Vasorelaxant Effect Induced by the Essential Oil of Ocotea duckei Vattimo Leaves and Its Main Constituent, Trans-caryophyllene, in Rat Mesenteric Artery

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Authors’ contributions
This work was carried out in collaboration among all authors. Author RMC designed the study, conducted the research, analyzed the results and drafted the manuscript. Authors TAFG, AFRR and DUOM analyzed the results and helped to review the manuscript. Author ESTE helped to draft the manuscript. Author IAM primary responsibility for the work, conceived and coordinated the study and helped to review the final version of manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the vasorelaxant effect induced by the essential oil of the leaves of O. duckei Vattimo (ODEO) and its main constituent, trans-caryophyllene, in rat superior mesenteric arteries.

Methodology: Isolated rat superior mesenteric rings were suspended by cotton threads for isometric tension recordings in Tyrode’s solution at 37°C, gassed with 95% O2 and 5% CO2 and different ODEO concentrations (0.1-300 μg/mL) or trans-caryophyllene (1-1000 μg/mL) were added cumulatively to the organ baths.

Results: Vasorelaxant effect induced by the essential oil of Ocotea duckei leaves (ODEO) and its main constituent, trans-caryophyllene (60.54 %), was evaluated in this work. In intact isolated rat superior mesenteric rings ODEO (0.1-300 μg/mL, n=6) induced concentration-dependent relaxation of tonus induced by phenylephrine (10 µM) or K+ -depolarizing solution (KCl 80 mM) (IC50=31±5, 5±0.4 µg/mL, respectively, n=6). The relaxations of phenylephrine-induced contractions were not significantly attenuated after removal of the vascular endothelium (IC50=25±5 µg/mL). ODEO antagonized the concentration-response curves to CaCl2 (10⁻⁶-3x10⁻² M) and Bay K 8644 (10⁻⁹-3x10⁻⁶ M). Furthermore, in nominally without calcium solution, ODEO significantly inhibited, in a concentration-dependent manner, transient contractions induced by 10 µM phenylephrine or 20 µM caffeine. Trans-caryophyllene induced vasorelaxations, however, this effect was 18.6 times less potent when compared to ODEO-induced vasorelaxations.

Conclusion: The relaxant effect induced by ODEO in rat superior mesenteric artery rings is endothelium-independent and seems to be related to both, inhibition of Ca²⁺ influx through L-type voltage-gated Ca²⁺-channels sensitive to dihydropyridines and inhibition of the calcium release from intracellular IP₃- and caffeine-sensitive stores.

Keywords: Ocotea duckei; essential oil; vasorelaxant effect; rat superior mesenteric artery; calcium channels.

1. INTRODUCTION

The genus Ocotea has a high occurrence in the Neotropics, being the richest in species of the Lauraceae family, comprising approximately 400 species, containing alkaloids, lignoids and flavonoids in their chemical constitution [1]. Among the species of the genus Ocotea we can highlight the Ocotea duckei Vattimo, popularly known as “louro de cheiro”, “louro pimenta” or “louro canela” [2]. The essential oil extracted from species of the genus Ocotea from the north and northeast regions of Brazil showed antipsychotic [3], Molluscicidal [4], antibacterial and cytotoxic [5], hypotensive [6], antiepileptic [7] and antiaggregant and activity [8]. Previous reports on the essential oil obtained by steam distillation from the roots, stems, leaves and fruits of O. duckei described the isolation of alkaloids and lignoids [6]. Furthermore, the essential oil of this plant presented 67 components [8], among them α-pinene, limonene, borneol, β-eudesmol, elemol, valencene and trans-caryophyllene, proved to be the major compound (60.54 %) [6,8,9]. In a previous pharmacological study, we demonstrated that the essential oil of O. duckei leaves induced marked hypotension that was not affected after atropine or L-NAME [10]. Similarly, reticulin, an alkaloid extracted from the essential oil of O. duckei, demonstrated a hypotensive effect, probably due to peripheral vasodilation mediated by muscarinic stimulation and activation of eNOS, as well as by blocking Cav1.2 channels [11]. In addition, we have also shown that yangambin, a lignan isolated from the essential oil of O. duckei, induces peripheral vasodilation by blocking voltage-gated Ca²⁺ channels [2]. Thus, considering that any pharmacological study relating the activity of this plant in rat resistance arteries was found in the literature, this work aimed to evaluate the vasorelaxant effect induced by the essential oil of the leaves of O. duckei Vattimo (ODEO) and its main constituent, trans-caryophyllene in rat superior mesenteric artery.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar 10 to 12-week-old rats (200-350 g) were used for all experiments. The animals were kept under temperature control conditions (21 ± 1°C) and lighting (lights on: 06:00-18:00 h). In addition, they had free access to food (PURINA®-Brazil) and tap water ad libitum. All of the experiments were approved by the Ethics
2.2 Chemicals

The drugs used were: (-)-trans-caryophyllene (trans-(1R,9S)-8-Methylene- 4,11,11-trimethylbicyclo [7.2.0] undec-4-ene), acetylcholine chloride (Ach), L-phenylephrine chloride (Phe), ethyleneeglycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), cremophor®, caffeine and 1,4-dihydro-2,6-dimethyl-5-nitro-4-[(trifluoromethyl)phenyl] pyridine-3-carboxylic acid methyl ester (±) (BAY K8644) (all from SIGMA). All compounds were freely dissolved in distilled water, except (±) BAYK 8644 which was dissolved in methanol 95%. Serial dilutions were made in distilled water. The final methanol concentration in the organ bath never exceeded 0.03% and was without effect when tested in control preparations (data not shown).

2.3 Essential Oil Extraction

O. duckei leaves were collected near the city of Santa Rita in the State of Paraiba, Brazil. A voucher specimen was deposited from Herbarium Prof. Lauro Pires Xavier under identification code Agra 4309. The essential oil was obtained of the fresh leaves (1000 g) by using a steam distillation process [12] in a Clevenger apparatus at 60°C for 1 h and stored at 4°C. When required, the oil was dissolved in a distilled water/cremophor solution and diluted to the desired concentrations (pH = 7.4). The final cremophor concentration in the organ bath never exceeded 0.01% and was without effect when tested in control preparations (data not shown).

2.4 Preparation of Isolated Rat Superior Mesenteric Artery Rings

The procedure was performed according to the protocol described by Assis et al. [13]. Briefly, the animals were euthanized and the superior mesenteric artery was removed and cleaned from connective tissue. Rings (1 – 2 mm) were obtained and suspended by cotton threads in organ baths containing 10 mL of Tyrode’s solution containing the following composition (mM): NaCl 138.161, KCl 4.0, CaCl\textsubscript{2} 2.0, MgCl\textsubscript{2} 1.05, NaH\textsubscript{2}PO\textsubscript{4} 0.42, NaHCO\textsubscript{3} 10.0, and glucose 5.6 (pH = 7.4); gassed with the carbogen mixture (95% O\textsubscript{2} and 5% CO\textsubscript{2}) and maintained at 37°C, except in the experiments involving caffeine, in which Tyrode’s solution was maintained at 22°C. The preparations were steadied under a resting tension of 0.75 g during 1 h. During this time the solution was changed each 15 min. to prevent the accumulation of metabolites. The isometric tension was recorded through a force transducer (FORT-10, WPI, Sarasota, FL, USA) coupled to an amplifier-recorder (Miobath-4, WPI, Sarasota, EUA). The endothelium was removed by softly rubbing of the intimal surface of the vessels. The existence of functional endothelium was assessed by the ability of acetylcholine (ACh) (10 μM) to elicit more than 80 % relaxation of mesenteric rings precontracted with phenylephrine (10 μM). The absence of the relaxant response to ACh was taken as an indication that the isolated artery rings were functionally denuded of endothelium.

2.5 Effect of ODEO and Trans-caryophyllene on Sustained Contractions Induced by Phenylephrine (10 μM) or KCl (80 mM) in Isolated Rat Superior Mesenteric Rings

The vasorelaxant effect of ODEO was initially observed during the tonic phase of contraction induced by Phe (10 μM). In this way, different ODEO concentrations (0.1, 0.3, 1, 10, 30, 100 and 300 μg/mL) or trans-caryophyllene (1, 3, 10, 100, 300 and 1000 μg/mL) were added cumulatively to the organ bath. Relaxations were measured by comparing the tension developed before and after the addition of ODEO and expressed as % relaxation. These experiments were carried out in the presence or absence of functional endothelium. The second set of the experiments, the rings in the absence of endothelium functional, were precontracted with K\textsuperscript{-} depolarizing solutions (KCl 80mM) and different concentrations of ODEO (0.1, 0.3, 1, 10, 30, 100, and 300 μg/mL) were added cumulatively to the organ bath. The relaxations were measured as previously described.

2.6 Effect of ODEO on Voltage-gated Ca\textsuperscript{2+} Channels

After the stabilization period, the rings without functional endothelium were washed with a nominally without Ca\textsuperscript{2+} solution (CaCl\textsubscript{2} was omitted) for 15 minutes and then exposed to a nominally without Ca\textsuperscript{2+} K\textsuperscript{-}depolarizing solution.

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for another 15 minutes. Then, a first cumulative concentration-response curve to CaCl$_2$ (10$^{-6}$, 3x10$^{-6}$, 10$^{-5}$, 3x10$^{-5}$, 3x10$^{-4}$, 10$^{-4}$, 3x10$^{-3}$, 10$^{-3}$ and 3x10$^{-2}$ M) was obtained. In these same preparations, ODEO (1, 10, 30, and 100 μg/mL) was individually pre-incubated for 15 minutes and a second cumulative concentration-response curve to CaCl$_2$ was obtained. The obtained curves were compared with those obtained in the absence of ODEO and results were expressed as percentages of the maximal response to CaCl$_2$ alone.

2.7 Effect of ODEO on CaV1.2 Channels

After stabilization period, a cumulative concentration-response curve to (±) Bay K 8644 (10$^{-10}$, 3x10$^{-10}$, 10$^{-9}$, 3x10$^{-9}$, 10$^{-8}$, 3x10$^{-8}$, 10$^{-7}$, 3x10$^{-7}$, 10$^{-6}$ and 3x10$^{-6}$ M), a direct activator of the CaV1.2 channels [14], was obtained. ODEO (0.01, 0.03, 0.1, 0.3, 1 and 3 μg/mL) was individually incubated for 15 minutes before the second cumulative curve to (±) Bay K 8644. This curve was compared with those obtained in the absence of ODEO and results were expressed as percentages of the maximal response to (±) Bay K 8644 alone. Because of the light sensitivity of (±) Bay K 8644, this experiment was conducted in the dark.

2.8 Effect of ODEO on Norepinephrine and Caffeine-sensitive Intracellular Calcium Stores

The effect of ODEO (0.1, 0.3, 1, 10, 30, and 100 μg/mL) on intracellular calcium stores sensitive to phenylephrine or caffeine was investigated using a protocol described by Dias et al. [11]. After the stabilization period, the preparations were exposed to a K$^+$-depolarizing solution (60 mM) for 3 minutes. Then, the preparations were washed with Ca$^{2+}$-free solution (CaCl$_2$ was omitted and 1 mM of EGTA was added) and 10 μM phenylephrine or 20 mM caffeine was added. Following, the preparations were washed with Tyrode’s solution and incubated with K$^+$-depolarizing solution (60 mM) for another 3 minutes to Ca$^{2+}$ reloading. This procedure was repeated until two similar transient contractions to the agonists had been obtained. This same procedure was performed after the incubation with ODEO (0.1, 0.3, 1, and 3 μg/mL) for 20 minutes before the application of phenylephrine or caffeine. This transient contraction was compared with those obtained in the absence of ODEO and results were expressed as percentages of the response induced by phenylephrine or caffeine alone.

2.9 Data Analysis

Values are expressed as mean ± SEM. When appropriate, student’s t-test or two-way analysis of variance (ANOVA) were done to evaluate the significance of the differences between means. The IC$_{50}$ values were calculated by non-linear regressions of individual concentration-response curves when appropriate. Statistical analysis was done by using Graph Pad Prism™ version 6.0 software.

3. RESULTS AND DISCUSSION

The major finding of the present work was that ODEO induces concentration-dependent relaxations in rat superior mesenteric artery that appears to be due to an inhibition of the Ca$^{2+}$ influx through CaV1.2 channels associated with an inhibition of the calcium release of the intracellular IP$_3$ and caffeine-sensitive stores.

3.1 ODEO Induces Vasorelaxation in Superior Mesenteric Artery Rings

In intact rings of the superior mesenteric artery of a rat pre-contracted with Phe, ODEO (0.1, 0.3, 1, 10, 30, 100, and 300 μg/mL) caused concentration-dependent relaxations (IC$_{50}$ = 31 ± 5 μg/mL) (Fig. 1).

It is well established in the literature that the endothelium is an important regulator of vascular tone by releasing relaxing factors derived from the endothelium (EDRFs), mainly products derived from NO and COX, such as PGI$_2$ [15]. To investigate the role of the endothelium in the vasorelaxant response induced by ODEO in rat superior mesenteric artery rings, we performed experiments in the absence of functional endothelium. Under these conditions, the vasorelaxant response induced by ODEO was not altered (IC$_{50}$ = 25 ± 5 μg/mL) (Fig. 1). This suggests that the appearance of the endothelium is not important for the expression of the vasorelaxant effect induced by ODEO and that a pathway independent of the endothelium is likely to be involved in this effect. Similar results have been demonstrated with Yangambin, a furufuran lignan isolated from the essential oil from O. duckei [2].
3.2 Vasorelaxing Effect of ODEO is Potentiated in Rings of Superior Mesenteric Artery Pre-contracted with K+-Depolarizing Solution (KCl 80 mM)

ODEO also induced concentration-dependent relaxations in endothelium denuded rings of rat superior mesenteric artery pre-contracted with K+-depolarizing solution (KCl 80 mM) (IC_{50} = 5.0±0.4 μg/mL) (Fig. 1). ODEO-induced relaxations were significantly more potent (p<0.05) than that observed in endothelium denuded rings pre-contracted with phenylephrine.

It is well known that the maintenance of smooth muscle contraction depends on Ca^{2+} entry from extracellular space through voltage and/or receptor-gated calcium channels [16-18]. It is well reported that increase of external K^+ concentration (KCl 80 mM) induces smooth muscle contraction through activation of CaV1.2 channels and subsequent release of calcium from the sarcoplasmic reticulum, without changing other signal transduction systems, while agonist linkage in theirs receptors, such as phenylephrine, induces smooth muscle contraction by activating receptor-operated calcium channels and subsequent release of calcium from the sarcoplasmic reticulum, through activation of IP_3 formation [10,19,20].

Thus, we could hypothesize that this effect could be due to a calcium channels blockade. To check this hypothesis, we performed experiments in intact rings precontracted with K+-depolarizing solutions (KCl 80mM). This set of experiments revealed that ODEO-induced vasorelaxations were significantly more potent than those in intact rings pre-contracted with phenylephrine, suggesting that the ODEO appears to be inhibiting Ca^{2+} influx through CaV1.2 channels.

3.3 ODEO Induces Vasorelaxant Effect by Blocking CaV1.2 Channels

To assess the ODEO antagonism on the voltage sensitive calcium channel, we verified the effect of different concentrations of ODEO on different stimulations of CaCl_2 and Bay K 8644. The concentration-response curves for CaCl_2 (Fig. 2a) and Bay K 8644 (Fig. 2b) were strongly inhibited by ODEO (1, 10, 30, and 100 μg/mL, and 0.01, 0.03, 0.1, 0.3, 1, and 3 μg/mL, respectively) in rat superior mesenteric rings, the maximum inhibition being obtained with ODEO 100 and 1 μg/mL, respectively.
Therefore, we can suggest that ODEO was able to antagonize the contractions induced by CaCl₂ in a concentration-dependent manner, confirming our hypothesis. (±) Bay K 8644 is a direct activator of CaV1.2 channels [14], so it was used in the absence and presence of ODEO, to evaluate the participation of this channel in the vasorelaxant mechanism induced by ODEO. Thus, (±) Bay K 8644 produced concentration-dependent contractions that were antagonized and abolished by ODEO, indicating that the voltage-gated Ca²⁺ channels subtype inhibited by ODEO appears to be of the CaV1.2 type. Activation of the phosphoinositide turnover by coupling the phospholipase C protein in response to receptor activation, such as alpha-adrenoceptors, is crucial for the cytoplasmic increase in calcium through the release of calcium from intracellular reserves and, consequently, the contraction of vascular smooth muscle [21-23].

### 3.4 ODEO Interferes with the Release of Ca²⁺ from Intracellular Stocks

Other calcium intracellular stores, such as caffeine/ryanodine sensitive, can be activated by caffeine, which activates the ryanodine receptor and leads to intracellular Ca²⁺ release [24]. This led us to investigate whether ODEO could also exert its effect on the calcium release from intracellular IP₃ and caffeine-sensitive stores. Thus, we performed experiments in that the rings were contracted by phenylephrine or caffeine, in Ca²⁺-free media, in the absence and presence of ODEO. Under this condition, ODEO inhibited transient contractions induced by phenylephrine and caffeine (Fig. 3), suggesting that the ODEO appears to be interfering in the calcium mobilization of both IP₃ and caffeine-sensitive intracellular stores. Interestingly, ODEO was more much able in inhibits the contractions induced by phenylephrine than by caffeine. It appears to be probably due to a major affinity of the ODEO by IP₃-sensitive calcium intracellular stores. However, further experiments are necessary to clearly elucidate this assumption.

These results agree with our previous studies [10], which demonstrated that in normotensive rats, ODEO induced marked hypotension that was not affected after atropine or L-NAME. Furthermore, ODEO-induced vasorelaxant response, at least in part, seems to account for the hypotensive effect.

### 3.5 Trans-caryophyllene, Major Constituent of ODEO, Induces Vasorelaxant Effect

Finally, trans-caryophyllene, the main constituent of ODEO and with calcium channel blocking activity [6,25], was also tested on isolated mesenteric rings. As shown in Fig. 4, the trans-caryophyllene (1, 3, 10, 100, 300 and 1000 μg/mL), was able to induce relaxation of the mesenteric artery rings with (IC₅₀ = 576 ± 7.4 μg/mL) and without endothelium (IC₅₀ = 418 ± 3.8 μg/mL). However, the concentration-

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**Fig. 2. Effect of ODEO on cumulative concentration response curves induced by CaCl₂ (a) (1, 10, 30, and 100 μg/mL) and Bay K 8644 (b) (0.01, 0.03, 0.1, 0.3, 1, and 3 μg/mL), in endothelium-denuded rat superior mesenteric rings.**

Values are mean ± SEM of 6 experiments.

* p<0.05, ** p<0.01 and *** p<0.001 vs control
response curves were significantly shifted to the right. Thus, the effect induced by trans-caryophyllene was 18.6 times less potent when compared to ODEO, suggesting that trans-caryophyllene, despite its blocking activity of calcium channels, does not appear to be entirely responsible for the vasorelaxant activity induced by essential oil.

**Fig. 3.** Effects of ODEO (0.1, 0.3, 1, 10, 30 and 100 μg/mL) on phenylephrine (10 μM) or caffeine (20 mM)-induced transient contractions in Ca²⁺-free media in isolated rat mesenteric rings.

Values are mean ± SEM of 6 experiments.

*** p<0.001 vs Phenylephrine

**Fig. 4.** Vasorelaxant effect of trans-caryophyllene (1, 3, 10, 100, 300, and 1000 μg/mL) in rings of rat superior mesenteric arteries, pre-contracted with phenylephrine (10 μM) in the control condition (intact rings) or after endothelium removal.

Values are mean ± SEM of 6 experiments.
4. CONCLUSION
The present study demonstrate that the relaxant effect induced by the essential oil of the leaves of *O. duckei* Vattimo in rings of rat superior mesenteric artery is endothelium-independent and appears to be due to both, an inhibition of Ca\(^{2+}\) influx through L-type voltage-gated Ca\(^{2+}\) channels sensitive to dihydropyridines and to an inhibition of the calcium release from intracellular calcium stores sensitive to IP\(_3\) and caffeine.

ETHICAL APPROVAL
All of the experiments were approved by the Ethics Committee on Animal Use from Federal University of Acre (UFAC), protocol number 38/2014.

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COMPETING INTERESTS
Authors have declared that no competing interests exist.

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