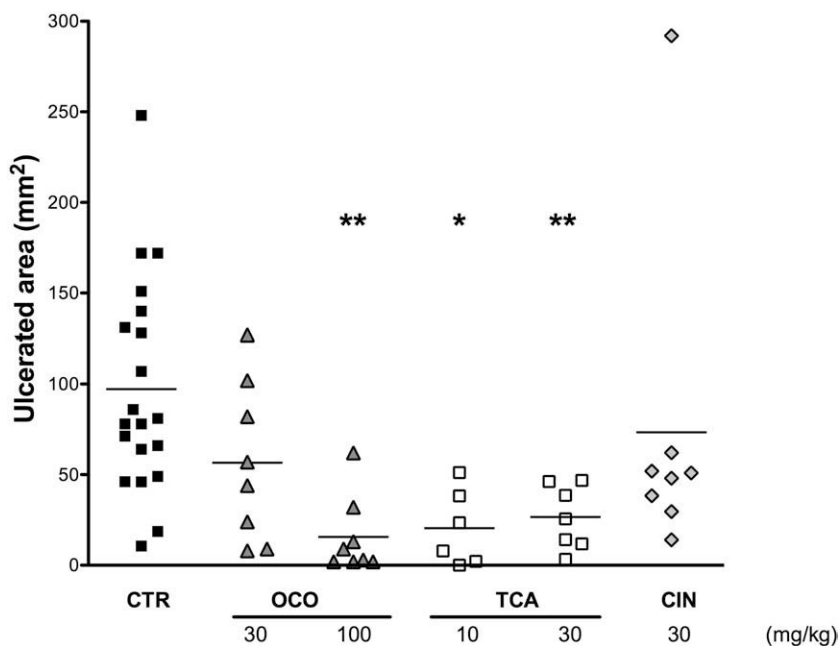


Fig. 6. Gastroprotective effect of *Ocotea quixos* essential oil (OCO) and its components trans-cinnamaldehyde (TCA) or methyl cinnamate (CIN) orally administered to rats 1 h before 1 ml 90% ethanol p.o. ** $P < 0.01$, one-way ANOVA followed by Tukey's comparison test.

dehyde provides significant anti-oedema activity while the consequences of the possible stimulation of TRPA1 ion channel seem to be apparently of secondary importance.

On the whole, the present findings provide the first evidence of a significant anti-inflammatory gastro-sparing activity of *O. quixos* essential oil. The pharmacological profile of *O. quixos* essential oil substantially appears to reflect and amplify the biological properties of its main component trans-cinnamaldehyde. This small molecule seems to possess an attractive and original activity being an anti-inflammatory agent endowed with gastroprotective and antiaggregating properties. The mechanisms underlying these responses could comprise, along with the inhibition of thromboxane A₂ receptors [6], the ability to interfere with the expression of inducible enzymes (iNOS, COX-2), as already reported in several experimental works [8,9,11,20], which have a primary role in the acute inflammatory response.

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