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Analysis of the Role of Nitric Oxide in the Relaxant Effect of the Crude Extract and Fractions from *Eugenia uniflora* in the Rat Thoracic Aorta

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Abstract

This study has evaluated the possible role played by the L-arginine-nitric oxide pathway in the vasorelaxant action of the hydroalcoholic extract from *Eugenia uniflora*, and fractions from the extract, in rings of rat thoracic aorta.

The addition of an increasing cumulative concentration of hydroalcoholic extract from *E. uniflora* (1–300 $\mu g\ mL^{-1}$) caused a concentration-dependent relaxation response in intact endothelium–thoracic aorta rings pre-contracted with noradrenaline (30–100 nM). The IC50 value, with its respective confidence limit, and the maximum relaxation (R_{max}) were 7·02 (4·77–10·00) $\mu g\ mL^{-1}$ and 83·94 ± 3·04%, respectively. The removal of the endothelium completely abolished these responses. The nitric oxide synthase inhibitors N^{ω}-nitro-L-arginine (L-NOARG, 30 μM) and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 30 μM), inhibited the relaxation (R_{max}) to − 10·43 ± 7·81% and − 3·69 ± 2·62%, respectively. In addition, L-arginine (1 mM), but not D-arginine (1 mM), completely reversed inhibition by L-NOARG. Methylene blue (30 μM), a soluble guanylate cyclase inhibitor, reduced the relaxation induced by the extract to 14·60 ± 7·40%.

These data indicate that in the rat thoracic aorta the hydroalcoholic extract, and its fractions, from the leaves of *E. uniflora* have graded and endothelium-dependent vasorelaxant effects.

Eugenia uniflora L. (Myrtaceae) is widely distributed in Brazil and some other South American countries. Infusions made from the leaves of this plant have been used as traditional remedies in folk medicine for the management of a number of diseases (Pio-Corrêa 1984; Simões et al 1986) and it has been frequently used as an antihypertensive. Preclinical studies have confirmed and also extended most of these popular uses in traditional medicine (Schmeda-Hirschmann et al 1987; Schapoval et al 1994). The constituents of *E. uniflora* have been chemically investigated (Rücker et al 1977; Weyerstahl et al 1988; Henriques et al 1993) and shown to include flavonoids and tannins (Schmeda-Hirschmann et al 1987; Alice et al 1991).

Preliminary data from our laboratory have recently demonstrated that the crude hydroalcoholic extract of the leaves of *E. uniflora* provokes hypotension in anaesthetized rats. Various agents have been shown to have relaxing action on vascular smooth muscle through endothelium-dependent nitric oxide/cGMP (Fitzpatrick et al 1995; Marín & Rodríguez-Martínez 1995). The aim of the current study was to determine the possible role played by the L-arginine-nitric oxide pathway in the vasorelaxant action of the hydroalcoholic extract from *E. uniflora* in rings of rat thoracic aorta. In addition, we have also studied the effect of other classes of drugs on the relaxation caused by *E. uniflora*.

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Materials and Methods

Plant material

Leaves of *E. uniflora* were collected in the morning, in Florianópolis (SC), Brazil, in the Spring of 1993. The plant was identified by A. Zanin and a specimen (reference number 25760) was deposited in the Herbarium of the Department of Botany, Universidade Federal de Santa Catarina. The dried powdered leaves were extracted with 80% ethanol (1 : 20 w/v) by maceration for 10 days at 4°C. The crude hydroalcoholic extract was filtered and concentrated under reduced pressure at 45–50°C. The extract (7·3%) was suspended in water and partitioned with dichloromethane (18%) and ethyl acetate (9·6%). The aqueous phase was lyophilized, yielding 73%. The hydroalcoholic extract and its fractions were submitted to phytochemical screening according to WHO methods (Akerele 1984). We obtained positive reactions for steroids or triterpenes, or both, in the dichloromethane fraction, for phenolic compounds and flavonoids in the ethyl acetate fraction, and for ellagitannins in the aqueous phase. Gallic acid and myricitin were isolated from the ethyl acetate fraction.

Pharmacological assays

Rings 3–4 mm long were obtained from the thoracic aortas of male Wistar rats (250–300 g). The rings, with or without functional endothelium (Furchgott & Zawadzki 1980), were placed in 5-mL baths of Krebs solution of composition (mM): NaCl 113, KCl 4·7, $CaCl_2$ 2·5, KH_2PO_4 0·9, $NaHCO_3$ 25, $MgSO_4$ 1·1, EDTA 0·025, ascorbic acid 0·01, glucose 11, and

oxygenated with carbogen (95% CO_2, 5% O_2) at 37°C. Initial tension was 1 g and the isometric recording was made with an F-60 force transducer coupled to a Narco Biosystem polygraph. After the equilibration period, the aorta was pre-contracted sub-maximally with noradrenaline (30–100 nM) and the integrity of endothelial layer was confirmed by a greater than 80% relaxation response to acetylcholine (1 μM), whereas removal of these cells was considered successful when acetylcholine failed to cause relaxation.

After washout and once tension returned to baseline (30 min), the preparations were again pre-contracted with noradrenaline (30–100 nM) and, in its presence with sustained tonic response, a cumulative relaxant concentration–response curve was obtained for the hydroalcoholic extract (1–300 μg mL^{-1}) from *E. uniflora*, and for the ethyl acetate and aqueous (0·3–300 μg mL^{-1}) fractions of the extract. After obtaining the first curve showing the effect of the extract, a second cumulative curve was obtained at 0·5, 2 and 4 h. The effect of acetylcholine- (1 μM) and sodium nitroprusside (1 μM)-induced relaxation was tested after this second cumulative curve. Acetylcholine was tested after 0·5, 2 and 4 h, sodium nitroprusside after 0·5 h only. The acetylcholine-induced relaxation was also tested after the second cumulative curve of ethyl acetate and aqueous fractions only at 0·5 h.

To test the effect of inhibition of nitric oxide synthesis, the cumulative relaxation responses to the hydroalcoholic extract from *E. uniflora* were obtained in the absence or in the presence of L-NOARG and L-NAME (30 μM). The effect of methylene blue (30 μM), which inhibits the activation of guanylate cyclase by nitric oxide, was also investigated.

The effect of nitric oxide inhibitors on the extract-induced relaxation was analysed in the presence of an excess of either L-arginine (1 and 10 mM) or D-arginine (1 mM), either co-incubated or pre-incubated in the bath.

To assess further the relative effect of cyclooxygenase products of arachidonic acid on the relaxation induced by the hydroalcoholic extract from *E. uniflora* in thoracic aorta, preparations were incubated with indomethacin (1 μM). The influences of tetraethylammonium (30 μM), a non-selective K^+-channel blocker, glibenclamide (1 μM), a blocker of ATP-sensitive K^+ channels, apamin (0·3 μM), a blocker of Ca^{2+}-sensitive K^+ channels, atropine (1 μM, a muscarinic blocker), pyrilamine (1 μM, an histaminergic H_1 blocker) and propranolol (1 μM, a β-adrenoceptor blocker) on the effect of the crude extract were also observed. The period of incubation for all these drugs was 30 min before testing the hydroalcoholic extract from *E. uniflora*. Control experiments, pre-contracted with noradrenaline, were also performed. Only one cumulative concentration–response curve of crude extract was obtained in each preparation in the absence or in the presence of these inhibitors. Relaxation was expressed as a percentage of the precontraction to noradrenaline.

Drugs

The drugs used were: acetylcholine chloride, L-arterenol hydrochloride (noradrenaline), indomethacin, sodium nitroprusside HCl, methylene blue HCl, L-NOARG, L-NAME, L-arginine, D-arginine, atropine sulphate salt, tetraethylammonium acetate, apamin, DL-propranolol hydrochloride, pyrilamine maleate, glibenclamide (Sigma, St Louis, MO).

The drugs were diluted in twice-distilled water or in phosphate-buffered saline. Indomethacin was dissolved in absolute ethanol: the final concentration of ethanol did not exceed 0·02%. Noradrenaline was dissolved in HCl and diluted with aqueous NaCl (0·9%) containing ascorbic acid (50 μg mL^{-1}).

Statistical analysis

IC50 and maximum relaxation (R_{max}) were calculated from individual concentration–response curves. Data were presented as mean ± s.e.m., except that IC50s were given as geometric means accompanied by their respective 95% confidence limits (Fleming et al 1972). Statistical analysis was performed by means of Student's unpaired *t*-test, where $P < 0.05$ was considered as indicative of significance.

Results

The cumulative addition of the hydroalcoholic extract from *E. uniflora* (1–300 μg mL^{-1}) and its fractions (0·3–300 μg mL^{-1}) to the bath preparation caused a concentration-dependent vasorelaxation response in endothelium-intact thoracic aorta rings pre-contracted with noradrenaline (30–100 nM). The removal of the endothelium completely abolished these responses. Figs 1a, b illustrate the vasorelaxation

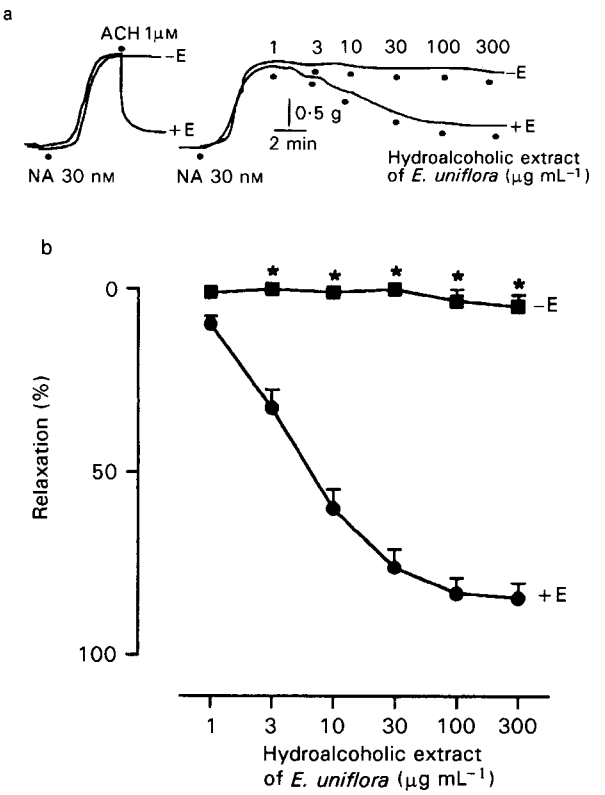

FIG. 1. a. Representative traces showing the relaxation mediated by the hydroalcoholic extract of *E. uniflora* in aorta ring preparations with (+E) and without (−E) endothelium. The vessels were pre-contracted with noradrenaline (NA). The failure of acetylcholine (ACh, 1 μM) to induce relaxation of these rings was taken as an indication of endothelium removal. b. Mean cumulative relaxant concentration–response to the hydroalcoholic extract from *E. uniflora* in the vessels. The ordinate scale shows the relaxation of the rings as a percentage of the contraction induced by noradrenaline. Data are presented as mean ± s.e.m. of five preparations; *$P < 0.001$.

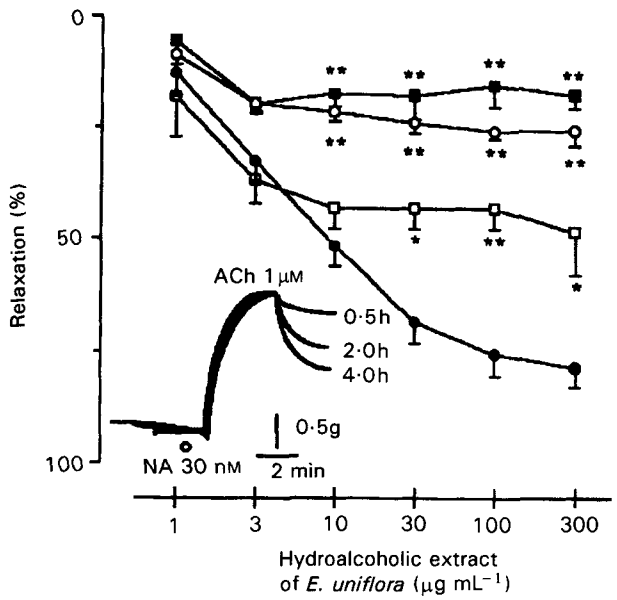

FIG. 2. Mean cumulative relaxant concentration–response curve for the hydroalcoholic extract from *E. uniflora* in the isolated aorta pre-contracted with noradrenaline (NA) in the presence of the vascular endothelium. First curves (●) and second curves recorded 0·5 h (○), 2 h (■) and 4 h (□) after the first curve. Inset shows acetylcholine (ACh, 1 μM)-induced relaxation 0·5, 2 and 4 h after second curve of effect of crude extract on rat thoracic aorta pre-contracted with noradrenaline (30 nM). Data are presented as mean ± s.e.m. of 4 to 10 experiments. $*P < 0.01$, $**P < 0.001$, significantly different from the first curve.

response caused by hydroalcoholic extract in the studied preparations. The IC50 (μg mL^{-1}) and R_{max} (%) were respectively: hydroalcoholic extract 3·83 (3·19–4·58) and 84·20 ± 3·94; ethyl acetate fraction 2·05 (0·62–6·80) and 83·30 ± 8·80; and aqueous fraction 1·99 (1·15–3·45) and 91·80 ± 2·44.

After determination of the relaxation caused by cumulative application of the hydroalcoholic extract from *E. uniflora*, the preparation was washed every 15 min for 0·5, 2 and 4 h. On cumulative application for a second time the hydroalcoholic extract no longer relaxed the arteries at 0·5 h (Fig. 2). Likewise, relaxation induced by acetylcholine (1 μM) was also inhibited (Fig. 2, inset). Sodium nitroprusside (1 μM), however, produced 100·00 ± 0·00% relaxation. The desensitization of the vessels to the hydroalcoholic extract from *E. uniflora* was partially reversible (48·46 ± 9·53%) only 4 h after the first application. The relaxation induced by acetylcholine (1 μM) was partially recovered 2 h (36·97 ± 6·42%) and 4 h (56·55 ± 7·49%) after the first application (Fig. 2 inset).

The ethyl acetate and aqueous fractions were, on the other hand, found to be active at relaxing these preparations on second cumulative application at 0·5 h, producing 44·05 ± 2·78% and 67·76 ± 4·06% relaxations, respectively. The relaxation induced by acetylcholine was 39·51 ± 9·24% and 74·85 ± 7·36% after the second application of the ethyl acetate and aqueous fractions, respectively.

Pretreatment of the arteries with L-NOARG and L-NAME (30 μM) caused powerful blockade, to − 10·43 ± 7·81% and − 3·69 ± 2·62%, respectively, (Fig. 3) of the relaxation (R_{max}) induced by the hydroalcoholic extract. In addition, L-arginine (1 mM), but not D-arginine (1 mM), completely reversed the inhibition by L-NOARG of the relaxation caused by the

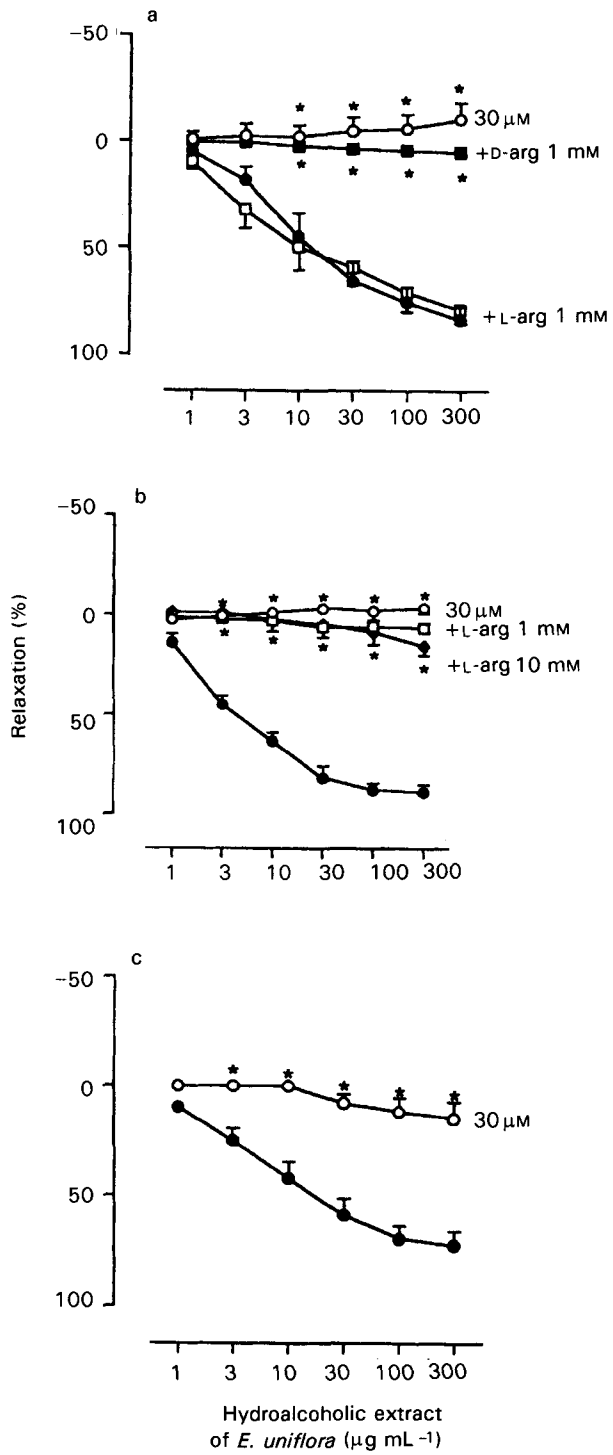

FIG. 3. Relaxant effect of the hydroalcoholic extract from *E. uniflora* on rat thoracic aorta pre-contracted with noradrenaline in the absence (●) and presence (○) of L-NOARG (a), L-NAME (b) and methylene blue (c). L-Arginine (□ and ◆; L-arg) restored the inhibition of extract-induced relaxation by L-NOARG, but not by L-NAME. D-Arginine (■; D-arg) did not reverse this inhibition by L-NOARG. Data are presented as mean ± s.e.m. of 5 to 8 experiments. $*P < 0.01$, significantly different from the corresponding control values.

hydroalcoholic extract (Fig. 3). The ability of L-NAME to block the relaxation induced by the hydroalcoholic extract from *E. uniflora* was not prevented by pretreatment with L-arginine (1 and 10 mM).

Pretreatment with methylene blue (30 μM) had inhibitory effects similar to those of L-NOARG and L-NAME (R_{max} to 14·60 ± 7·40%; Fig. 3).

The IC50 and R_{max} (%) of the cumulative relaxation curve of the hydroalcoholic extract from *E. uniflora* was not affected by preincubation with indomethacin (1 μM), tetraethylammonium (30 μM), glibenclamide (3 μM), apamin (0·3 μM), pyrilamine (1 μM), propranolol (1 μM) or atropine (1 μM).

Discussion

These results indicate that the hydroalcoholic extract of leaves from *E. uniflora* has a relaxant effect on rings of rat thoracic aorta. The relaxation produced by the extract and its fractions appears to involve the endothelium-derived relaxing factor first described by Furchgott & Zawadzki (1980), and subsequently identified as nitric oxide or a derivative of nitric oxide (Palmer et al 1987).

The vasodilatation caused by the hydroalcoholic extract was significantly attenuated by L-NOARG and L-NAME (30 μM), inhibitors of L-arginine in the nitric oxide pathway. Nitric oxide synthesis can be competitively inhibited by analogues of L-arginine such as N^{ω}-nitro-L-arginine and its methyl ester (Wakabayashi et al 1994). In rat aorta, it has been demonstrated that nitric oxide is the predominant endothelium-derived relaxing factor (Zygmunt et al 1994), and it is particularly sensitive to the inhibitory effect of L-NOARG on the relaxant response (Vargas et al 1991).

The endogenous substrate L-arginine (1 mM), but not D-arginine (1 mM), competitively inhibited the ability of L-NOARG (30 μM) to block the production of nitric oxide stimulated by the hydroalcoholic extract from *E. uniflora*. L-NAME also blocked the production of nitric oxide stimulated by this crude extract. Pretreatment with L-arginine (1 and 10 mM) did not, however, prevent this blockade by L-NAME. In the rabbit corpus cavernosum, on the other hand, L-arginine (300 μM) prevented the inhibitory action of L-NAME (10 μM) on the relaxing response to the hydroalcoholic extract from *E. uniflora* (data not shown). Randall & Griffith (1991) found that L-arginine did not influence the inhibitory effects of L-NAME in the rabbit ear. It is possible that there are differences between the mode of action of L-NAME at the level of the enzymes responsible for the synthesis of nitric oxide. The possibility of heterogeneity in the mechanisms of synthesis of nitric oxide is also suggested by the in-vivo findings of Gardiner et al (1990) that the administration of L-arginine reverted the haemodynamic changes induced by L-NAME in the mesenteric and renal beds of the rat but not, apparently, in the hindquarters. It is also possible that the mechanisms responsible for the conversion of L-arginine to nitric oxide would be influenced by the animal species or the biological preparations used.

Methylene blue (30 μM) inhibited the relaxant response of the rat aorta to the hydroalcoholic extract from *E. uniflora*. Methylene blue blocks guanylate cyclase and induces a reduction in the basal level of cGMP (Inoue et al 1991). In our experiments the extracts were incapable of relaxing vascular rings with intact endothelium after K^+-induced contraction (40, 60, 80 mM) (data not shown). Furchgott (1983) also found that endothelium-derived relaxing factor was more effective against receptor-mediated contractions than KCl-induced contractions. On the second cumulative application of the hydroalcoholic extract, the arteries were no longer relaxed 0·5 h and 2 h after the first determination of the curve of the relaxation induced by the hydroalcoholic extract. When desensitization to the hydroalcoholic extract was developed, relaxation by sodium nitroprusside was not affected. The desensitization induced by the hydroalcoholic extract did, however, affect relaxation by acetylcholine, suggesting that this desensitization interferes with the activity of the endothelial layer.

This interference does not appear to have been a result of a lesion in the endothelium. In our experiments, we were unable to supply pharmacological proof that the extract caused lesion in endothelium, because preincubation with increasing concentrations of the hydroalcoholic extract (30–300 $\mu g\ mL^{-1}$) reduced the maximum contractile response of isolated thoracic aorta to noradrenaline (10–300 nM) (data not shown). It was also observed that in the 4 h after obtaining the first curve there was partial recovery of the relaxant effect of the extract, probably indicating non-interference in the integrity of the endothelial cells. The hydroalcoholic extract might, therefore, induce an inhibitory action on binding, on the utilization of the substrate L-arginine (Chiesi & Schwaller 1995), or on other biochemical events.

Another important point to consider is that this inhibitory action disappeared after fractionation of the hydroalcoholic extract, indicating that the polyphenols (flavonoids and ellagitannins) present in these fractions could have stimulated endothelial nitric oxide synthase activity. In addition, the results of this study accord with those from previous work by Russel & Rohrbach (1989) and Fitzpatrick et al (1993), who observed that the stimulation of endothelial nitric oxide synthase activity by polyphenols leads to a vasodilatory effect on these arteries. The desensitization experiments suggest that it is the formation of nitric oxide that becomes tachyphylatic rather than its effect. The lack of tachyphylaxis with the ethyl acetate and aqueous fractions might suggest different components in these extracts which cause relaxation via different mechanisms.

In summary, this study demonstrates that in the rat thoracic aorta the hydroalcoholic extract of the leaves of *E. uniflora*, and its fractions, have a graded and endothelium-dependent vasorelaxant effect, an action which seems to be predominantly mediated by release of nitric oxide or nitric oxide-derived substances and activation of the cGMP pathway. Such results might account for the beneficial effects of *E. uniflora* reported in traditional medicine. Chemical studies are in progress to isolate and characterize the active constituent(s) responsible for such effects.

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