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In-vitro vasodilatory activity of the hexanic extract of leaves and stems from *Piper truncatum* Vell. in rats

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Abstract

Several plants from the Brazilian Tropical Forest are used in folk medicine for treatment of hypertension and asthma. In this study, we investigated the effects of hexanic extracts of leaves (HLE) and stems (HSE) from *Piper truncatum* on the contractility of cardiac, vascular and tracheal smooth muscles. Twitches of cardiac muscles obtained with electrical stimulation were recorded before and after exposure to increasing concentrations of hexanic extracts. HLE and HSE respectively reduced significantly the amplitude of twitches to 57.05 ± 11.63 and $61.58 \pm 5.70\%$ of control in the presence of $100\,\mu g\,mL^{-1}$. Contractile response to a single concentration of adrenaline (epinephrine) was measured before and after exposure of rat aorta rings to HLE and HSE. Both extracts inhibited aorta contraction in a concentration-dependent manner. The concentration of 50% inhibitory effect (IC50) was 32.3 ± 13.8 and $47.0 \pm 23.8\,\mu g\,mL^{-1}$ for HLE and HSE, respectively, in aorta with intact endothelium. HLE and HSE also reduced the acetylcholine-precontracted trachea in a concentration-dependent manner with maximal effect observed at 250 and $350\,\mu g\,mL^{-1}$, respectively. Vasodilatation and trachea relaxation induced by HLE and HSE could explain the use of *Piper* extracts to reduce blood pressure and bronchospasm.

Introduction

The Piperaceae family, circumscript to the order Piperales, is widely distributed in the tropical and subtropical regions of the world. Plants of the genus *Piper*, one of the most representative of the family (dos Santos et al 2001), are largely used in folk medicine as anti-inflammatory and analgesic agents (Domínguez & Alcorn 1985), in the treatment of asthma, bronchitis, fever and gastrointestinal diseases (for a review see Parmar et al 1997; di Stasi et al 2002). Other studies on *Piper* species revealed compounds with cytotoxic (Murakami et al 2000; Cheng et al 2003), bactericidal (Dorman & Deans 2000; Setzer et al 2000) and antifungal (Navickiene et al 2000; Lopez et al 2002; Vasques-da-Silva et al 2002) activity. In addition to an antihypertensive activity (Parmar et al 1997), plants of this family were also shown to induce relaxation of smooth muscle (Seitz et al 1997; Martin et al 2000) and to have affinity for central nervous receptors (Dinh et al 2002). However, until now, no pharmacological properties of *Piper truncatum* Vell. have been studied. As part of a Program to investigate the biological properties of these species, the purpose of this study is to evaluate the effects of hexanic extracts of *P. truncatum* leaves (HLE) and stems (HSE) on the contraction of cardiac and smooth (aorta and trachea) muscles. Both HLE and HSE have a negative inotropic effect in cardiac and smooth muscles.

Materials and Methods

Plant material

Piper truncatum Vell was collected from Tijuca Forest, Rio de Janeiro, Brazil. Botanical identification was carried out at Nucleo de Pesquisa de Produtos Naturais

by Dr Elsie Franklin Guimarães and a voucher specimen (RB-389602) was deposited in the Herbarium of Jardim Botânico, Rio de Janeiro, Brazil. Fresh leaves (930 g) and stems (1620 g) were extracted separately with pure hexane at room temperature. The extraction process was performed over 3 days during which time the hexane was changed every 24 h. The solutions were concentrated under reduced pressure to yield the hexane extract from leaves (5.9 g) and from stems (1.2 g).

Preparation of papillary muscles

The following protocols were approved by Animal Care and Use Committee at Universidade Federal do Rio de Janeiro. Left papillary muscles were dissected from Wistar rats, 200–250 g, for isometric tension recording. They were mounted in vertical chambers in which one end of each muscle was attached to a force transducer (Grass, FT 03) and the other end fixed to the bottom. Chambers were filled with Tyrode solution (composition in mM: NaCl 130; KCl 5; $MgCl_2$ 1; NaH_2PO_4 0.5; $NaHCO_3$ 24; dextrose 5.6; $CaCl_2$ 2.5) and were oxygenated with carbogen gas (95% O_2–5% CO_2) at $37 \pm 1°C$. Muscular twitches were obtained with electrical stimulation at a rate of 1 Hz and 2 ms duration. The electrical signal was digitalized (Digidata 1322; Axon Instruments, Inc.), conditioned by a Cyberamp (Axon Instruments, Inc.) and then displayed and stored on a computer for future analysis using Axoscope software (Axon Instruments, Inc.). Increasing concentrations of hexanic extracts were added into the Tyrode solution and twitches were recorded before and after extract exposure.

Preparation of aortic and tracheal rings

Thoracic aorta and trachea were dissected from male Wistar rats, 200–250 g, cleaned of connective tissue and prepared for isometric tension recording. Aorta and trachea were cut in 2- to 3-mm rings and were placed in a vertical chamber (internal volume 10 mL) filled with saline solution (composition in mM: NaCl 120; KCl 5.9; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$ 18; $CaCl_2$ 2.5; glucose 11 (pH 7.4)) and oxygenated with carbogen gas at $37 \pm 0.5°C$. Each aorta ring was mounted between two hooks in which one was attached to a force transducer (Grass mod. FT-03). The transducer signal was conditioned by a Cyberamp (Axon Instruments, Inc.) and then displayed and stored on a computer for future analysis using Axoscope software (Axon Instruments, Inc.). Preparations were stabilized under 1 g resting tension for 2 h before initiating the experimental protocol. Contractile response to a single concentration of adrenaline (epinephrine) (10 μM) was measured before and after exposure of aorta rings to increasing concentrations of plant extract. Adrenaline-induced contracture was also observed at the beginning and end of each experiment followed by exposure to acetylcholine (10 μM) to test the integrity of endothelium. Endothelium was considered intact if acetylcholine-induced relaxation of precontracted aorta was greater than 50% (Togna et al 2001). In some experiments, the extract was tested in aorta in which the endothelium was mechanically removed. Removal of functional endothelium was confirmed by the lack of relaxation (< 50%) in the presence of acetylcholine. The trachea rings were also connected to a force transducer for isometric tension recording. Rings were equilibrated under 0.5 g resting tension for 30 min and then were contracted by exposing to acetylcholine (10 μM). During the plateau phase, increasing concentrations of leaves or stems extract (50, 100, 150, 250, 350 $\mu g\,mL^{-1}$) were added to the preparation. The hexanic extract of *Piper truncatum* Vell. was dissolved in dimethyl sulfoxide (DMSO) as stock solution of 50 $mg\,mL^{-1}$. Control experiments, performed in presence of DMSO alone, at the same concentrations of the extract tested, demonstrated that the solvent did not affect the contractile response of isolated papillary muscle, aorta or trachea. Adrenaline and acetylcholine were obtained from Sigma Chemical Co. (St Louis, MO).

Statistics

Relaxation induced by *Piper truncatum* Vell. was expressed as percentage of maximal tension observed in the presence of adrenaline. All data were expressed as mean ± s.e.m. and differences between different concentrations were considered statistically significant when $P < 0.05$ using paired Student's *t*-test. For comparison between two groups, Mann–Whitney test was used for non-normal distribution of the data. The dose–response curves for HLE or HSE were fitted to the contractile response data using the equation: $y = y_o + ae^{-bx}$, where y was the percentage of isometric tension.

Results

Effect of hexanic extract of *P. truncatum* leaves (HLE) and stems (HSE) on isometric tension in papillary muscle

Decrease in the isometric tension of papillary muscles was observed when they were exposed to increased concentrations of HLE. Figure 1A shows representative tracings of twitches of electrically stimulated papillary muscles in the absence and presence of different concentrations of HLE (25–450 $\mu g\,mL^{-1}$). The inhibitory effect of HLE and HSE was concentration dependent (Figure 1B, C). HLE significantly reduced the amplitude of twitches to 57.05 ± 11.63 ($n = 8$, $P < 0.01$) and $6.64 \pm 5.26\%$ ($n = 8$, $P < 0.01$) of control in the presence of 100 and 450 $\mu g\,mL^{-1}$, respectively. Similar results were observed with HSE. At 100 and 450 $\mu g\,mL^{-1}$ of HSE, the amplitude of twitches was decreased to 61.58 ± 5.70 ($n = 8$, $P < 0.01$) and $19.57 \pm 5.77\%$ ($n = 8$, $P < 0.01$) of control, respectively. Extract of *P. truncatum* seems to be an effective negative inotropic agent because it significantly reduced cardiac contractile performance. Reduction of contractility by HLE or HSE was not reversed after 30 min washout.

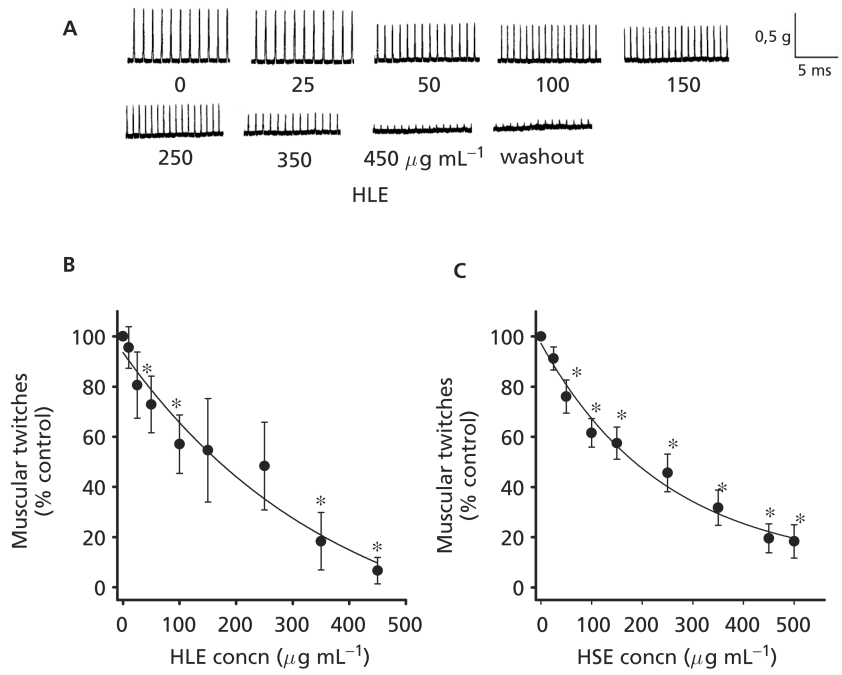

Figure 1 Effect of hexanic extracts of leaves (HLE) and stem (HSE) from *P. truncatum* on rat cardiac muscle contractility. A. Typical tracings of isometric tension in papillary muscle from Wistar rat before and after exposure to HLE (25–450 $\mu g\,mL^{-1}$) and 30 min after washout. B and C. Muscular twitches as percentage of control (before addition of extract) vs HLE and HSE concentration, respectively. Data are means ± s.e.m. of 8 experiments. $^*P < 0.05$, compared with control value.

Effect of HLE and HSE on isometric tension in vascular smooth muscle

The activity of *P. truncatum* hexanic extract on vascular smooth muscle was investigated in thoracic aortic rings pre-contracted with adrenaline. HSE promoted relaxation of pre-contracted aorta in a dose-dependent manner (Figure 2A). Complete relaxation of smooth muscle was observed in the presence of 250 $\mu g\,mL^{-1}$ of HSE when aorta had intact endothelium. The concentration of HSE necessary to reduce by 50% the maximal contraction induced by adrenaline (IC50) in aorta with intact endothelium was 47.0 ± 23.8 $\mu g\,mL^{-1}$ (Table 1). Similar results were observed with HLE. The IC50 for HLE (32.3 ± 13.8 $\mu g\,mL^{-1}$) in aorta with intact endothelium (Table 1) was not significantly different from HSE ($P = 0.88$). The same experimental protocol was repeated in aortic rings with disrupted endothelium. No significant difference was observed in the dose–inhibitory response curves for intact and for endothelium-free aorta in the presence of HSE (Figure 2C). Mechanical removal of endothelium did not alter the IC50 values of either extract for inhibiting the adrenaline-induced contraction. Vasodilatation induced by HLE and HSE was totally reverted after 30 min washout.

Effects of HLE and HSE on isometric tension in trachea smooth muscle

To investigate whether the inhibitory effects of *P. truncatum* hexanic extracts were selective to vascular smooth muscle, rat trachea muscles were exposed to increasing concentrations of HLE or HSE (50–350 $\mu g\,mL^{-1}$). HLE reduced the acetylcholine-precontracted trachea in a concentration-dependent manner (Figure 3A). Maximal contracture was completely inhibited by 250 $\mu g\,mL^{-1}$ of HLE or 350 $\mu g\,mL^{-1}$ of HSE. Concentration–response curves (Figure 3B, C) showed that the relaxing effect of the extracts on acetylcholine-precontracted trachea was very similar with an IC50 of 175.6 ± 38.7 $\mu g\,mL^{-1}$ and 213.0 ± 28.7 $\mu g\,mL^{-1}$ for HLE and HSE, respectively (Table 1). Maximal relaxation was observed at 250 $\mu g\,mL^{-1}$ of HLE by which the contracture was reduced to 14.60 ± 10.13% of control (Figure 3A). HSE completely inhibited acetylcholine-induced contracture at 350 $\mu g\,mL^{-1}$ (Figure 3B). At the end of each experiment, trachea was washed for 30 min and responsiveness to acetylcholine was again tested. The contractile response of trachea was totally reverted after that period of washout.

Discussion

This study was designed to examine the effects of hexanic extracts from leaves (HLE) and stems (HSE) of *Piper truncatum* on the contractility of cardiac and vascular and tracheal smooth muscles.

In recent years, several studies have investigated the activity of compounds isolated from plants on muscle contractility. Kaurenoic acid from *Viguiera robusta*

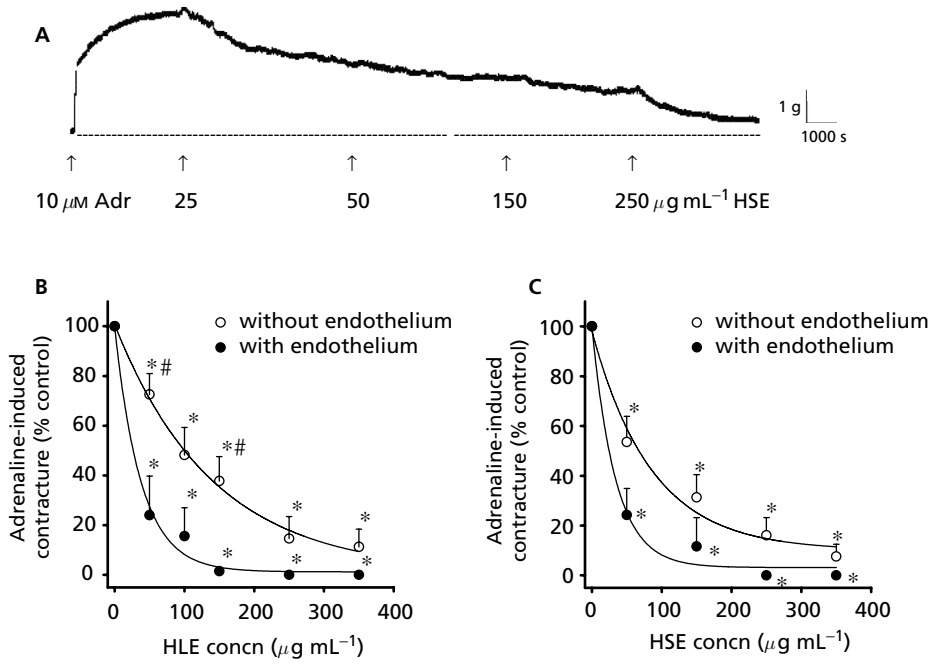

Figure 2 Effect of hexanic extracts of *P. truncatum* on contracture induced by adrenaline in rat aortic rings. A. Representative tracing of tension in response to 10 μM adrenaline (Adr). Hexanic extract of stem (HSE) from *P. truncatum* caused significant relaxation of pre-contracted isolated aorta. B and C. Curves fitted through the data points in the absence and presence of increasing concentrations of HLE and HSE, respectively. Data presented are mean ± s.e.m. of 8 experiments. $*P < 0.05$, compared with control value; $\#P < 0.05$, compared with aorta with intact endothelium.

Table 1 Reduction of contractile response by HLE and HSE in rat cardiac and smooth muscles

	IC50 (μg mL^{-1})	
	HLE	HSE
Aorta	32.3 ± 13.8	47.0 ± 23.8
Papillary muscle	171.8 ± 43.4	206.0 ± 27.3
Trachea	175.6 ± 38.7	213.0 ± 28.7

Percentage of contractile response inhibition was plotted against HLE or HSE concentrations to determine the concentration that reduced by 50% the maximal contraction (IC50). Data are expressed as mean ± s.e.m. of 8 experiments. There was no significant difference in IC50 between HLE and HSE in all three different muscles tested.

(Asteraceae) inhibited the contractility of rat carotid artery (Tirapelli et al 2002) and extracts of *Magifera indica* stem bark and *Pluchea ovalis* roots have a similar effect on rat tracheal smooth muscle (Agbonon et al 2002). Inotropic effects of extracts of *Psidium guajava* L. (guava) leaves on the guinea-pig atrium have also been reported (Conde-Garcia et al 2003). However, much less is known about the effects of species of the Piperaceae family on muscle contractility. Kavain, the principal biologically active compound of *Piper methysticum*, was shown to decrease the carbachol-induced contraction of guinea-pig ileum (Seitz et al 1997) and rat tracheal ring preparations (Martin et al 2000).

We showed that HLE and HSE of *Piper truncatum* significantly inhibited twitches from papillary muscles in a concentration-dependent manner, and reduced adrenaline-induced contraction on rat aortic rings and acetylcholine-induced contraction on rat trachea. However, reduction of cardiac muscular twitches and trachea contractile response were less pronounced than the relaxation observed in vascular smooth muscle. In fact 250 μg mL^{-1} of the extracts decreased the contraction of the papillary muscle to about 50% of the electrically induced response. The same concentration of extracts had a much more pronounced effect in reducing the contractility of the aorta. Studies with other plant extracts showed a reversible inhibitory effect on vessels (Tirapelli et al 2002) and tracheal (Agbonon et al 2002) muscle contractility. In our study, the effect of *P. truncatum* extracts was also totally reverted by a 30 min washout of the preparation.

The mechanism of action of plant extracts and their components on muscle contraction is still under investigation. Recently, it was proposed that the relaxing effect of shikonin/alkannin (SA), a naphthoquinone analogue isolated from roots of *Macrotomia euchroma*, on rat aortic rings was endothelium-dependent (Hu et al 2004). It was also shown that flavonoid compounds from a variety of plants induce an endothelium-dependent (NO-mediated) vasodilatation (Stoclet et al 1999). However, although flavonoids were present in extracts of *Piper* species (Parmar et al 1997), our data demonstrated that the

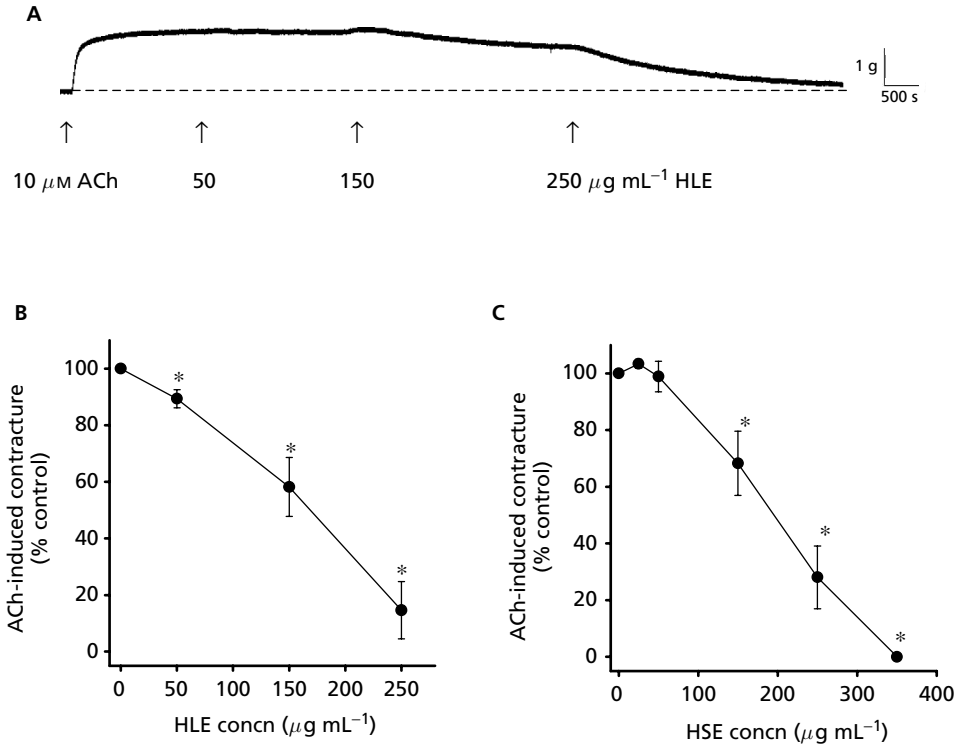

Figure 3 Effect of hexanic extracts of *P. truncatum* on the contractile response induced by acetylcholine on rat trachea. A. Representative recording of contracture induced by 10 μM of acetylcholine (ACh) before and after exposure of increasing concentrations of HLE. Contracture as percentage of control (before addition of extracts) vs HLE (B) and HSE (C) concentration. Data presented are mean ± s.e.m. of 8 experiments. $^*P < 0.05$, compared with control value.

relaxation induced by hexanic extracts of *P. truncatum* in isolated rat aortic rings was not dependent on the presence of functional endothelium. This indicated that the relaxing effect of *P. truncatum* probably is not related to the presence of flavonoids. The results also suggested that vasodilatation of aorta could be mediated by a mechanism other than endothelium-derived nitric oxide (NO) generation, probably by a direct effect on smooth muscle cells.

Bronchodilatory agents can be tested using cholinergic agonists such as acetylcholine (Takagi et al 1992) to induced bronchoconstriction in tracheal smooth muscle. Our results indicate that *P. truncatum* extracts inhibited the tracheal response induced by stimulation of muscarinic receptor with acetylcholine. Although relaxation observed in trachea seems to be less pronounced than that in vascular tissue, these findings suggest that *P. truncatum* extracts are not selective to vascular smooth muscle. Complementary studies should be performed to identify the mechanism of action of extracts in cardiac and smooth muscles.

Conclusions

An early study of plants popularly used in the Brazilian Tropical Atlantic Forest has shown that Piperaceae belongs to the group of families with the highest number of reported medicinal species. However, although in Brazilian folk medicine extracts from *Piper* species are used for the treatment of hypertension and asthma, no studies had been done to investigate the pharmacological basis of this use. Our results showed that crude extracts from leaves (HLE) and stem (HSE) of *Piper truncatum* significantly reduced adrenaline-induced contraction on rat aortic rings, acetylcholine-induced contraction of rat trachea and the contractility of cardiac muscle. Vasodilatation of aorta induced by HLE and HSE is likely to be mediated by a direct effect on smooth muscle cells because it was not dependent on the integrity of vascular endothelium. Thus, our findings suggest that the use of *P. truncatum* crude extracts in the treatment of hypertension and asthma in traditional medicine could be explained by their broncho-vasodilatory activity. The similarity in the results obtained with the two extracts indicate that the active compound responsible for the investigated activities should be present on both leaves and stem extracts.

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