Mechanisms involved in the endothelium-dependent vasodilatory effect of an ethyl acetate fraction of *Cyathea phalerata* Mart. in isolated rats' aorta rings

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ABSTRACT

The species *Cyathea phalerata* Mart. is a tree fern, commonly known as "xaxim", which is found in tropical and subtropical areas of Brazil. The present study investigated the mechanisms related with the vasorelaxant effects of an Ethyl Acetate Fraction (EAF) obtained from *C. phalerata* in rats' thoracic aorta rings. In pre-contracted vessels, EAF (0.1–1000 μg/mL) caused a concentration-dependent relaxation. The endothelium denudation, the nitric oxide (NO) synthase and guanylyl cyclase inhibitor reduced the vasodilation, indicating the participation of NO/cGMP pathway in its effect. The relaxation of EAF was abolished in the absence of extracellular Ca^{2+} and was significantly decreased in the presence of Ca^{2+} entry blocker, suggesting that Ca^{2+} influx plays an important role in EAF effect and probably in eNOS activity. However, the PI3K/Akt pathway is not responsible for eNOS phosphorylation/activation. The vasodilator effect of EAF was partially inhibited by KCl 40 mM and almost totally abolished with L-NOARG + KCl 40 mM, indicating also the role of hyperpolarization in its effect. Calcium activated K^+ channels are not involved in the EAF-induced hyperpolarization. The COX inhibitor, indomethacin, slightly reduced the vasodilation induced by EAF. In addition, EAF did not alter the relaxant effects of NO-donor, indicating that the relaxant activity cannot be attributed to free radical-scavenging properties. In conclusion, the present study showed that the EAF, causes an endothelium-dependent vasorelaxant effect in aorta that mainly involves the NO-cGMP pathway, hyperpolarization and prostanoids. The vasorelaxant activity of EAF can be attributed to the occurrence of polyphenol compounds.

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1. Introduction

The use of herbal remedies, supplements, and alternative therapeutic items has been described in the traditional medicine for thousands of years.[1,2] In the plant kingdom, Pteridophytes represent a group of vascular cryptograms, presenting 13,600 species, many of which are tree ferns found in tropical and subtropical countries.[3] Several ferns present health benefits and are used in the form of food, tea and drugs. They are rich sources of bioactive phytochemicals and have a wide variety of biological properties.[4,5]

Among the Pteridophytes, the genus *Cyathea* has proved to have great medicinal relevance. Several classes of compounds were identified in the *Cyathea* genus, for example: steroids, saponins, tannins, fernene, filicene and hopane triterpenes, phenolic acids (coumaric and caffeic), protocatechuic acids and flavonoids, specially kaempferol glycosides.[6–10] Studies about pharmacological properties from the *Cyathea* genus are still limited. The methanolic extract from the bark of *Cyathea gigantea* presented antioxidant and anti-inflammatory effects in acute carrageenan-induced paw edema in rats.[11] Kiran and colleagues[12] showed that methanolic extract from the leaves of the same species was hepatoprotective against acetaminophen-induced liver damage in rats. More

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recently, Janakiranam and Johnson[6] showed the cytotoxic effects of three different species from *Cyathea* (*C. crinite, C. nilgirensis* and *C. gigantea*) in a human breast adenocarcinoma cell line (MCF-7).

The specie *Cyathea phalerata* Mart. is a tree fern, commonly known as "xaxim", which is found in tropical and subtropical areas of Brazil. In popular medicine, it is used as expectorant, anti-inflammatory and for the treatment of varicose veins and hemorrhoids.[13,14] In previous studies, several compounds were identified and isolated from *C. phalerata* stem extracts, for example, 4-*O*-β-D-glucopyranosyl caffeic acid, 4-*O*-β-D-glucopyranosyl *p*-coumaric acid, 3,4-spyroglucopyranosyl protocatechuic acid, sitosterol- β −D glucoside, β-sitosterol, kaempferol, vitexin, ethylgalactoside and the major compound kaempferol-3-neohesperidoside.[13–15]

There are few literature data about the biological and pharmacological effects of *C. phalerata.* In 2007, Brighente et al.[13] showed the *in vitro* antioxidant power of leaves and stem extracts from *C. phalerata* and its isolated compound kaempferol-3-*O*-a-L-rhamnopyranosyl-(1−2)-b-D-glucopyranosylcaffeic. Our research group previously demonstrated the antioxidant potential *in vitro* of an extract and fractions from *C. phalerata* and the hepatoprotective effects of ethyl acetate fraction (EAF) against oxidative stress induced by carbon tetrachloride in mice.[14] Moreover, the flavonoid, kampferol-3-neohesperidoside, isolated from *C. phalerata,* has hypoglycemic effect in diabetic rats[16] and mimics the action of insulin by promoting glucose uptake in a cultured cell line of skeletal muscle.[17] More recently, Andrade and colleagues[18] verified that an extract from aerial parts of *C. phalerata* can inhibit monoamine oxidase A activity, indicating that it may be a potential candidate antidepressant and anti-neurodegenerative drug.

Although some studies report pharmacological activities of *C. phalerata* that can suggest its beneficial potential to the cardiovascular system, for example, the antioxidant activity, there are no studies about its vascular effects. Based on this, the purpose of the present study was to investigate the effects of EAF from *C. phalerata* on vascular tone and the mechanism by which EAF relaxes vascular smooth muscle in isolated rat thoracic aortic rings.

2. Material and methods

2.1. Drugs

Acetylcholine (Ach), apamin, atropine, *H* [1,2,3]oxadiazolo [4,3-alpha]quinoxalin (ODQ), indomethacin, lanthanum, L-arginine, Nʷ-nitro-L-arginine (L-NOARG), phenylephrine, Phorbol-12,13-dibuyrate (PDBU), superoxide dismutase (SOD), tetraethylammonium (TEA) and wortmannin were purchased from Sigma (St. Louis, MO, USA). Potassium chloride and sodium nitroprusside were purchased from Nuclear (São Paulo, Brazil). All drug solutions and EAF (dry power) were dissolved in distilled water, except PDBU which was dissolved in ethanol and wortmannin which was dissolved in dimethylsulfoxide.

2.2. Plant material and extraction procedure

Cyathea phalerata Mart. was collected in March 2002 in Palhoça city, Santa Catarina State, Brazil and identified by Professor Lana da Silva Sylvestre. A voucher specimen RBR 4287 was deposited in the herbarium of the Botany Department at the Universidade Federal Rural do Rio de Janeiro, Seropedica, Brazil. The fresh plant stalk was cut into small pieces and extracted by maceration for 15 days at room temperature with 8:2 ethanol:water. The extracts were concentrated in a rotavapor and left to rest at 5 °C for 24 h. The aqueous portion was extracted with ethyl acetate to obtain the ethyl acetate fraction (EAF) (for details see[14]).

2.3. Characterization of ethyl acetate fraction (EAF)

The EAF from *C. phalerata* was characterized as described by Hort et al.[14] Based on spectroscopic analysis, nine compounds were identified and characterized: *p*-coumaric acid, sitosterol-β-D-glucoside, kaempferol-3-neohesperidoside, 4-*O*-β-D-glucopyranosyl, 3,4-spyroglucopyranosyl protocatechuic acid, β-sitosterol, 4-*O*-β- D-glucopyranosyl caffeic acid, kaempferol, ethylgalactoside and vitexin. The main component of EAF is the kaempferol-3-neohesperidoside representing 51.25%.

2.4. Animals

Male Wistar rats (250–350 g) were kept at 22 ± 2 °C under a 12 h light/12 h dark cycle and received food and water *ad libitum*. The experiments were approved by the Institutional Ethics Committee of the Federal University of Santa Catarina (approval number 002728/2005−41/UFSC) in agreement with the guidelines of the Brazilian National Council for Control of Animal Experimentation.

2.5. Tissue preparation

Thoracic aorta was isolated as described previously by Andriambeloson et al.[19] Briefly, rats were killed by an overdose injection of ketamine plus xylazine via an intramuscular pathway and exsanguinated. The aorta was cleaned of fat and connective tissue, cut into rings 3–4 mm in length and mounted using two wires inserted through the lumen of the vessel in an organ chamber in Krebs-Henseleit solution (composition in mM): NaCl, 113; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 0.9; $NaHCO_3$, 25; $MgCl_2$, 1.1; glucose, 11; pH 7.4. The rings were maintained continuously gassed with 95% O_2 and 5% CO_2 and at 37 °C, and under a resting tension of 1 g. Tension changes were recorded by a force transducer connected to a computerized data acquisition system (Soft and Solutions/KIT-CAD8, Brazil). Seven to nine rings were used to each experimental protocol and at least 4 different animals were used in each test.

2.6. Experimental protocol

The rings remain at an equilibrium period for 60 min. After that, they were pre-contracted with phenylephrine (Phe, 1 μM) and the presence of functional endothelium was verified by the capacity of acetylcholine (Ach, 1 μM) to cause more than 75% relaxation. After washout and the return of tension to the basal levels, the preparations were again contracted with Phe (1 μM) or phorbol-12,13-dibutirate (PDBU; 15–20 nM), according to the experimental protocol. After the contractile response reaches a plateau, increasing concentrations of EAF (1–1000 μg/mL) were added cumulatively. The maximal volume of EAF added to the bath organ was 50 μL in a total chamber volume of 5 mL and the medium time of exposure to each dose was 5 min. To evaluate the participation of functional endothelium in relaxant effect of EAF, experiments were performed in rings without endothelium. In this case, the endothelial layer was mechanically removed and considered denuded when acetylcholine exhibited relaxation was <10%.

To involvement of nitric oxide (NO)/cGMP pathway in the EAF effect was investigated in the presence of L-NOARG (10 μM), an inhibitor of nitric oxide synthase (NOS) and ODQ (1 μM), a guanylyl cyclase inhibitor. The drugs were added to the bath 20 min before the addition of Phe. An excess of L-arginine (1 mM) was added to the medium 10 min before L-NOARG (10 μM) to evaluate the effect of L-NOARG on the EAF-induced relaxation. The involvement of the enzyme Phosphoinositide 3-kinase (PI3K), which phosphorylates the endothelial NOS (eNOS), was investigated by adding wortmannin (30 nM) to the medium 20 min before the addition of Phe.

The involvement of extracellular Ca^{2+} in the relaxation induced by EAF was analyzed in rings with or without $CaCl_2$ in the medium or in the presence of a Ca^{2+} blocker (Lanthanum, 100 μM), in vessels pre-contracted with PDBU (15–20 nM). To prepare the Ca^{2+}-free medium, $CaCl_2$ was removed, and 0.1 mM EGTA was added to the Krebs solution. To evaluate the involvement of prostanoids and muscarinic receptor on the effect of EAF, cyclooxygenase inhibitor indomethacin (1 μM) and a muscarinic antagonist atropine (1 μM) were added to the bath 20 min before contraction with Phe. The participation of potassium channels at the EAF effect was evaluated using high potassium solution (KCl 40 mM), tetraethylammonium (TEA, 500 μM) (a non-selective potassium channels blocker) and apamin (100 nM) (a blocker of calcium activated potassium channels). In vessels incubated in KCl 40 mM, Phe was not added to the preparation.

To investigate if EAF could enhance the biologic activity of NO, concentration-response curves for Sodium nitruprusside (SNP, 0.01–300 nM) were performed in Phe-contracted vessels without endothelium, in the absence or presence of EAF (30 and 100 μg/mL), added 15 min prior to SNP addition. Superoxide dismutase (SOD, 100 U/mL), which inhibits $O_2^{\bullet-}$ generation, was used to confirm the influence of extracellular $O_2^{\bullet-}$ in the SNP effect.

2.7. Statistical analysis

All the data were expressed as mean ± standard error of the mean (SEM) values. Statistical comparisons between the groups was assessed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Differences with P values < 0.05 were considered statistically significant.

3. Results

The cumulative additions of EAF from C. phalerata, caused a concentration-dependent vasodilator effect in intact thoracic aorta rings pre-contracted with Phe. Phe induced a contraction of 1.52 ± 0.11 g. The maximal relaxation (R_{max}) value obtained in endothelium intact was 59.67 ± 2.66%. However, in endothelium denuded preparations, EAF induced vasodilatation was almost completely abolished, indicating that the vasodilator effect is dependent on endothelium-derived relaxing factors (Fig. 1A).

To investigate the involvement of nitric oxide (NO) in the vasorelaxant activity, experiments were conducted in the presence of NO synthase inhibitor (L-NOARG). Pretreatment of the rings with L-NOARG caused a reduction of the EAF-induced relaxation, which was reversed by the excess of L-arginine (L-ARG). In addition, guanylyl cyclase inhibitor (ODQ) also decreased the vasodilator effect of EAF (Fig. 1B).

In the PDBu-pre-contracted vessel, EAF produced a similar relaxation in thoracic aorta rings when compared to rings that were contracted with Phe. The relaxation of EAF was reduced when extracellular Ca^{2+} was removed from the medium and when the Ca^{2+} entry blocker, lanthanum, was added (Fig. 1C). These results suggest that Ca^{2+} influx has an important participation in EAF effect and probably in the eNOS activity. The catalytic activity of eNOS is also modulated by phosphorylation; however, the inhibitor of eNOS phosphorylation (wortmannin) did not affect the relaxant activity of EAF (Fig. 1D).

In order to investigate the participation of cholinergic receptors, cumulative concentration-response curve (CCR) for EAF was obtained in the presence of atropine (muscarinic antagonist). According to Fig. 2A, atropine potentiates the vasorelaxant effect of EAF.

The role of prostanoids in the vasorelaxant effect of EAF was evaluated using a cyclooxigenase inhibitor (indomethacin). In the presence of this drug, the CCR of EAF was slightly shifted to the right, but the maximal effect was not modified (Fig. 2B). The participation of potassium channels was evaluated in the presence of high concentrations of potassium. Fig. 3A shows that the vasodilator effect of EAF was reduced in the presence of KCl and completely abolished when L-NOARG was added together with KCl. These results indicate that both NO system and potassium are responsible for the fraction effect. Moreover, the presence of a non-selective potassium channel blocker (TEA), the vasodilation induced by EAF was reduced. Still, calcium-activated K^+ channels do not participate in the vasodilator action of EAF, since the presence of apamin did not modify its effect (Fig. 3B).

Fig. 4 shows the CCR for Sodium Nitroprusside (SNP) in aortic rings devoid of endothelium and contracted with Phe, in the absence and presence of EAF. These results show that EAF (30 and 100 μg/mL) did not affect SNP-induced vasodilatation. When aortic rings were incubated with SOD, which reduces $O_2^{\bullet-}$ levels, the CCR was shifted to the left and SNP effect was potentiated.

4. Discussion

This study has shown for the first time that a fraction obtained from C. phalerata, which is constituted by a mixture of phenolic compounds, caused vasodilation in aorta rings. The mechanisms of the endothelium-dependent relaxation response to EAF were also investigated. Our results clearly demonstrated that the vasorelaxant response to EAF is mediated by NO/cGMP pathway and endothelium-derived hyperpolarizing factor (EDHF), and to a lesser extent by prostanoids.

In the cardiovascular system, endothelial cells play a wide variety of critical roles in the control of vascular function, including the regulation of vascular tone.[20,21] Our results show that EAF-induced vasodilation is dependent of endothelial cells, because this effect was abolished when the endothelium was removed. This is in agreement with other studies that have demonstrated that extracts rich in flavonoids cause endothelium-dependent vasorelaxation.[19,22–24] Moreover, previous reports demonstrated that kaempferol, the aglycone from the main constituent of EAF, kaempferol-3-neohesperidoside, induces vasodilation in an endothelium-dependent manner.[25,26] Although some evidence indicates the vasoactive effects of kaempferol, other components found in EAF also have vasodilator properties. Some studies have already demonstrated that caffeic,[19,27] coumaric[19,28] and protocatechuic acids[29] are vasodilators in isolated blood vessels. Based on this, we cannot discard the possible effects from other components than kaempferol from EAF or a synergism among the compounds.

Regulation of vasodilation by endothelium is evoked by three main components: NO, the endothelium-derived hyperpolarizing and prostacyclin (PGI_2) factor.[30] NO is synthesized from oxygen and L-arginine by eNOS and diffuses into the vascular smooth muscle cells where it stimulates soluble guanylate cyclase (GC), that produces cyclic GMP and causes subsequent relaxation of vascular muscle cells.[20,31] The results from the present study suggest that the vasodilator of EAF depends, at least in part, on the release of NO from endothelial cells, since L-NOARG, a NOS inhibitor, and ODQ, a GC inhibitor, significantly decreased the its effects. EAF may stimulate eNOS and induce relaxation of vascular smooth muscle by increasing cGMP levels in these cells. Similar results were found in studies which showed that eNOS inhibitor reduces the vasodilator effect from different flavonoid rich extracts[22,32] and kaempferol.[25]

The activity of eNOS is modulated by both calcium-dependent and calcium-independent ways. Different neurohumoral mediators can activate specific receptors coupled to G protein, such as cholinergic muscarinic receptors, of the endothelial membrane and
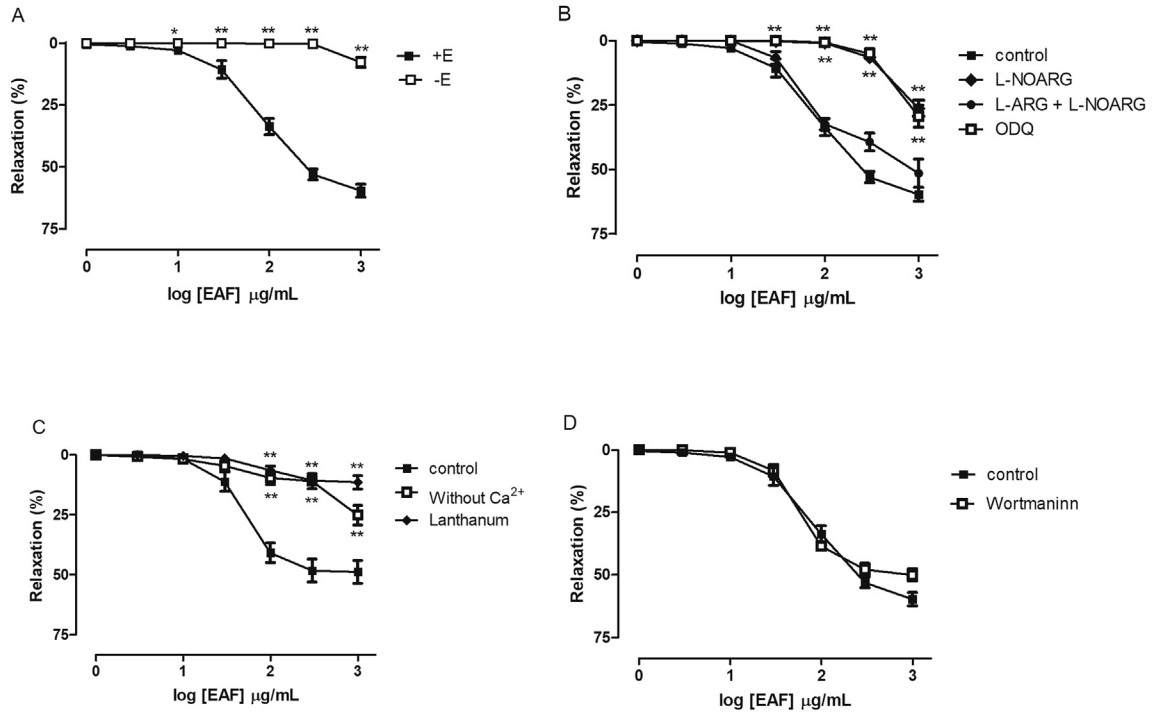
Fig. 1. (A) Concentration-response curve of EAF-induced vasodilatation (1–1000 μg/mL) in rats' isolated thoracic aorta rings pre-contracted with Phe (1 μM) with intact (+E) and denuded (-E) endothelium. (B) Effect of L-NOARG (10 μM), ʟ-arginine (10 mM) + L-NOARG (10 μM), ODQ (1 μM) on the vasodilator effect of EAF (1–1000 μg/mL) in rats' isolated thoracic aorta rings pre-contracted with Phe (1 μM). (C) Effect of the presence (control) or absence of calcium (without Ca^{2+}) in physiologic solution, and lanthanum (100 μM) on the vasodilator effect of EAF (1–1000 μg/mL) in rats' isolated thoracic aorta rings pre-contracted with PDBu (15–20 nM). (D) Effect of wortmaninn (30 nM) on the vasodilator effects of EAF (0.1–1000 μg/mL) in rats' isolated thoracic aorta rings pre-contracted with Phe (1 μM). Values represent the mean ± S.E.M. of 7–9 experiments. *p < 0.05; **p < 0.01 indicates the difference when compared to intact endothelium (+E) or control group (one-way ANOVA followed by Tukey pos hoc test).
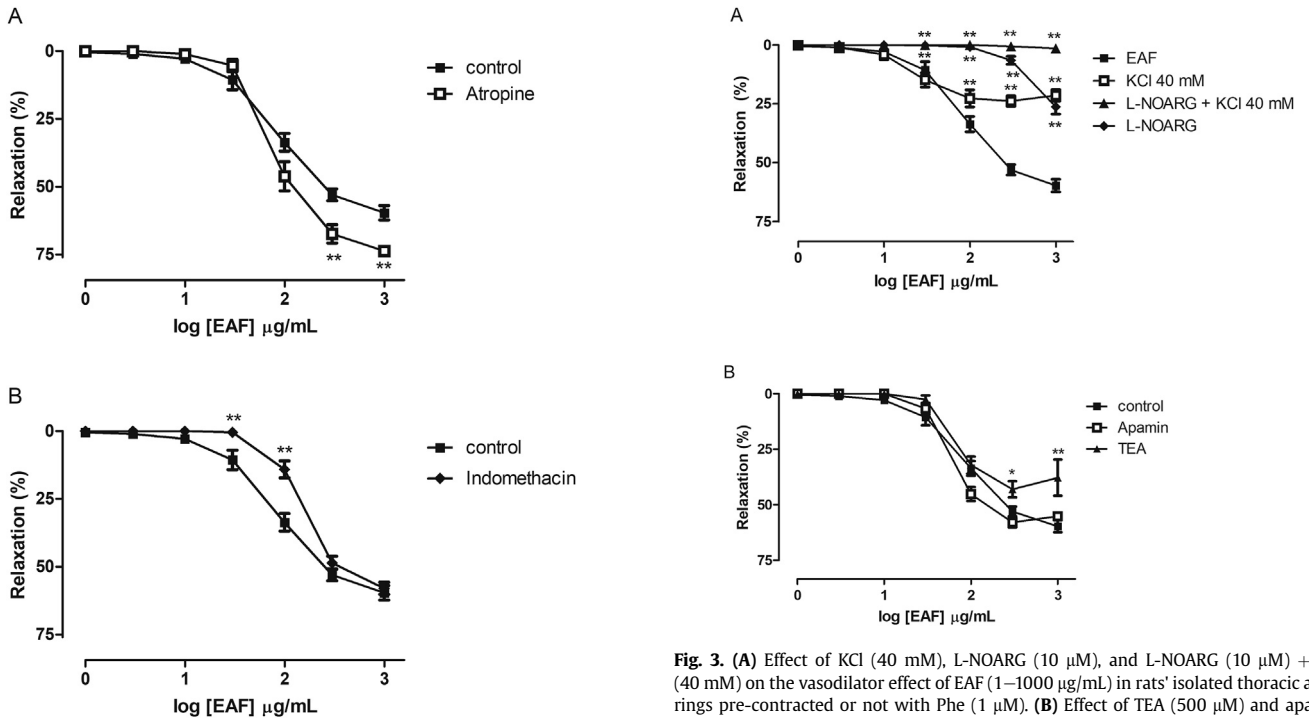

Fig. 2. Effect of Atropine (1 μM) (A) and Indomethacin (1 μM) (B) on the vasodilator effects of EAF (0.1–1000 μg/mL) in rats' isolated thoracic aorta rings pre-contracted with Phe (1 μM). Values represent the mean ± S.E.M. of 6–8 experiments. **p < 0.01 indicates the difference when compared to intact control group (one-way ANOVA followed by Tukey pos hoc test).
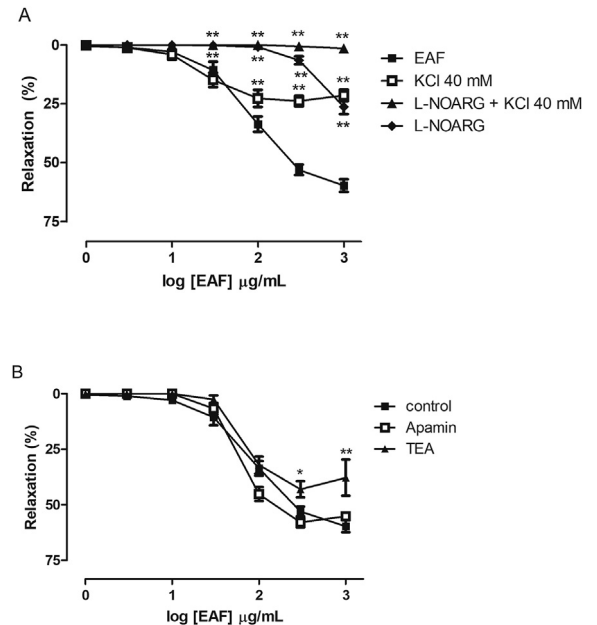

Fig. 3. (A) Effect of KCl (40 mM), L-NOARG (10 μM), and L-NOARG (10 μM) + KCl (40 mM) on the vasodilator effect of EAF (1–1000 μg/mL) in rats' isolated thoracic aorta rings pre-contracted or not with Phe (1 μM). (B) Effect of TEA (500 μM) and apamin (100 nM) on the vasodilator effects of EAF (0.1–1000 μg/mL) in rats' isolated thoracic aorta rings pre-contracted with Phe (1 μM). Values represent the mean ± S.E.M. of 7–9 experiments. *p < 0.05; **p < 0.01 indicates the difference when compared to control group (one-way ANOVA followed by Tukey pos hoc test).
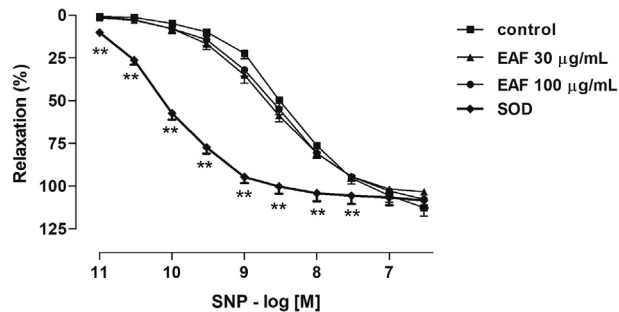
Fig. 4. Concentration-response curves for SNP-induced relaxation in endothelium-denuded rats' isolated thoracic aorta rings pre-contracted with Phe (1 μM) in the absence (control) or presence of EAF (30 or 100 μg/mL) and in the presence of SOD (100 U/mL). Values represent the mean ± SEM of 7–9 experiments. **p < 0.01 indicates the difference when compared to control group (one-way ANOVA followed by Tukey *post hoc* test).

increases the intracellular concentration of calcium ions (Ca^{2+}). Ca^{2+} binds to calmodulin (CaM) that activates eNOS to produce NO.[31,33] In our study, it was investigated whether EAF can induce NO production through the activation of cholinergic muscarinic receptors. When aorta rings were incubated with atropine, a muscarinic receptor antagonist, EAF-induced vasodilation was potentiated, indicating that EAF does not seem to activate NOS via the stimulation of cholinergic muscarinic receptors. Previous studies, reported that atropine cause endothelium-dependent vasodilation in different blood vessels, including the aorta.[34–36] Therefore, it is possible that the vasorelaxant effect of atropine can contribute to the improvement observed in the vasodilation induced by EAF.

The agonist stimulated NO release from endothelial cells also requires an influx of extracellular Ca^{2+} to stimulate eNOS. The removal of Ca^{2+} from the extracellular medium abolishes the agonist-induced NO formation and the subsequent vasodilation.[37,38] In our data, it was demonstrated that when extracellular Ca^{2+} was removed from the physiological solution and Ca^{2+} entry was blocked with lanthanum the EAF-induced vasodilation decreased. These results suggest that Ca^{2+} influx plays a critical role in the vasorelaxant effect of EAF.

In addition to the modulation of calcium-dependent activity, the catalytic activity of eNOS is modulated by phosphorylation on threonine, serine and tyrosine residues.[39] Some studies established that phenolic compounds, such as flavonoids, affect the phosphorylation of Akt in a PI3K-dependent manner, which consequently phosphorylate eNOS, increasing the NO formation.[40,41] Whether the NO release induced by EAF was mediated by the PI3K/Akt signaling pathway was investigated. It was observed that prior incubation with PI3K blocker, wortmaninn, did not attenuate EAF-induced vasodilation, indicating that the PI3K/Akt pathway is not responsible for eNOS phosphorylation/activation.

In addition to NO, PGI_2, a prostanoid produced by cyclooxygenase pathway, is also a relaxing factor derived from endothelium. The vascular relaxation induced by PGI_2 is mediated by cAMP that leads to Ca^{2+} outflow from the cytosol and decrease the sensitivity of the contractile machinery to Ca^{2+}. Moreover, PGI_2 evokes hyperpolarization in various vasculatures through the activation of different types of potassium channels.[21,42] In the present study, we found that indomethacin, a cyclooxygenase inhibitor, affects slightly the vasodilator effect of EAF, without affecting the maximal relaxation. Based on this result it seems that prostanoids have a smaller role in the vasodilator effects when compared to the effects triggered by NO and endothelium-derived hyperpolarizing factor (EDHF).

Besides controlling the vascular tonus by the release of NO and PGI_2, the endothelium uses other pathways that causes hyperpolarization of the smooth muscle cells. This property was the origin of the term "endothelium-derived hyperpolarizing factor" (EDHF). EDHF-mediated responses involve an increase in the intracellular Ca^{2+} in the endothelial cells followed by opening SK_{Ca} and IK_{Ca} channels (small and intermediate conductance Ca^{2+}-activated K^+ channels respectively). Following SK_{Ca} activation, smooth muscle hyperpolarization is preferentially evoked by electrical coupling through myoendothelial gap junctions, while, following IK_{Ca} activation, K^+ efflux can activate smooth muscle inward Na^+/K^+-ATPase or rectifier K^+ channel.[43] The hyperpolarization of smooth muscle causes vasodilation by reducing the open probability of voltage-dependent Ca^{2+} channels and the turnover of intracellular phosphatidylinositol, thus decreasing Ca^{2+} intracellular concentrations.[43,44]

Previous studies reported that phenolic compounds cause endothelium-dependent EDHF-mediated relaxation in different vascular beds.[45,46] According to our results, the vasodilator effect of EAF may involve hyperpolarization though K^+ channels, since 40 mM KCl and TEA (a nonselective K^+ channel blocker) reduced the vasodilation induced by EAF in aorta rings. Moreover, SK_{Ca} channels are not involved in the EAF effect, since apamin did not affect EAF-induced relaxation. Corroborating our data, Xu and colleagues[47] showed that kaempferol potentiated the bradykinin-induced relaxation mediated by endothelium-dependent hyperpolarization and this vascular effect of kaempferol involved the activation of large-conductance-activated potassium channel. Mahobiya et al.[48] also showed that SK_{Ca} channels are no responsible for the relaxant effects of kaempferol in pulmonary arterial rings. Additionally, the inhibitory effect of L-NOARG and high K^+ on the EAF induced vasodilatory activity was further increased when both were incubated simultaneously. These data indicate that both NO and EDHF are responsible for the vasodilatory effects of the fraction.

As previously reported, EAF presented an important antioxidant activity, through *in vitro* and *in vivo* experiments. Of particular importance, EAF presented an *in vitro* superoxide anion ($O_2^{\bullet-}$)-scavenging capacity in the nitroblue tetrazolium (NBT) reduction test.[14] Antioxidants capable of sequestering free radicals, such as $O_2^{\bullet-}$ increase the bioavailability of NO and consequently its vasodilatory effect. $O_2^{\bullet-}$ reacts with NO forming peroxynitrite and is much less effective as a guanylate cyclase activator, resulting in a reduction in NO bioavailability.[49] In order to investigate whether the vasodilatory effect produced by the EAF would be triggered by its antioxidant activity, experiments were carried out using an NO donor, SNP. Our results showed that EAF was not able to scavenge $O_2^{\bullet-}$ because it did not potentiate SNP effects. Thus, the vasodilatory activity would not be directly related to the antioxidant activity of EAF, and should be mainly due to the activation of the NO/cGMP pathway and EDHF. These data are in agreement with studies by Andriambeloson et al.[50,51] which suggested that the endothelium-dependent vasodilatory activity of polyphenols in wine is not due to their $O_2^{\bullet-}$ radical-scavenging capacity but to the increase in NO resulting from the interaction with targets located in the endothelial cells.

In conclusion, the present study demonstrates that the EAF, obtained from *C. phalerata,* mediates an endothelium-dependent vasorelaxant effect in the rat thoracic aorta that mainly involves the NO/cGMP pathway, hyperpolarization and prostanoids. Moreover, it was evidenced that the mechanism of the NO/cGMP pathway is probably by a calcium dependent way; however, the PI3K/Akt pathway is not responsible for eNOS phosphorylation/activation. Still, calcium channels seem to play an important role in EAF effect since that EDHF pathways also depends of intracellular calcium increase in endothelial cells. These findings provide a

mechanistic explanation for the vasodilatory effects of *C. phalerata* and its possible benefit effects to cardiovascular system. Nevertheless, further pharmacological and toxicological studies are needed to better understand their antihypertensive potential.

Conflicts of interest

None.

Footnotes

None.

Funding sources

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