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Antiplatelet and antithrombotic activities of essential oil from wild *Ocotea quixos* (Lam.) Kosterm. (Lauraceae) calices from Amazonian Ecuador

Vigilio Ballabeni [a], Massimiliano Tognolini [a], Simona Bertoni [a], Renato Bruni [b], Alessandra Guerrini [b], Gabriela Moreno Rueda [c], Elisabetta Barocelli [a,*]

[a] *Dip. di Scienze Farmacologiche, Biologiche e Chimiche Applicate, Università di Parma, Viale G.P.Usberti 27/A, 43100 Parma, Italy*
[b] *Dip. di Biologia Evolutiva e Funzionale, Sez. Biologia Vegetale e Orto Botanico, Università degli Studi di Parma, Viale G.P.Usberti 11/A, 43100 Parma, Italy*
[c] *Fundacion Chankuap', Soasty y Domingo Comin, Macas, Ecuador*

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Abstract

Ocotea quixos essential oil was shown to possess significant inhibitory activity of platelet aggregation and clot retraction in rodent plasma. This study is aimed at fully characterizing the antiplatelet activity of the whole essential oil and its main components *trans*-cinnamaldehyde and methyl cinnamate also in human plasma, at investigating the mechanism underlying such activity and at evaluating the potential antithrombotic activity of subacute treatment of mice with *Ocotea* essential oil. *In vitro Ocotea* essential oil and *trans*-cinnamaldehyde inhibited arachidonic acid-, U46619-, ADP-, phorbol12-myristate13-alcetate-, collagen-induced platelet aggregation and thrombin-induced clot retraction in human and rodent plasma; *Ocotea* oil and *trans*-cinnamaldehyde competitively antagonized contractions induced by thromboxane A_2 receptor agonist U46619 in rat isolated aortic ring (K_B = 18 and 3.2 μg ml^{-1}, respectively). *In vivo Ocotea* oil, orally administered in a subacute treatment (30–100 mg kg^{-1} day^{-1} for 5 days) to mice, prevented acute thrombosis induced by collagen-epinephrine intravenous injection. This antithrombotic activity was not accompanied by pro-haemorragic side effect, as detected by the inactivity in bleeding test, thus showing a favourable safety profile compared to the conventional antiplatelet agent, acetylsalicylic acid. Present findings indicate that *Ocotea* essential oil possesses potent and safe antithrombotic activity attributable to its antiplatelet and vasorelaxant effects. The main constituent *trans*-cinnamaldehyde seems to be the primary responsible for this activity through a putative mechanism involving the inhibition of thromboxane A_2 receptors.

Keywords: *Ocotea quixos*; Antiplatelet activity; Experimental thrombosis; Bleeding; Thromboxane A_2 antagonism

1. Introduction

The genus *Ocotea* includes more than 350 tropical and subtropical aromatic shrubs or trees distributed within the Americas and in Southern Africa. Most of them are important timber trees subjected to be felled off because of the deforestation process of the rainforest habitat. They constitute a large source of essential oils with a predominant phenylpropanoid pathway and some of them, i.e. those from *O. pretiosa, O. sassafras, O. caudata* and *O. cymbarum,* have a renewed market position mainly in the perfume industry [1].

Ocotea quixos (Lam.) Kosterm. (Lauraceae) (=*Nectandra cinnamomoides* Nees., = *Laurus quixos* Lam.) is a small tree (5–20 m) with greenish floral buds, white flowers, dimorphic fruits and coriaceous leaves with reddish venations, bright glossy green on adaxial surface and yellowish on the abaxial. Once considered endemic of the rainforests of Ecuador, it has recently been collected also in southern Colombia and Peru [2,3]. It has been traditionally esteemed for its aromatic properties since the Incaic times and appreciated as appetizer, eupeptic, disinfectant and as local anaesthetic [4,5]. Its leaves are also used to make an anti-diarrheic infusion. Every two years the tree produces big, woody flower calices, locally called Ishpink (Quechua, Shuar, Cofan), Ocatuhue viqui (Huaorani) or Flor de Canela. Due to their cinnamon-like smell, Ishpingo calices are traditionally used by indigenous people of the Amazon as a cinnamon-substitute spice and to aromatize sweets and cakes: the essential oil obtained from the floral calices is, in fact, mainly composed of odoriferous phenylpropanoids like *trans*-cinnamaldehyde and methyl cinnamate [6]. Recent

* Corresponding author. Tel.: +39 0521 905093; fax: +39 0521 905091.
E-mail address: barocell@unipr.it (E. Barocelli).

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studies demonstrate that *Ocotea* essential oil possesses antioxidant and antimicrobic activities that are generally displayed by volatile fragrances secreted by several plants [6–8]. Furthermore, it has to be mentioned that phenylpropanoid rich essential oils have been reported as modulators of pain or inflammation [9–11] and as influencers of blood-clotting [12–14]. This last activity results particularly interesting in the light of the high incidence of thromboembolic diseases [15] and the limited tolerability of the main antiplatelet agents currently used [16]. Indeed, nowadays the recognition of herbal antithrombotic remedies, with documented efficacy and safety, is considered an important goal for the herbal and pharmaceutical industries.

Recently, in a screening survey on the antiplatelet activity of 23 different essential oils we provided evidence that *O. quixos* essential oil prevents *in vitro* platelet aggregation and clot retraction [17]. Thus, as a completion of that study, the present investigation mainly focuses on the full characterization of *in vitro* antiplatelet activity of *Ocotea* essential oil in rodent and human plasma. Vaso-relaxant properties in rat aortic ring preparation and cytotoxic effect in platelets are also investigated. Pharmacological properties of oil main constituents, *trans*-cinnamaldehyde and methyl cinnamate, are evaluated in order to assess their contribution to whole oil activities. Finally, *in vivo* antithrombotic and prohemorrhagic properties of subacute treatment with *O. quixos*. essential oil are assessed in mice.

2. Materials and methods

Phorbol 12-myristate 13-acetate (PMA), arachidonic acid (AA), adenosine diphosphate (ADP), thiazolyl blue tetrazolium bromide (MTT), isopropanol, sodium citrate, phenylephrine, calf collagen type III (for *in vivo* experiments), *trans*-cinnamaldehyde, methyl cinnamate, methocel, and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, USA). Collagen for *in vitro* experiments was from Menarini (Firenze, Italy). Acetylsalicylic acid (ASA) as lysine acetylsalicylate used for *in vivo* experiments was from Sanofi–Synthelabo. U46619 and SQ29548 were from Caymanchem (Ann Arbor, USA).

Male guinea pigs (300–350 g), male Wistar rats (250–300 g) or male Swiss mice (30–35 g), purchased from Charles River, Italy, were used applying experimental procedures supervised and approved by the Ethical Committee of the University of Parma and by "Ministero della Salute" (DL116/92).

2.1. Plant material

O. quixos (Lam.) Kosterm. (Lauraceae) calices were obtained from Fundacion Chankuap' (Macas, Ecuador) from three different stocks collected in January 2002 from wild trees on the outskirts of the Wasak'entsa reserve in eastern Ecuador (77°.15′ W/2°.35′ S). A voucher specimen of the spice was taxonomically determined and deposited in the Dipartimento delle Risorse Naturali e Culturali, University of Ferrara as ISH01.

2.2. Essential oil

Isolation and analysis of essential oil: the finely ground calices (500 g) were submitted for 8 h steam distillation according to European Pharmacopoeia methods and 3.74 g of essential oil were obtained from 200 g of crude drug (yield 1.9 ± 0.25%). The essential oil content was determined on a volume to dry weight basis. The oil was then dried over anhydrous sodium sulphate and stored at −20 °C.

2.3. NMR spectroscopy

The ^{13}C NMR spectra were recorded were recorded on a Varian Gemini-400 spectrometer operating at 100.58 MHz and 303 K. The essential oils (80 mg 0.8 ml^{-1}) were dissolved in deuterated chloroform (50 mg ml^{-1}) into a 5 mm NMR tube. Spectra were run using a Varian standard pulse sequence "s2pul". The time domain size was 17 K, spectral width 25000 Hz, Fid resolution 1.43 Hz. Chemical shifts (ppm) and peak attribution of ^{13}C NMR spectra were made according with those of literature [18,19] and pure standards (namely *trans*-cynnamaldehyde, benzaldehyde, limonene, α-pinene, linalool, α-terpineol, γ-terpinene, 1,8-cineole) and comparing their relative intensity according to previously determined abundance in the essential oil [6]. Multiplicities were obtained by 45, 90 and 135° DEPT experiments, while relative intensity was determined as relative percentage of NMR peak height. Supplemental NMR data were provided for fingerprinting purposes, while quantitative determinations were previously obtained via gas-chromatography [6].

2.4. In vitro assays

Human blood was obtained from volunteer donors after informed consent and anticoagulated with sodium citrate 3.8% (1 part citrate plus 9 part blood).

Animal blood, from male guinea pig or Wistar rat, was obtained by cardiac puncture after CO_2 euthanasia; it was collected in plastic tubes and anticoagulated with sodium citrate 3.8% (1 part citrate plus 9 part blood). Human or animal blood was centrifuged for 15 min at 180 × *g* to obtain platelet rich plasma (PRP) or 10 min 2000 × *g* to obtain platelet poor plasma (PPP).

2.4.1. Platelet aggregation

PRP from guinea pig was used to perform aggregation in the Aggrecorder PA 3220 (Menarini, Firenze) following Born's turbidimetric method [20]. Aggregation was recorded as the percent change in light transmission: the baseline value was set using PRP and maximal transmission using PPP. PRP was preincubated at 37 °C for 5 min with solvent (DMSO, final concentration 0.5%) or the compound under study before addition of the platelet agonist. Maximal aggregation was obtained stimulating platelets with 3 μM ADP, 50 μM arachidonic acid, 5 μg ml^{-1} collagen, 0.5 μM PMA (protein kinase C stimulant) or 1 μM U46619 (TxA_2 receptor agonist). Tests were performed within 3 h to avoid platelet inactivation. The effects of test oil

and acetylsalicylic acid (up to 300 μg ml^{-1}) were expressed as percent inhibition compared to control samples. 0.5% DMSO did not interfere with platelet aggregation.

Aggregation of PRP from human blood was induced by 5 μM ADP and 2 μM U46619 following the aforementioned method.

2.4.2. Clot retraction

PRP from rats was diluted with Tyrode buffer (137 mM NaCl, 20 mM Hepes, 5.6 mM glucose, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4, pH 7.4) to obtain a final concentration of 200.000 plts μl^{-1} and the assay was performed according to Davidson and Henry [21].

Briefly, 447.5 μl aliquots of the above platelet suspension was added to siliconized glass tubes and incubated 10 min with 2.5 μl solvent (DMSO, 0.5% final concentration) or the compound under study at 37 °C. Fibrin clot retraction was induced by addition of 50 μl thrombin 20 U ml^{-1}. Pictures of clots were taken 2 and 60 min after thrombin addition by means of the NIH Image 1.67e software. Data were expressed as percentage of retraction = ((area t_0 − area t)/area t_0) × 100, where t_0 was the area of the clot 2 min after thrombin addition and t was the area at the test time.

2.4.3. Aortic ring relaxation

Thoracic aorta from Wistar rats was carefully removed and placed in Henseleit–Krebs solution. Adhering fat and connective tissues were removed and rings of the aorta with endothelium were prepared according to Luscher and Vanhoutte's method [22]. Each ring (about 5 mm in length) was mounted in an organ bath containing 20 ml of Henseleit–Krebs solution (134 mM NaCl, 3.4 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM KH_2PO_4, 16 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 7.7 mM glucose) bubbled with 95% O_2 and 5% CO_2 gas at 37 °C. After 1 h of incubation period under 3.0 g resting tension, rat aorta with or without endothelium was contracted with 1 μM phenylephrine. The contractile response was isometrically recorded on a polygraph via a force-displacement transducer (Basile, Varese, Italy). The endothelium integrity was verified evaluating NO-dependent relaxation by Ach 100 μM. In denuded aorta experiments, the endothelium was mechanically removed by gently rubbing the ring intima surface with a cotton ball. The absence of Ach-induced relaxation indicated endothelium destruction. The spasmolytic effect of the substances under study was evaluated adding cumulative concentrations starting from 1 to 300 μg ml^{-1} on pre-contracted aorta rings [23]. In another pool of experiments, cumulative concentrations of U46619 (1 nM to 1 μM) were added to the organ bath to induce thromboxane receptor-mediated contraction. Dose-response curves were repeated in the presence of the studied substances at the appropriate concentrations. SQ 29548, a selective thromboxane-A_2 antagonist, was used as reference compound.

2.4.4. Platelets viability

Platelets viability was quantitated by measuring the activity of acid phosphatase, a platelet enzyme whose activity is stable independently of platelet stimulation and is not released from vital platelets. A modification of the method described by Bellavite at al. was used [24]. Briefly, 150 μl of platelet rich plasma was added to each well of a microtiter plate in presence of different concentrations of test compounds dissolved in DMSO (final concentration 0.5%) or in the presence of 1% triton or 0.5% DMSO. After 30 min of incubation the acid phosphatase activity was measured by using the substrate *p*-nitrophenyl phosphate at a final concentration of 5 mM. This reaction was allowed to proceed for 30 min at 37 °C in the dark and stopped with 150 μl of 2 N NaOH per well. Optical density at 405 nm was measured. Platelet viability was calculated assuming untreated platelet as 100% viability and 1% triton treated platelets as 0% viability. 0.5% DMSO had no toxic effect when incubated for 30 min.

2.4.5. Binding assay

The binding to human platelets TP receptors was determined by Cerep Inc. (Courtaboeuf, France) according to Hedberg et al. [25]. The radioligand used was [^{3}H] SQ 29548 (5 nM) and 50 μM U44069 was used to determine the non-specific binding. The assays were run in the absence and in the presence of 100 μg ml^{-1} *Ocotea* essential oil or 30 μg ml^{-1} *trans*-cinnamaldheyde, incubated with platelet homogenates for 60 min at 22 °C. The percentage of displacement of [^{3}H] SQ 29548 specific binding is given as the average of three determinations.

2.5. In vivo assays

Male Swiss mice were orally treated for 5 days, once a day, with 10, 30 or 100 mg kg^{-1} *Ocotea* essential oil or lysine acetylsalicylate (ASA). Control animals received only vehicle (methocel 0.5%). The last treatment was performed 3 h before the experiments. Animal body weights were recorded at the beginning and at the end of the treatment to assess the subacute tolerability.

2.5.1. Acute pulmonary thromboembolism

A modification of Di Minno's method was used [26]. Pulmonary acute thromboembolism was induced in mice by rapid intravenous injection into a tail vein of a mixture of 12 mg kg^{-1} collagen and 1 mg kg^{-1} epinephrine in order to induce about 85% of paralysis in the control group. The loss of the righting reflex for 30 s was considered as indication of paralysis. The occurrence of paralysed animals was recorded for 15 min after thrombotic mixture injection. The number of lethal events within 5 days from thrombotic challenge was recorded for each group of treatment.

2.5.2. Bleeding test

The tail transection bleeding was determined applying modified Dejana's method [27]. Briefly, tails of mice under light diethyl ether anaesthesia were transected at 2 mm from the tip and immersed in 1 ml of 37 °C saline for 2 min. Erythrocytes were lysed by adding 20 ml of triton 5% and absorbance of the solution was read at 560 nm (Jenway, mod. 6300, Dunmow, Essex, England). The amount of haemorrhage was estimated by linear regression analysis of a standard curve obtained from

samples of mice blood properly diluted and it was expressed as blood lost $\mu l\,2min^{-1}$.

2.6. Data analysis

All the results are expressed as means ± standard error of the mean (S.E.M.) or with indication of confidence limits. Differences between groups were analysed by Student's *t* test for *in vitro* data and by χ^2 test for pulmonary thromboembolism or by Mann–Whitney test for bleeding assay. $^*P < 0.05$ and $^{**}P < 0.01$ were indicative of significant and highly significant difference, respectively.

IC_{50} (concentration giving 50% inhibition of agonist induced maximal response) was calculated by interpolation of the linear part of the dose–response curve and K_B was calculated following Furchgott's method [28].

3. Results and discussion

3.1. Essential oil composition

The composition of the essential oil obtained from *Ocotea* floral calices showed that *trans*-cinnamaldehyde and methyl cinnamate were the most abundant constituents, accounting for 27.8 and 21.6%, respectively, while minor amounts of other phenylpropanoids (benzaldehyde, 3.1%), terpenes (limonene, α-pinene, *p*-cimene, 8.1, 3.1 and 4.8%, respectively) and aliphatic alcohols (linalool, 4- and α-terpineol, 3.2, 2.2 and 2.9%, respectively) were calculated via gas-chromatography [6]. The fingerprinting of the essential oil was achieved by means of ^{13}C NMR and spectral data are provided in Fig. 1 while the chemical shifts and relative intensity of constituents in ^{13}C NMR spectrum are given as Supplementary data published online alongside the electronic version of the article in Elsevier website.

The application of NMR techniques to phytocomplex fingerprinting is gaining popularity both for its efficacy, clarity and non-destructive characteristics [29,30]. Moreover, it allows an easy comparison of different batches of essential oils from a same plant source, which are subject to great quali-quantitative variations according to a plethora of factors [31]. However, the routinely use of ^{13}C NMR spectroscopy in essential oils analysis is not well established at present, mainly as a consequence of the low availability of specific comparative data, with rare exceptions regarding the most common commercial oils [18].

3.2. In vitro assays

In a previous screening work, *Ocotea* calices oil evidenced a significant antiplatelet effect towards aggregation induced by arachidonic acid, U46619 and ADP in guinea pig PRP [17]. The whole oil was about 5-fold less potent than ASA (IC_{50} oil = 47 $\mu g\,ml^{-1}$ versus IC_{50} ASA = 10 $\mu g\,ml^{-1}$) in inhibiting platelet aggregation induced by arachidonic acid but it was able to completely block the aggregation induced by U46619 and ADP at concentration of 67 and 70 $\mu g\,ml^{-1}$, respectively, whereas ASA failed to significantly inhibit aggregation up to 300 $\mu g\,ml^{-1}$. In the present study *Ocotea* was about 10-fold less potent than ASA in inhibiting collagen-induced aggregation in guinea pig PRP (IC_{50} oil = 163 $\mu g\,ml^{-1}$ versus IC_{50} ASA = 12 $\mu g\,ml^{-1}$) and, unlike ASA, showed a slight but significant antiplatelet effect also towards PMA induced aggregation (Table 1). These results are consistent with those obtained in human PRP where essential oil showed slightly lower potency (Table 1). As concerns clot retraction process, a physiological event known to be controlled pharmacologically only by few $\alpha_{IIb}\beta 3$ ligands, it was inhibited by *Ocotea* oil, displaying an IC_{50} of 19 $\mu g\,ml^{-1}$ and it was not affected by ASA.

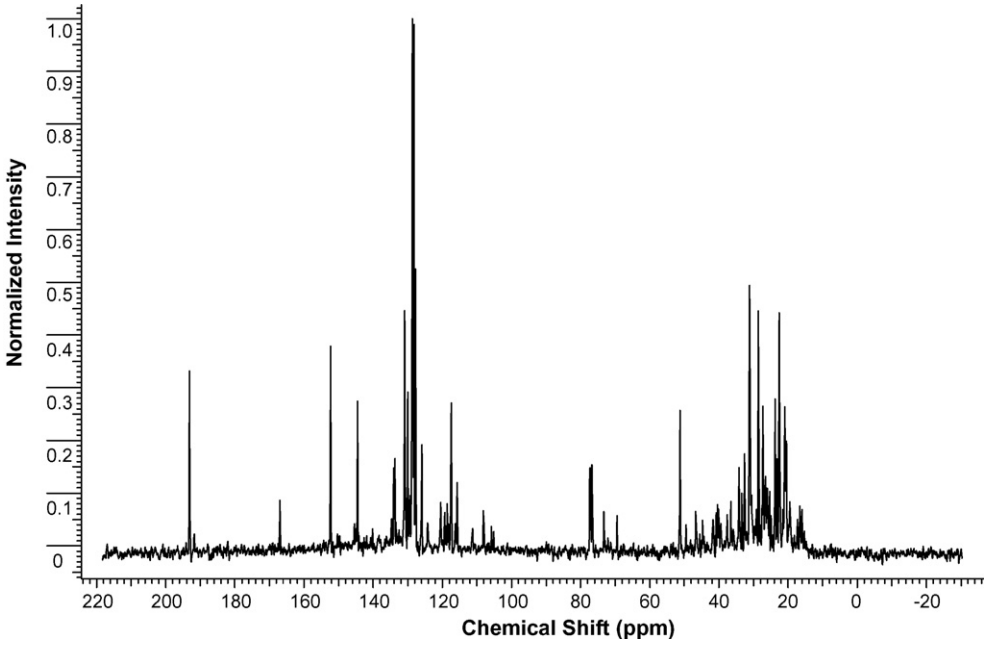

Fig. 1. Fingerprint of *Ocotea* calices essential oil: ^{13}C NMR spectrum.

Table 1
Inhibitory potency of compounds against platelet aggregation induced by arachidonic acid, U46619, ADP, collagen and PMA in guinea pig and human PRP

Compounds	Platelet aggregation guinea pig PRP					Platelet aggregation human PRP	
	AA 50 μM	U46619 1 μM	ADP 3 μM	PMA 0.5 μM	Collagen 5 μg/ml	ADP 5 μM	U46619 2 μM
Ocotea oil	47 (23–95)*	67 (57–78)*	70 (36–136)*	406 (223–738)	163 (118–225)	128 (82–199)	115 (77–170)
Trans-cinnamaldehyde	17 (13–23)	28 (18–44)	76 (60–96)	762 (510–1140)	37 (15–87)	57 (33–99)	84 (46–152)
Methyl cinnamate	162 (148–176)	112 (89–142)	b	b	93 (38–227)	a	a
ASA	10 (7–14)*	a*	b*	a	12 (8–13)	b	a

The IC_{50} values are expressed as $\mu g\,ml^{-1}$ and 95% confidence limits are indicated in brackets. Each IC_{50} value was calculated from 4 independent dose–response curves. (a) Inactive up to 300 $\mu g\,ml^{-1}$; (b) IC_{50} not calculable, because maximal inhibition is lower than 50%; (*) see [17].

The main components of the oil, *trans*-cinnamaldehyde and methyl cinnamate, were also studied. It is likely that *trans*-cinnamaldehyde is the constituent responsible for most part of *Ocotea* oil antiplatelet activity; in fact, it displays comparable or higher potency than the whole phytocomplex in inhibiting the aggregation induced by all the agonists both in guinea pig and human plasma (Table 1). Moreover, *trans*-cinnamaldehyde completely blocked also clot retraction with an IC_{50} of 25 $\mu g\,ml^{-1}$, roughly comparable with that of *Ocotea* oil. On the contrary, methyl cinnamate showed a modest antiplatelet activity, since it was a weak inhibitor of AA-, U46619- and collagen-induced aggregation with negligible activity towards ADP or PMA aggregation and thrombin-evoked clot retraction.

In a previous work, a reduction of collagen-induced platelet aggregation by *trans*-cinnamaldehyde has been described and primarily attributed to suppression of AA release from membrane phospholipids [32]. In the present study, the remarkable inhibitory potency displayed by *trans*-cinnamaldehyde against platelet aggregation caused by AA and U46619 indicates that thromboxane A_2 receptor or downstream events are the possible targets of *trans*-cinnamaldehyde antiplatelet activity. The involvement of thromboxane A_2 receptor is consistent with binding data of *Ocotea* and *trans*-cynnamaldeide to human TxA_2 receptors, as determined in a commercial screening (Cerep, France). In this case *Ocotea* essential oil 100 $\mu g\,ml^{-1}$ provided 52% inhibition of [^{3}H] SQ 29548 specific binding while *trans*-cinnamaldehyde 30 $\mu g\,ml^{-1}$ gave 17% inhibition.

It is worth noting that essential oil as well as *trans*-cinnamaldehyde prevents also ADP-induced platelet aggregation, therefore, the involvement of additional mechanisms, independent of arachidonate cascade (and related to intracellular increase of cAMP) could not be excluded. Furthermore, the scarce potency exhibited by *Ocotea* essential oil and *trans*-cynnamaldehyde against PMA-induced platelet aggregation suggests that the antiplatelet activity likely derives from the interaction of both compounds with cellular targets upstream protein kinase C activation. It has to be taken into consideration that several mechanisms of action have been so far proposed to account for the different activities demonstrated by *trans*-cinnamaldehyde in a variety of biological substrates [33–38]. Further researches will be needed to ascertain whether someone of these mechanisms can be involved in the effects described in the present study.

The thrombotic process is usually accompanied by vascular constriction due to mediators released from platelets (thromboxane A_2, serotonin) and deficiency of local vasodilatory factors as endothelial-derived nitric oxide. For this reason, in addition to clot destabilization or prevention of platelet aggregation, also vasodilation could be a beneficial effect to be exploited to facilitate the perfusion of the tissues and to limit the consequences of ischemic events.

In order to investigate the effects of *Ocotea* oil on vascular function, the essential oil was studied on isolated rat aortic rings with or without endothelium. *Ocotea* oil was able to relax the aorta contracted with phenylephrine 1 μM at concentrations effective in platelet inhibition; in intact rings it was as potent as in denuded tissues (IC_{50} = 86 $\mu g\,ml^{-1}$ vs. 110 $\mu g\,ml^{-1}$) suggesting the involvement of endothelium independent mechanisms and no interference with nitric oxide release (Table 2).

Also in this case *trans*-cinnamaldehyde seems to be the principal responsible for the vasodilatory property of the oil being about 3 times more potent than the phytocomplex (Table 2). A relaxant effect on extravasal smooth muscle (guinea pig ileum and trachea) has been already reported for *trans*-cinnamaldehyde but the mechanism remains to be elucidated [39].

The rat aorta model was also used to study the interference of *Ocotea* oil and *trans*-cinnamaldehyde on thromboxane A_2 receptor TP [40]. The oil, dose dependently shifted to the right the concentration–response curve of U46619 with a K_B of 18.5 $\mu g\,ml^{-1}$, behaving as the competitive antagonist of TP receptors SQ29548 (K_B = 8 nM). *Trans*-cinnamaldehyde showed a competitive antagonism too (K_B = 3.2 $\mu g\,ml^{-1}$) whereas methyl cinnamate was completely ineffective up to 30 $\mu g\,ml^{-1}$ (Fig. 2). Taken together, these results led us to hypothesize that inhibitory activity shown by *Ocotea* essential oil against platelet aggregation and vasoconstriction is largely due to its primary component *trans*-cinnamaldehyde in most part through the apparent inhibition of thromboxane receptors.

Table 2
Spasmolytic effect of compounds on phenilephrine (1 μM) induced contraction of rat aortic ring with or without endothelium

Compounds	With endothelium IC_{50} ($\mu g\,ml^{-1}$)	Without endothelium IC_{50} ($\mu g\,ml^{-1}$)
Ocotea oil	86(49–150)	110 (84–144)
Trans-cinnamaldehyde	31(24–38)	41(33–50)
Methyl cinnamate	153(146–160)	107(81–141)

Each IC_{50} value was calculated from 4 independent dose–response curves.

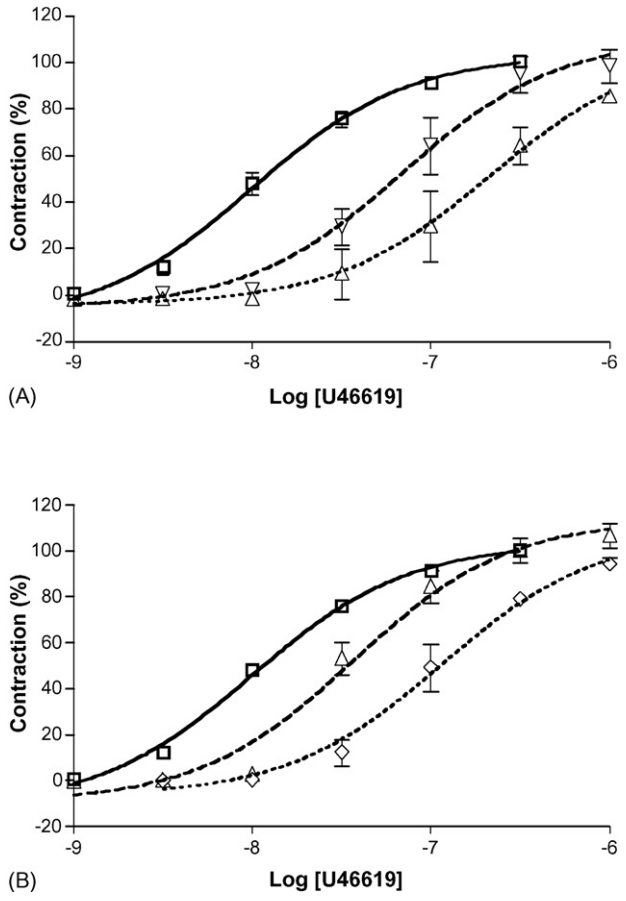

Fig. 2. Contraction of rat aortic ring induced by U46619 in the absence (□) or in the presence of (A) *Ocotea* oil (▽) 30 μg ml^{-1}; (△) 100 μg ml^{-1}; (B) *trans*-cinnamaldehyde (△) 10 μg ml^{-1}; (◊) 30 μg ml^{-1}. Data are means of 4 distinct experiments and standard errors are indicated as vertical bars.

Regarding *in vitro* cytotoxicity, *Ocotea* essential oil resulted to be substantially non-toxic in platelet viability assay. In fact, it did not affect platelets viability when incubated up to 300 μg ml^{-1} at variance with *trans*-cinnamaldehyde and methyl cinnamate that significantly reduced cells viability at the same concentration (Fig. 3). This finding enables us to exclude the occurrence of cellular toxicity when *Ocotea* essential oil was used in platelet aggregation test.

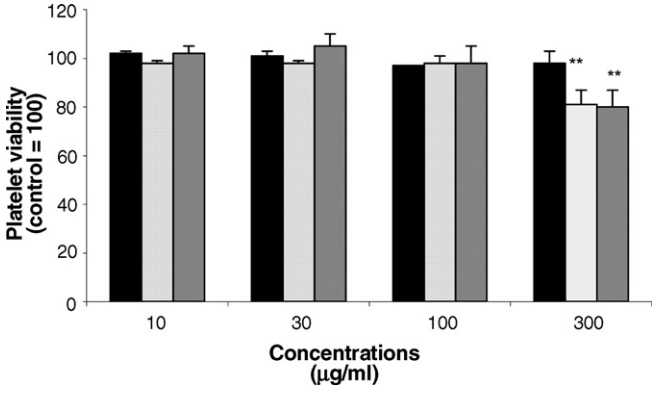

Fig. 3. Effects of *Ocotea* oil (■), *trans*-cinnamaldehyde (□) and methyl cinnamate (▩) on platelets viability. Each histogram represents the mean of three individual experiments, standard errors are indicated as vertical bars. $^{**}P < 0.01$ vs. control.

3.3. In vivo assays

In *in vivo* studies, animals were orally treated for 5 days with vehicle (methocel), *Ocotea* oil or ASA at 3 different dosage levels. During the period of treatment compounds affected neither body weight nor behaviour with respect to vehicle treated mice (data not shown). At the end of the week animals were challenged with collagen/epinephrine mixture to produce acute pulmonary thromboembolism. In control group 29 animals out of 34 underwent paralysis due to pulmonary thromboembolism (85%) and 19 out of 32 dead within 5 days (59%). ASA administered at 100 mg kg^{-1} day^{-1} significantly reduced paralysis by 37% and death by 55%. Lower concentrations of ASA (30 mg kg^{-1} day^{-1}) were inactive. On the other hand, *Ocotea* oil showed to possess high and dose-dependent antithrombotic activity. Indeed, paralysis events (Fig. 4) were

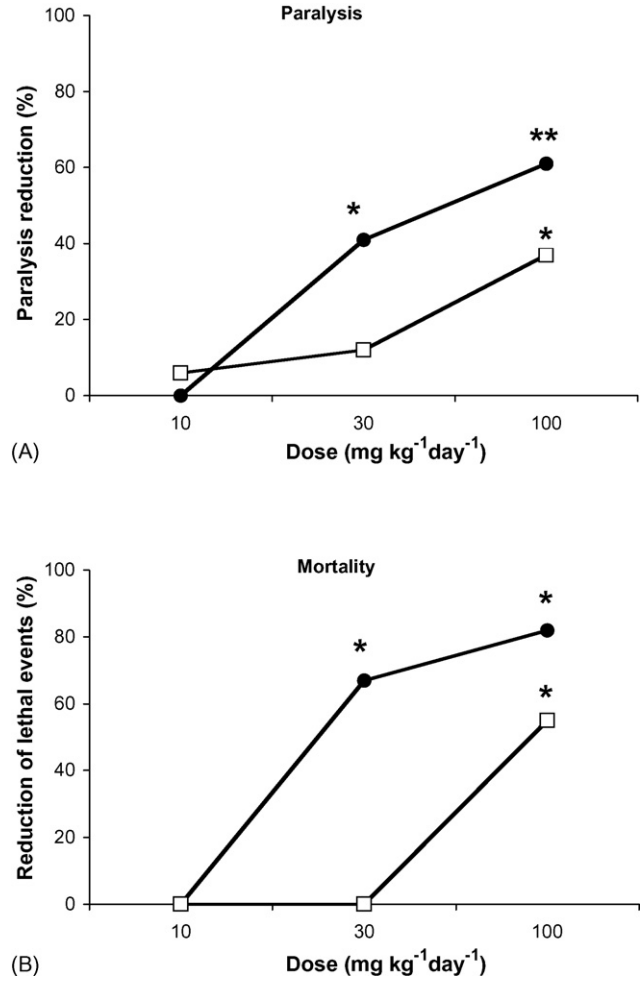

Fig. 4. Acute pulmonary thromboembolism induced by i.v. shot injection of a mixture of 12 mg kg^{-1} collagen and 1 mg kg^{-1} epinephrine in mice, orally treated for 5 days with vehicle alone, aspirin (□) or *Ocotea* oil (●) (10, 30 or 100 mg kg^{-1} day^{-1}). (A) Reduction of paralysis events compared to control group. (B) Reduction of mortality after 5 days from injection compared to control $^{*}P < 0.05$, $^{**}P < 0.01$ by χ^2 test.

significantly decreased by 61% and 41%, respectively at 100 and 30 mg kg^{-1} day^{-1}. Death within 5 days (Fig. 4) was reduced of 81% and 66%, respectively. No significant effects were detected at 10 mg kg^{-1} day^{-1}. *Ocotea* oil up to 100 mg kg^{-1} day^{-1} did not show any significant pro-hemorrhagic effect in bleeding test (blood lost within 2 min was 4.2 ± 1.7 μl vs. 4.9 ± 1 μl for vehicle treated mice), differently from animals treated with 100 mg kg^{-1} day^{-1} ASA that increased the amount of blood lost (13.1 ± 5.9 μl).

4. Conclusion

In conclusion, *Ocotea quixos* calices essential oil is an antithrombotic phytocomplex free of prohemorragic side effect and devoid of systemic toxicity when administered in a subacute treatment. The antithrombotic activity could be related to its ability to block both platelet aggregation and clot retraction and to inhibit vasoconstriction. *Trans*-cinnamaldehyde could be the primary oil's constituent responsible for these activities.

For antiplatelet and vasorelaxant activity of *Ocotea* essential oil and *trans*-cynnamaldeide, a mechanism of action involving the inhibition of TP receptors is speculated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phrs.2006.09.009.

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