VASCULAR SMOOTH MUSCLE RELAXANT PROPERTIES OF THE LEAF METHANOL EXTRACT OF *BIDENS PILOSA* LINN (ASTERACEAE)

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Summary

The present study investigated the mechanism underlying the vasorelaxant effect of the leaf methanol extract of *Bidens pilosa* on the rat isolated aorta and identification of the phenolics and stilbene compounds found in the plant extract. Powerful antioxidant compounds known to promote vascular relaxation were found in the leaves of *B. pilosa*. The vasorelaxant activity of the plant extract is most likely due to its phenolics and stilbenes. The leaf methanol extract of *B. pilosa* was more potent in relaxing norepinephrine (NE)- than KCl-induced contractions. The relaxant effect of the plant extract was not dependent on the presence of endothelium and was not antagonized by inhibition of nitric oxide (NO), prostaglandine synthesis or potassium channel blockers. The extract did not antagonize NE-induced intracellular calcium entry. The plant extract caused the relaxation of tonic, but failed to inhibit the phasic, component of the contraction induced by NE in rat aorta. Therefore, the vasorelaxant profile of *B. pilosa*, is not that of typical Ca^{2+}-entry blockers which preferentially inhibit the contraction induced by strong depolarisations. All of these findings indicated that *B. pilosa* methanol extract relaxes rat thoracic aorta by virtue of its Ca^{2+} channel blocking properties in vascular smooth muscles.

Keywords: *Bidens pilosa*, vasorelaxation, aorta, rat, Ca^{2+} channels.

Introduction

Bidens pilosa Linn (Asteraceae) is an erect annual herb with yellow disk-florets and white ray-florets. The leaves are pinnate (rarely simple), glabresent; the leaftet-margins are regularly serrate. The fruits are strongly adherent. *B. pilosa* is highly reputed in folk African traditional medical practice, especially in the western region of Cameroon as well as in parts of Central America, as a potent hypotensive agent (1). The Kallawaya in the Bolivian Andes take it in mate in combination with the juice of *Valeriana officinalis* and of peas to lower blood pressure (2). In some parts of Africa, it is used in the treatment of malaria, otitis, chronic ulcers, diabetes, diarrhoea and dysentery (3, 4). It is also used as a diuretic and choleretic (5). Phytochemical studies of the plant have revealed mainly polyacetylenes and flavonoids. Several flavonoids have been isolated, among them the chalcones and a series of corresponding glycosides in the leaves (6, 7, 8, 9, 10) with antimicrobial and anti-inflammatory properties (11, 12). Jäger et al. (13) demonstrated that *B. pilosa* extract inhibits prostaglandin-synthesis. Prostaglandins are involved in the regulation of the vascular tone and therefore the blood pressure. Recently, Ghiadoni et al. (14) found that in the developmental stage of hypertension, a dysfunctional endothelium can produce prostanoïds such as thromboxane A2, which causes vasoconstriction and platelet aggregation, and/or oxygen free radicals, which can destroy NO and cause vascular damage. It has also been reported that flavonoid compounds present in various plants may produce beneficial effects in cardiovascular diseases such as atherosclerosis, coronary artery disease and hypertension (15, 16) by increasing production of NO which could induce vasodilatation, platelet antiaggregation and inhibition of LDL oxidation. We recently reported that the leaf methanol extract of *B. pilosa* causes a reduction of blood pressure in hypertensive animals (17). Substances that induce a fall in blood pressure could act via one of several vasodilator mechanisms (18, 19).

The present study was designed to investigate the mechanism of the vasorelaxant effect of the leaf methanol extract of *B. pilosa* on rat isolated aorta. The roles of endothelium and calcium exchanges were more particularly explored. We also present here the results of the identification and quantification of phenolic and stilbene compounds in the leaves of *B. pilosa*.

Materials and Methods

Plant processing, and extract/ fraction preparation

Plant material was collected around the campus of the University of Yaounde I, Cameroon, in November. The voucher specimen is deposited at the Yaounde National Herbarium and registered under N° HNC/58742. The leaves of the plant species were air-dried and ground into a fine powder.

The methanol extract of *B. pilosa* was obtained by extracting exhaustively the powdered dried leaves (650 g) with methanol/dichloromethane ($MeOH/CH_2Cl_2$ 1:1). The solvent was evaporated at 80°C in a vacuum desiccator and sequentially washed with MeOH to give about 20g of dry extract. Four grammes of this extract were dissolved in 100 ml of distilled water to give a final concentration of 40 mg/ml. Dilutions of this extract in water were used in our experiments.

For fraction preparation, 100 g of dried powder were extracted exhaustively with 1 L of distilled water at boiling temperature for 30 min. The resulting solution (440 mL) was filtered, freeze-dried and partitioned between water and n-butanol. The n-butanol extract obtained was evapored under reduced pressure in a rotavapor. The dryness of the resulting extract was achieved in a vacuum dessicator with a yielded of 9.5 g. Part of this extract (6 g) was subjected to columm chromatography and eluted with water-MeOH (90 : 10). Fractions were freeze-dried and monitored by TLC coated with cellulose MN 300 and similar fractions were combined.

Standards and HPLC analysis

For tanins and phenolic acids: (-)-epicatechin, caffeic acid and rutin were obtained from Aldrich (St. Quentin Fallavier, France). Epigallocatechin, Epicatechingallate, Ellagic acid and Vanillic acid were obtained from Extrasynthese (Genay, France). Caftaric acid was provided by Dr Ursa Vorshek.

HPLC analysis with UV detection was used. A Hewlett-Packard Model 1090 with three low-pressure pumps and a diode array detector coupled to a Hewlett-Packard Chem Station was used for solvent delivery and detection. A Hewlett-Packard column packed with Nucleosil 100 C18 (250 x 4 mm, 5 µm particle size) was used for the stationary phase with a flow of 0.7 mL/min. The solvents used for separation were as follows: solvent A, 50 mmol/L dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B, 20% A with 80% acetonitrile; solvent C, 200 mmol/L orthophosphoric acid adjusted with ammonia to pH 1.5. Elution was performed with a gradient previously described (20). Detection was achieved at 280, 313, 365 and 520 nm.

For Stilbene compounds, monomers or oligomers and Astilbin

Trans resveratrol was purchased from Sigma (St Quentin Fallavier, France), Trans-piceid was obtained from cell suspension cultures as previously described (21, 22). Pallidol, (E)-ε-viniferin and astilbin were obtained from merlot stalks (23). Cis-piceid, Cis-resveratrol, Resveratrol-transdehydromer-O-ß-glucoside were isolated from *Vitis vinifiera* cells cultures (21, 24).

Separation and quantification of stilbenes were carried out by HPLC. A Hewlett-Packard Model 1090 with three low-pressure pumps and a diode ray detector coupled to a Hewlett-Packard Chem Station was used for solvent delivery and detection. A Hewlett-Packard column packed with Nucleosil 100 C18 (250 x 4 mm, 5 µm particle size) thermostated at 30°C was used as stationary phase with a flow rate of 0.5 mL/min. The solvents used for the separation were as follows: solvent A, Acetic acid in H_20, pH 2.4; solvent B, 20% phase A with 80% Acetonitrile; elution was performed with a gradient previously described (25). Detection was carried out at 280, 286, 306 and 321nm.

Tissue preparation and experimental design

Male Wistar rats (250 – 300 g) were killed by decapitation and isolated aortic segments mounted for isometric recording as previously described (26). Briefly, the thoracic aorta was carrefully excised and adhering fat and connective tissue removed. Vessels were cut into rings 3 - 4 mm in length. Some rings were denuded of endothelium by gentle rubbing of the luminal surface with cotton thread. Two stainless steel wires hooks were inserted into the lumen of the aortic ring. One wire was attached to an isometric transducer connected to a Physiograph Narco Bio-System and the other anchored to a plastic holder. The ring was then placed in a 10 ml organ bath at 37°C containing oxygenated (95% O2 and 5% CO2) Krebs buffer solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1. The initial resting tension applied to the aortic ring was 1g; the aortic rings were equilibrated for 90 min. During this time, the Krebs buffer was changed at 15 min. intervals. Tissues were contracted submaximally with norepinephrine (NE, 10^{-8} M) and then relaxed with acetylcholine (ACh., 10^{-5} M) to test for the intactness of the endothelium. For experiments with endothelium, a relaxation lower than 84% of NE-induced response was taken as an indicator that the endothelium was partially destroyed and the corresponding segment was discarded. For experiments without endothelium (see below) only segments showing no ACh-induced relaxation were used.

After ACh testing, the aortic rings were washed with Physiological salt solution (PSS) three times during the next hour, prior to the next sequence. After equilibration, the following experiments were performed:

In a first group of experiments, the effects of various concentrations of the methanol extract of *B. pilosa* were determined on precontracted preparations.

Aorta rings were initially exposed to a single concentration of KCl (60 mM) or NE (10^{-6} M). Control experiments had shown that in both cases contraction was maximal within 10 min. and stable for at least 240 min. After the contractile response had plateauded, *B. pilosa* extract was added in progressively increasing cumulative concentrations. Rings were allowed to reach a new steady-state tension before each successive addition of the extract (aprox. 50 min.). Results were expressed as the percentage relaxation of the initial contraction induced by NE or KCl. Relaxation of aortic rings with or without endothelium were compared following application of the leaf methanol extract of *B. pilosa*.

In the second group of experiments, tissues containing intact endothelium were used to test the ability of (a) a nitric oxide synthase inhibitor, N^w-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), (b) a prostaglandin synthesis inhibitor, indomethacin (10^{-4} M), (c) a blocker of ATP-sensitive K^+ channels, glibenclamide (10^{-6} M), or (d) a non specific K^+ channel inhibitor, tetraethylammonium (TEA, 10^{-6} M), to antagonize the effect of *B. pilosa* extract. The experimental design was similar to that described in the first set of experiments. The contractile agent was NE for L-NAME or indomethacin, and KCl for glibenclamide or TEA. L-NAME and indomethacin were added 15 min. before NE, while glibenclamide and TEA were added 20 min. after KCl (27).

In the third group of experiments, we studied the effects of the methanol extract of *B. pilosa* on (a) intracellular calcium mobilization or (b) extracellular calcium entry. For studying NE-induced mobilisation of intracellular Ca^{2+} (27), aortic rings were incubated in normal PSS, then initially exposed to KCl (80 mM) until the contractile response reached a steady tension (control contraction), then washed in PSS for 15 min. The media were then changed to Ca^{2+}-free PSS (1 mM EGTA) and tissues incubated for 60 min. in the presence or absence of *B. pilosa* extract. The aortic rings were then stimulated by 10^{-6} M NE for 5 min. to produce a typical phasic contraction. The response to NE was expressed as percentage of the control KCl-induced contraction. For studying NE-stimulated Ca^{2+} entry, aortic rings were initially contracted with 10^{-6} M NE in normal PSS (control contraction). After washing, rings were incubated in Ca^{2+} -free PSS (1 mM EGTA) with verapamil for 10 min. The aortic rings were then incubated for 60 min. in the presence of 10^{-6} M NE (which produced a transient phasic contraction), and in the absence or presence of various concentrations of *B. pilosa* extract. The concentration of $CaCl_2$ in the bathing media was then increased to 2.5 mM and the tonic contraction recorded. The contractile responses to $CaCl_2$ were expressed as percentages of the initial control NE-induced contraction.

In the fourth set of experiments, the relaxation kinetics of rings with intact endothelium were studied by contracting them with NE (10^{-6} M) for 30 minutes and then allowing them to relax in the presence of a single concentration of *B. pilosa* fractions. The times taken for 100% relaxation (t_{100}) and the minimal concentration capable of inducing total relaxation were determined.

Drugs

Norepinephrine, Acetylcholine, EGTA, nifedipine, indomethacin, N^w-nitro-L-arginine methyl ester, glibenclamide and tetraethylammonium were purchased from Sigma Chemical Company, St. Louis, MO, USA. Ascorbic acid (0.57 mM) was added to each solution of NE, made up freshly every day. Glibenclamide, EGTA and nifedipine were initially dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution and further dilutions were made in PSS. The final DMSO concentrations did not produce significant effects on contractile responses.

Expression of results and statistical analysis

Results are given as means ± SEM. The number of replications is indicated by n. The one way analysis of variance (ANOVA) followed by the student's *t*-test was used to determine the statistical significance of differences between treatments. Probability values (p) less than 0.05 were considered to be significant.

Results

Phytochemical analysis of B. pilosa fractions

The phenolic composition of *B. pilosa* extract and liquor is given in Tables 1and 2. Powerful antioxidant compounds such as gallotanins, phenolics acids, cinnamates and stilbenes compounds are present in this plant variety. We identified 12.3% of phenolics present in the extract with a high level of ellagic acid, caffeic acid, caftaric acid, epigallocatechin and epicatechin, rutin, trans-piceid, viniferin, resveratrol-transdehydromer-O-ß-Glucoside and astilbin. In comparison, the liquor contained ten times less of phenolics (1.23%), with the presence of caffeic acid, caftaric acid, rutin, viniferin, resveratrol-transdehydromer-O-ß-Glucoside and astilbin.

Table 1: Phenolic compounds found in liquor or extract of *B. pilosa*

Phenolic compounds	*B. pilosa* liquor (mg/g)	*B. pilosa* extract (mg/g)
Ellagic acid	ND	10.43
Caftaric acid	2.44	8.64
Vanilic acid	0.99	4.05
Caffeic acid	2.8	9.73
Epigallocatechin	ND	9.79
Epicatechingallate	0.94	2.72
Epicatechin	ND	7.53
Rutin	2.83	11.33
Trans-resveratrol	0.036	0.39
Trans-piceid	0.46	7.46
Cis-resveratrol	0.087	1.08
Cis-piceid	ND	ND
Viniferin	1.06	9.46
Astilbin	0.68	12
Resveratrol-transdehydromer-O-βglucoside	ND	28.58
Total amount	12.323	123.18

ND : Not detected

Table 2: Stilbene compounds found in fractions of *B. pilosa*

Stilbene compounds	F 40 (mg/g)	F42 (mg/g)	F 43 (mg/g)	F45 (mg/g)
Trans-resveratrol	0.97	2.92	4.16	1.63
Trans-piceid	ND	ND	ND	1.63
Cis-resveratrol	ND	ND	0.14	0.64
Cis-piceid	ND	ND	0.09	0.35
Viniferin	ND	ND	ND	ND
Astibin	0.97	2.92	4.16	ND
Pallidol	ND	0.34	26.45	13.59

ND: Not detected.

Effects of *B. pilosa* extract on NE-induced contraction

The relaxation induced by the methanol extract of *B. pilosa* or fractions from n-butanol extract on intact aortic rings was delayed and gradual in the presence of NE (table 3). The plant extract provoked a slowly evolving relaxation which achieved its maximum over a period of 40 to 150 min. depending on the extract concentration. The minimal concentration capable of provoking total vasorelaxation was 30µg/mL. Figure 1 illustrates the effect of cumulative concentrations of the leaf methanol extract of *B. pilosa (*25 – 200 µg/mL) on isolated rat aorta precontracted by NE in the presence or absence of endothelium. As shown, the relaxation was not endothelium-dependent.

Table 3: Effect of the methanol leaf extract of *B. pilosa* and the fraction from n-butanol on NE-induced contraction of intact aortic rings

B. pilosa extract	Concentration (µg/mL)	% Relaxation	Time for total relaxation (min)
Methanol extract	30	22 ± 5	-
	100	102.96 ± 4.68	60 – 90
	200	118.35 ± 8.49	40 – 50
F 84 – 104	200	111.57 ± 3.08	60 -90
F 50 –70	200	109.41 ± 9.87	40 – 60
F 84/85	200	137.88 ± 4.09	60 – 80
F 59 – 70	200	79.41 ± 3.57	-
F 37	20	45.49 ± 1.97	-
	30	51.03 ± 5.46	-
	40	53.48 ± 2.16	-
	200	110.27 ± 6.38	30 – 45
F 44	30	41.62 ± 5.64	-
	40	108.58 ± 4.69	70 - 80
	100	114.18 ± 2.33	60- 80
	200	119.71 ± 8.39	30 – 40
F 41	30	108.58 ± 4.69	80 – 95
F 46	30	111.71 ± 3.58	80 – 100
F 42	30	103.05 ± 3.65	100 –120
F 45	30	118.68 ± 9.84	90 – 100
F 39	30	106.08 ± 6.49	120 –150
F 37	30	118.36 ± 5.78	100 – 130
Trans-piceid	30	24.61 ± 3.96	-
Trans-resveratrol	30	107.36 ± 8.79	40 – 60
Isoquercitrine	15	40.07 ± 5.68	-
Quercetin	15	78.85 ± 2.58	-
Rutine	15	2.07 ± 1.35	-

The responses are expressed as percent of the maximal relaxation. n = 4.

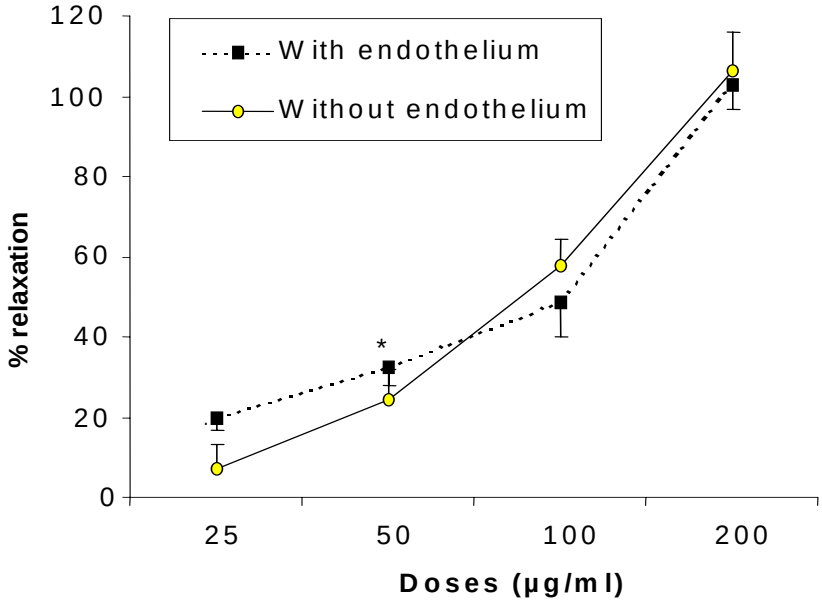

Figure 1: Effects of *B. pilosa* extract added cumulatively to aortic rings with and without endothelium precontracted by norepinephrine. Each point represents the mean ± SEM; n = 15. **p <0.01 vs aorta without endothelium.

Effects of the methanol extract of *B. pilosa* extract on KCl-induced contraction.

In the presence of KCl, the relaxation induced by the plant extract was immediate and was obtained with higher concentrations (0.5 – 8 mg/ml). Figure 2 shows that cumulatively increased concentrations of *B. pilosa* extract in aortic rings precontracted by KCl (60 mM) resulted in a concentration-dependent relaxation in endothelium-intact and-denuded aorta.

B. pilosa extract showed an about 20 to 40 fold higher potency to relax contractions induced by NE as compared to those induced by KCl (active minimal concentration 30 µg/mL and 0.5 mg/mL, respectively, on intact aortic rings). In the same experimental conditions, the relaxation induced by the calcium entry blocker, nifedipine (3 x 10^{-8} M) was higher for KCl- than for NE-induced contractions, either in the presence (93 ± 3% and 34 ± 2%) or in the absence (90 ± 2 % and 26 ± 1%, respectively) of endothelium. As well, the vasorelaxing activity of *B. pilosa* extract was not influenced by L-NAME (10^{-4} M), indomethacin (10^{-4} M), glibenclamide (10^{-6} M) or TEA (10^{-6} M) (data not illustrated).

Effects of *B. pilosa* on receptor-dependent intracellular calcium mobilization or cellular calcium entry

Intracellular calcium mobilization was explored by measuring the response to NE in Ca^{2+}-free medium (1 mM EGTA). In these conditions, NE produced a phasic contraction which average 32 ± 2% (n = 14) of the control. Pretreatment (60 min.) with the leaf methanol extract of *B. pilosa* (50 – 200 µg/mL) did not significantly influence NE response (Fig. 3).

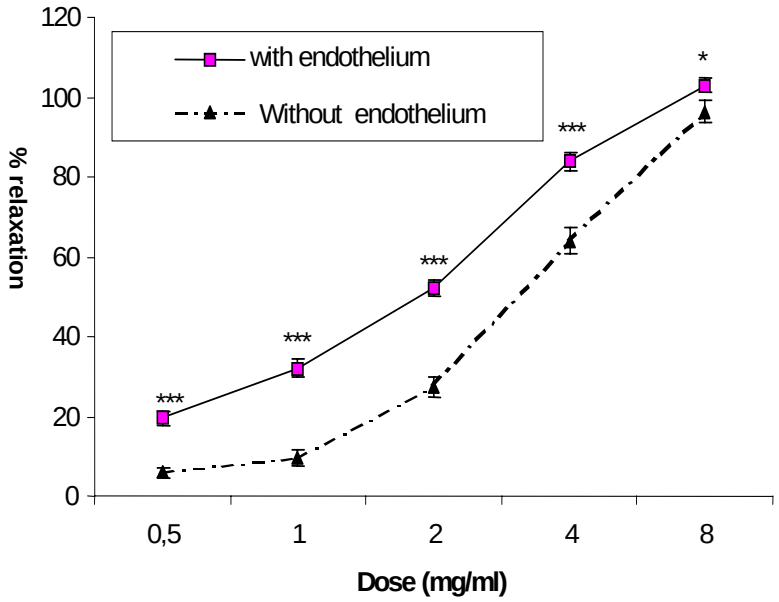

Figure 2: Effects of *B. pilosa* extract added cumulatively on KCl-induced contraction of rat aortic rings with and without endothelium. Each point represents the mean ± SEM; n = 25. **p < 0.01 vs aorta without endothelium.

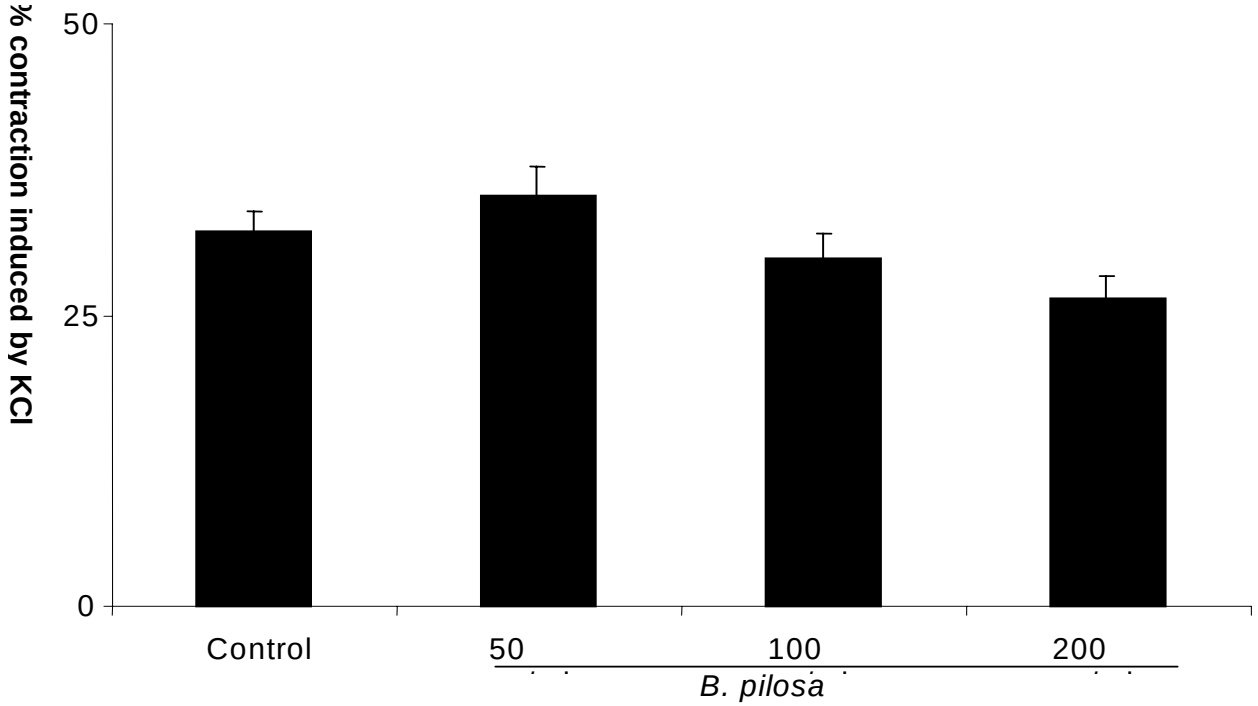

Figure 3: Effects of *B. pilosa* extract on the phasic contractions of the aortic rings induced by norepinephrine in depolarizing Ca^{2+}-free high-K^{+} medium. *B. pilosa* extract was added 60 min. before induction of the phasic tension using 10^{-6} M norepinephrine. Responses are expressed as percentages of the initial 80 mM KCl-induced contractions. Each bar represents the mean ± SEM; n = 14.

Extracellular calcium entry through receptor-dependent calcium channels was explored by measuring the effect of the addition of verapamil to calcium-free medium (1 mM EGTA) in the presence of NE. In these conditions, the addition of 2.5 mM $CaCl_2$ produced a sustained tension which averaged 62 ± 3% (n = 24) of the control contraction. As shown in Figur 4, the leaf methanol extract of *B. pilosa* (50 - 200 µg/ml) partially inhibited in a concentration-dependent manner the tonic contraction induced by the addition of calcium in the presence of NE. The maximum active tonic response was 51 ± 2% and 32 ± 3% ($p < 0.05$), respectively, at the concentration of 100 and 200 µg/mL.

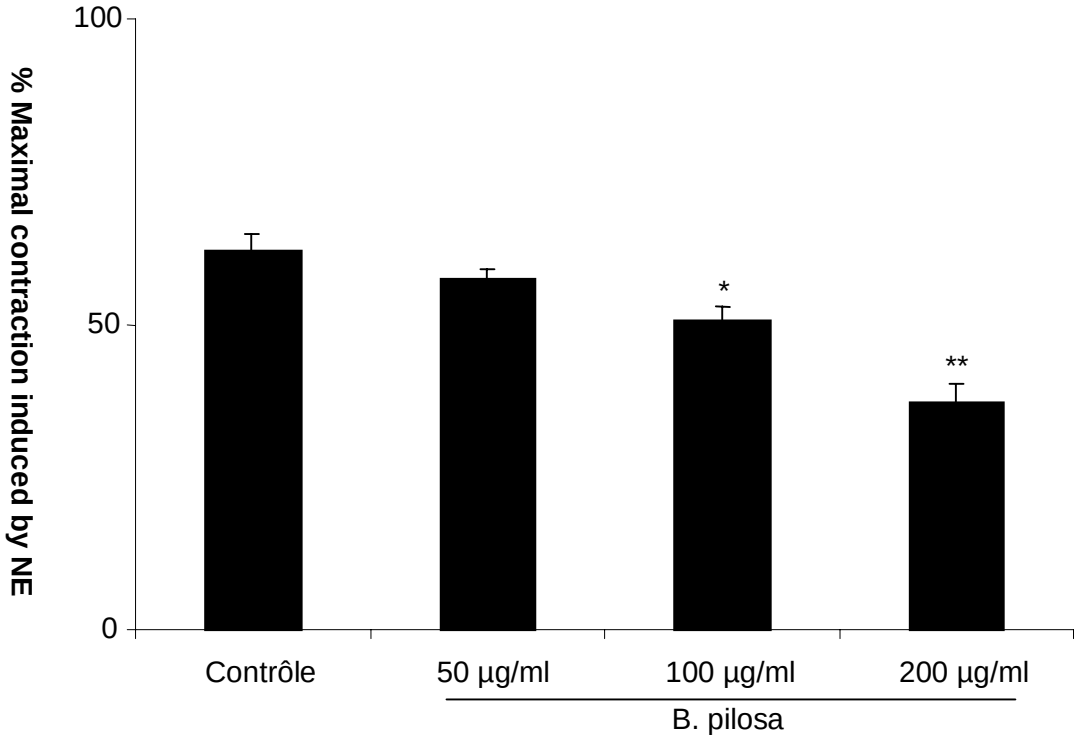

Figure 4: Effects of *B. pilosa* extract on the Ca^{2+}-entry component of norepinephrine-induced contractions. Aortic rings were first contracted with norepinephrine 10^{-6} M in Ca^{2+}-free PSS supplemented with 10^{-5} M verapamil. 25 min. later, a sustained contraction was provoked by restoring extracellular Ca^{2+} (2.5 mM). Responses are expressed as percentage of the initial control norepinephrine-induced contractions. Each bar represents the mean ± SEM, n = 24. *p < 0 .05; **p < 0.01; as compared to Control.

Discussion

The aim of the present study was to investigate the mechanism of the vasorelaxant effect of the leaf methanol extract of *B. pilosa* and to identify the active principles (mainly polyphenolic and stilbene compounds) of the leaves of *B. pilosa*. The vasorelaxant activity of the plant extract may be largely attributed to its content of phenolics and stilbenes. Since it is well known that these compounds have vasodilatory effects (15, 28, 29). In order to test whether the vasorelaxant effect of *B. pilosa* might be due largely to the presence of polyphenolic and stilbene compounds, the effects of some commercially available key substances, quercetin, rutine, isoquercitrine, trans piceid and trans resveratrol, on rat aortic rings were examined. In rat aortic rings, none of these compounds developed the same vasorelaxant kinetics as *B. pilosa* extract. Resveratrols and flavonoids found in the plant extract have been demonstrated to possess free radical scavenging and lipid antiperoxidation activity.

They are known to promote vascular relaxation in disease of the vascular wall involving inflammation (30, 29).

The leaf methanol extract of *B. pilosa* was more potent in relaxing NE-than KCl-induced contraction as shown by the difference in the magnetude of the active concentrations (25 – 200 µg/mL and 0.5 – 8 mg/mL, respectively). Its relaxing activity was not dependent on the presence of endothelium, or antagonized by inhibitors of NO or prostaglandin synthesis, or potassium channel blockers. However, *B. pilosa* extract did not antagonize NE-induced intracellular calcium entry.

These data confirm our previous results obtained on the rat aorta using the aqueous instead of the methanol extract (31). Among vasorelaxant agents, sodium nitroprusside (SNP) was also found to inhibit high-K^+-induced contraction of rat aorta rings at a concentration higher than that needed to inhibit the NE-induced contraction (32); another similarity between the *B. pilosa* extract and SNP is that both products induce endothelium-independent vasorelaxation. However, in contrast to *B. pilosa* extract, the effect of SNP is significantly reduced by the non specific K^+ channel inhibitor, tetraethylammonium (33), suggesting that their mechanism of action is not similar. As well, the fact that the sensitive blocker of ATP-dependent K^+ channels, glibenclamide (34) did not influence the vasorelaxant activity of *B. pilosa* extract indicates that its mechanism is distinct from that of K_{ATP} opener vasodilators.

Another possibility was that *B. pilosa* extract would antagonize voltage-dependent Ca^{2+} channels, similarly to the dihydropyridine, nifedipine or the phenylalkylamine, verapamil. In our hands, however, nifedipine was more potent in relaxing KCl-than NE-induced contractions ; similar results were observed in a previous study (26). Therefore, the vasodilator profile of *B. pilosa* extract is not that of a typical Ca^{2+}-entry blocker.

This report shows that the leaf methanol extract of *B. pilosa* was particularly effective in relaxing NE-induced contractions. However, the extract caused the relaxation of tonic, but failed to inhibit the phasic component of the contraction induced by NE in rat aorta. The phasic component of the contraction induced by alpha 1-adrenoceptors in Ca^{2+} free PSS, has been attributed to IP_3-mediated release of Ca^{2+} from intracellular stores (35), whereas the tonic component is thought to be related to an increase in extracellular Ca $^{2+}$ entry, mostly via L-type calcium channels sensitive to Ca^{2+}-entry blockers (36). Recent data (37) have shown that, in addition to the calcium entry blocker, nitrendipine and the receptor-operated calcium entry antagonist, SKF 96365, the Na/Ca exchange inhibitor, amiloride was particularly effective in antagonizing calcium-induced contration in the presence of NE, i.e. under conditions similar to those used in the present work. These results suggest that, in addition to voltage-dependent calcium channels, various mechanisms are involved in the NE-induced calcium entry. Future studies will explore the mechanism of *B. pilosa* extract relaxing activity in view of these possible mechanisms.

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