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Original Article

Sesquiterpene lactones from *Hedyosmum brasiliense* induce *in vitro* relaxation of rat aorta and *corpus cavernosum*

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ABSTRACT

Hedyosmum brasiliense Miq., Chloranthaceae, has been used in Southern Brazil as a sedative, anti-inflammatory, and aphrodisiac. In this study, endothelium-intact and endothelium-denuded rat aortic rings and strips of *corpus cavernosum* were used to investigate the relaxant effects of an hexane fraction of leaves of *H. brasiliense* and its sesquiterpene lactones 13-hydroxy-8,9-dehydroshizukanolide, podoandin, and elemanolide 15-acetoxy-isogermafurenolide. The incubation of hexane fraction of leaves of *H. brasiliense* resulted in significant relaxation of endothelium-intact aortic rings previously contracted by phenylephrine. In addition, 13-hydroxy-8,9-dehydroshizukanolide and podoandin displayed a clear concentration-dependent ability to relax endothelium-intact (~85 to 90%) and endothelium-denuded (~45 to 55%) rat aortic rings. A less pronounced vascular relaxation was recorded when 15-hydroxy-isogermafurenolide was tested. Interestingly, in tissues previously incubated with the nitric oxide synthase inhibitor L-NAME (100 μM), both 13-hydroxy-8,9-dehydroshizukanolide and podoandin had their effects in endothelium-intact vessels reduced to the same degree of relaxation observed in endothelium-denuded aortic rings. Podoandin, 13-hydroxy-8,9-dehydroshizukanolide, and 15-acetoxy-isogermafurenolide (100 μM) were also able to relax precontracted *corpus cavernosum* strips by 49.5 ± 3.9%, 65.9 ± 7.3% and 57.9 ± 5.5%, respectively. Our results demonstrated that 13-hydroxy-8,9-dehydroshizukanolide, podoandin and 15-acetoxy-isogermafurenolide, isolated from *H. brasiliense*, generate both endothelium-dependent and -independent relaxation of rat aortic rings, as well as being able to induce *in vitro* relaxation of rat *corpus cavernosum*. Importantly, the endothelium-dependent effect is fully dependent on nitric oxide production. Considering that penile erection depends on both relaxation of cavernosal smooth muscle and inflow of blood for the cavernous bodies, this is the first study reporting experimental evidence supporting the aphrodisiac properties of *H. brasiliense*.

Introduction

Hedyosmum brasiliense Miq. is a member of the Chloranthaceae family widely distributed in Southern Brazil. It is popularly known as "cidrão", "cidreira" and "erva-de-bugre", among others. In folk medicine, it is indicated for the treatment of migraine, depression and diseases of ovary, and has been used as sedative, anti-inflammatory, and aphrodisiac (Reitz, 1965). Experimental evidence demonstrated that solutions obtained from *H. brasiliense* present analgesic (Trentin et al., 1999a), hypnotic-, anxiolytic- and antidepressant-like effects in rodents (Tolardo et al., 2010; Goncalves et al., 2012). Nevertheless, at least to our knowledge, the aphrodisiac effect has never been investigated.

Sesquiterpene lactones are a group of secondary metabolites found in several species, such as Acanthaceae, Amaranthaceae, Apiaceae, Aristolochiaceae, Burseraceae, Coriariaceae, Illiciaceae, Magnoliaceae, Menispermaceae, Lamiaceae, Lauraceae, Polygonaceae, Winteraceae, and Chloranthaceae (Picman, 1986; Kawabata and Mizutani, 1988; Cao et al., 2008; Hu et al., 2013). These compounds are well known for their antifeedant and protective roles in plants, mainly against infections by viruses, bacteria, and fungi (Nawrot and Harmatha, 2012; Chadwick et al., 2013). The potential therapeutic effects of sesquiterpene lactones have been widely investigated in animal models, revealing anti-inflammatory (Merfort, 2011; Ferrari et al., 2013), antimicrobial (Ordonez et al.,

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2011; Ciric et al., 2012), antitumor (Zhang et al., 2005), and antiviral effects (Ozcelik et al., 2009; Rossini et al., 2012; Mohammed et al., 2014), among others. In addition, their biological effects include vascular relaxation as demonstrated by several studies (Campos et al., 2003; Hong et al., 2005). The structure of sesquiterpene lactones consists of three isoprene units and a lactone group, which may accordingly be classified with their carbon skeletons. There are about 100 types of carbonskeletons, and although the majority of the sesquiterpene lactones are classified into seven main groups known as germacranolides, guaianolides, elemanolides, eremofilanolides, eudesmanolides, pseudoguaianolides and xanthanolides (Schmidt, 2006), phytochemical studies have shown lindenanolides as the main chemical markers of the Chloranthaceae family (Kawabata and Mizutani, 1988). Importantly, some of the biological effects found after the administration of solutions obtained from *H. brasiliense* in rodents were, at least in part, dependent on the activity of sesquiterpene lactones, such as the lindenanolide onoseriolide or 13-hydroxy-8,9-dehydroshizukanolide (HDS) (Trentin et al., 1999a), and the guaianolide podoandin (Tolardo et al., 2010; Goncalves et al., 2012).

Given the popular usage of *H. brasiliense*, its high amounts of sesquiterpene lactones and the putative involvement of these compounds to explain some of the popular uses of this plant, we designed this study to investigate the ability of HDS, podoandin, and the recently described elemanolide 15-acetoxy-isogermafurenolide (IGM-A; Amoah et al., 2013), to induce *in vitro* relaxation of rat *corpus cavernosum* and aorta.

Materials and methods

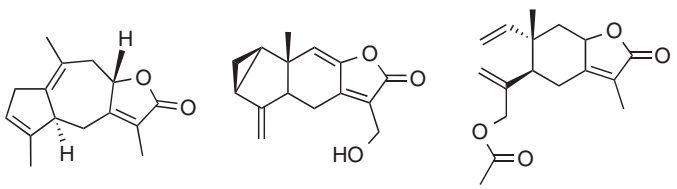
Plant material

Hedyosmum brasiliense Miq., Chloranthaceae, was collected from the municipal area of Antonio Carlos in the Santa Catarina state (Brazil). It was identified by the botanist Professor Dr. Ademir Reis and a voucher specimen (number 2031) was deposited at the Lymann Bradford Smith Herbarium (Univali, Itajaí, SC, Brazil).

Preparation of the hexane fraction and isolation of compounds

Fresh leaves (5 kg) of *H. brasiliense* were subjected to extraction with bi-distilled ethanol for 15 days. The solvent was subsequently removed under reduced pressure using a rotary evaporator. The recuperated solvent was used to re-extract the plant material twice, thereby resulting into a total of 210 g of crude extract. The crude extract (190 g) was subjected to liquid:liquid partition to yield hexane (16 g), CH_2Cl_2 (4 g), EtOAc (13 g), and the residual aqueous (74 g) fraction. All fractions were stored at −18 °C. About 2 g of the hexane fraction (HFHB) was kept for biological and pharmacological tests and the rest was subjected to flash silica gel column chromatography (240–400 mesh). It was eluted with a gradient of 0–70% CH_2Cl_2 in hexane (200 ml) followed by 0–70% EtOAc in CH_2Cl_2 (200 ml), yielding eight sub-fractions (A–H).

The sub-fraction B was recrystallized to afford the guaianolide podoandin (**1**; 300 mg). It was identified comparing its spectroscopic data with already published data (Blay et al., 2000; Kubo et al., 1992). There was a spontaneous crystallization of the previously described (Trentin et al., 1999b) lindenanolide 13-hydroxy-8,9-dehydroshizukanolide (**2**, HDS; 40 mg) from sub-fraction C. The elemanolide 15-acetoxy-isogermafurenolide (**3**, IGM-A; 35 mg) was isolated from sub-fraction F, as previously detailed (Amoah et al., 2013).



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Drugs and reagents

Acetylcholine (ACh) chloride, phenylephrine hydrochloride (PE), and Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), as well as the salts used for the preparation of the physiological saline solution (PSS) were obtained from Sigma–Aldrich Co. (St Louis, MI, USA). All reagents used for preparation of the hexane fraction and the isolation of sesquiterpenes were of analytical grade. The stock solution of HDS, podoandin, and IGM-A were dissolved in pure DMSO. A concentrated solution of hexane fraction was prepared using DMSO and deionized water (1:10). All working solutions of HDS, podoandin, and IGM-A, which were applied in the *in vitro* experiments were, however, prepared in physiological saline solution.

Animals

Male Wistar rats (3–4 months) were provided by the Central Vivarium facilities of the Universidade Federal de Santa Catarina. The animals were maintained under controlled light–dark cycle (12/12 h) and temperature (22 ± 2 °C), with free access to water and chow. This study was performed in accordance with the NIH guidelines for animal experimentation. In addition, the Institutional Ethics Committee for Animal Use (CEUA) of the Universidade Federal de Santa Catarina (UFSC, Brazil) approved all procedures adopted in this study (authorization number 5371190815).

Isolation of corpus cavernosum *and aorta*

Immediately before the experiments, the animals were euthanized by anesthesia overdose (ketamine and xylazine, *i.p.*) and had their thoracic aorta and penis carefully excised and kept in physiological saline solution (PSS, composition in mM: 130.3 NaCl, 4.7 KCl, 1.6 $CaCl_2{\cdot}2H_2O$, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 14.9 $NaHCO_3$, 5.5 D-glucose) warmed at 37 °C. After removing adherent tissues, the aorta was cut into rings of approximately 3–4 mm. The dorsal penile vein and spongy tissue were carefully removed from the penis and the *corpus cavernosum* was divided into two longitudinal strips. Both aortic rings and strips of *corpus cavernosum* were mounted in organ baths containing PSS (37 °C, continuously aerated with 95% O_2/5% CO_2) and connected to force–displacement transducers for recording of isometric force. The experiments using the aortic rings were conducted in endothelium-intact and endothelium denuded preparations. To remove the endothelium, the lumen of the vessels was gently rubbed with a small wire before the setup in the organ baths. A stabilization period of 60 min was allowed for all preparations, which were maintained at a resting tension of 3 g (aortic rings) or 250 mg (strips of *corpus cavernosum*). The isometric tension was recorded using a digital data acquisition system (PowerLab®) coupled to a computer running the software LabChart v. 7.1 (both from ADInstruments, Castle Hill, Australia).

In vitro *evaluation of the effects of* H. brasiliense *and its isolated sesquiterpene lactones in corpus cavernosum and aorta*

After the stabilization period of 60 min both *corpus cavernosum* and aortic rings were exposed to high potassium nutritive solution containing 80 mM or 120 mM KCl, respectively. The tension was recorded for 15 min and the preparations were washed and allowed to stabilize during 30 min. The strips of *corpus cavernosum* were then exposed to phenylephrine (PE; 10 μM), and after reaching the tonic phase of contraction, the preparations were incubated with cumulative concentrations of HFHB (3, 10, 30, 100 and 300 μg/ml), HDS, podoandin, or IGM-A (10 nM to 100 μM, for all compounds). Time-matched experiments where the preparations were exposed to the same amounts of the vehicle used to dilute the HFHB or the isolated compounds were used as control. The resulting relaxation was recorded and evaluated. Importantly, each strip of *corpus cavernosum* was incubated only with one of the compounds or fractions investigated in this study.

The same protocol was followed in rat aortic rings. However, once these experiments were performed in both endothelium-intact and endothelium-denuded preparation, these vessels underwent a first exposition to PE (1 μM) followed by addition of acetylcholine (ACh; 1 μM). The inability of ACh to induce relaxation in PE-contracted preparations was used to confirm the lack of functional endothelium. On the other hand, aortic rings whose ACh-induced relaxation was above 80% were considered endothelium-intact preparations. After the evaluation of the endothelial functionality, a new interval of 60 min was allowed for stabilization, followed by addition of PE (1 μM) and cumulative concentrations of HFHB (3, 10, 30, 100 and 300 μg/ml), HDS, podoandin, or IGM-A (10 nM to 100 μM, for all compounds). In addition, the effects of HDS, podoandin and IGM-A were also evaluated in endothelium-intact aortic rings previously incubated with 100 μM L-NAME for 20 min. For all experiments, the relaxation induced by HFHB, HDS, podoandin, and IGM-A, in preparations with or without functional endothelium, as well as in the presence or absence of L-NAME, were recorded and compared among the groups.

Importantly, for control parameters we performed time-matched experiments in which both *corpus cavernosum* strips and aortic rings were exposed to the same amounts of the vehicle used to dilute the HFHB or the isolated compounds.

Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.) of 5–6 preparations (removed from different animals) per group. The graphs and the statistical analysis were performed with the program GraphPad Prism® version 6.0d for Mac (GraphPad Software, La Jolla, CA, USA). Comparisons were made by one way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

The first set of results obtained in this study revealed that the hexane fraction prepared with leaves of *H. brasiliense* (HFHB) presented minor effects on the vascular tone of rat aortic rings that had its endothelial surface layer damaged prior to contraction induced by the selective α-1 adrenergic agonist phenylephrine (PE). Indeed, the highest concentration of HFHB tested in our experiments (300 μg/ml) decreased the vascular tone of PE-contracted vessels by 22.3 ± 4.5%, which was not statistically different from the relaxation obtained when these endothelium-denuded (E−) vessels were exposed to the same amounts of dimethyl sulfoxide, the vehicle used to dissolve the HFHB (Fig. 1A). Nonetheless, a robust relaxation was obtained when the HFHB was tested in endothelium-intact (E+) aortic rings. In these preparations, the addition of 100 and 300 μg/ml of the HFHB into the organ baths reduced the vascular tone by 33.4 ± 5.3 and 70.2 ± 8.6%, respectively, which were significantly higher than the 10.2 ± 2.7 and 13.6 ± 4.8% maximum relaxation induced by vehicle only (Fig. 1B).

Considering the pronounced vascular relaxation obtained with the HFHB and the previously described main components isolated from this fraction (Amoah et al., 2013), we did evaluate the potential role of the sesquiterpene lactones HDS, podoandin and IGM-A in HFHB-induced aorta relaxation. Notably, the final concentration of DMSO present in the working solutions containing these compounds did not influence the tone of pre-contracted aortic rings, as confirmed by time-matched experiments performed during the pharmacological tests (Fig. 2, diamond-shaped symbols). Both HDS and podoandin displayed a clear concentration-dependent ability to relax both endothelium-intact and endothelium-denuded rat aortic rings. However, as demonstrated in Fig. 2A and B (open circles) a more intense vascular relaxation was reached in vessels with functional endothelium. For instance, at the final

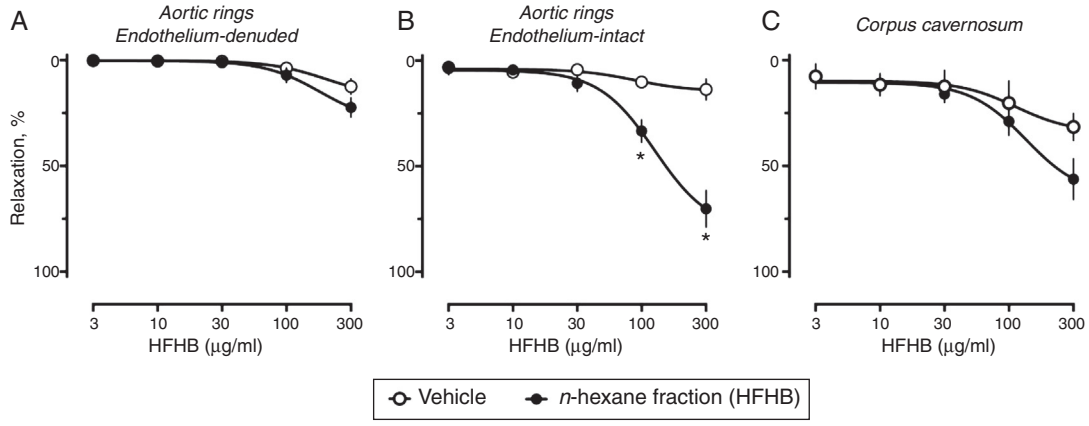

Fig. 1. Effects of the hexane fraction of *Hedyosmum brasiliense* (HFHB) on the tone of isolated rat aortic rings and strips of *corpus cavernosum*. The preparations were maintained in classical organ bath apparatus for recording of the isometric tension. The effects of cumulative concentrations of HFHB was evaluated in endothelium-denuded (A) and endothelium-intact (B) rat aortic rings, as well as in strips of rat *corpus cavernosum* (C) previously contracted by phenylephrine. Each point represents the mean ± S.E. of 5–6 preparations from different animals per group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by *t*-test subjected to Bonferroni correction. * Indicates a value of $p < 0.05$ when compared with the vehicle group.

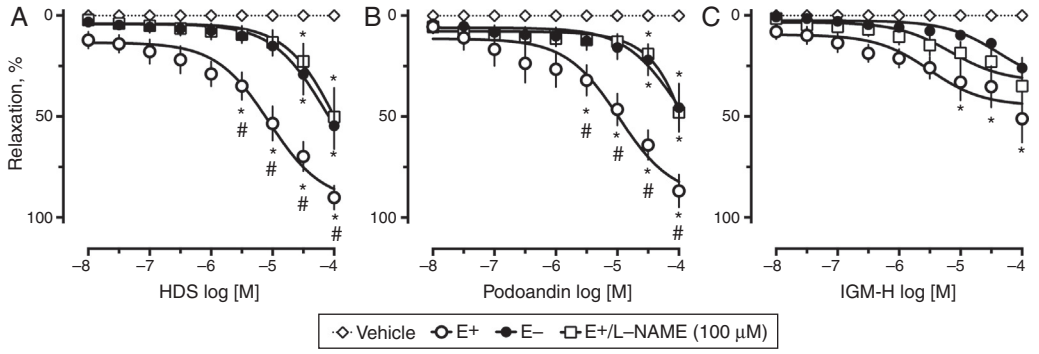

Fig. 2. Endothelium-dependent and -independent vascular relaxation induced by sesquiterpene lactones isolated from *Hedyosmum brasiliense*. Cumulative concentrations of HDS (A), podoandin (B), and IGM-A (C) were obtained in endothelium-denuded (E+; closed circles), endothelium-intact (E−; open circles), or endothelium-intact rat aortic rings previously incubated with the nitric oxide synthase inhibitor L-NAME (squares), and contracted by phenylephrine (1 μM). The dashed line (diamond-shaped symbols) shows the lack of influence of the vehicle used to dissolve the compounds on the vascular tone. Each point represents the mean ± S.E. of 5–6 preparations from different animals per group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by *t*-test subjected to Bonferroni correction. * Indicates a value of $p < 0.05$ when compared with the vehicle group. # Indicates a value of $p < 0.05$ when the E+ group was compared with the E+/L-NAME group.

concentration of 100 μM, HDS and podoandin were able to relax endothelium-intact vessels by 90.1 ± 5.9% and 86.8 ± 8.0%, respectively, while in endothelium-denuded preparations, the maximum relaxation obtained were 54.6 ± 5.9% and 46.6 ± 5.7%, respectively. Interestingly, there were no differences between HDS and podoandin in their potency to induce vascular relaxation, either in endothelium-intact or endothelium-denuded aortic rings. For instance, the calculated EC_{50} (concentration that generated the half maximal response) found in endothelium-intact vessels were 8.7 (4.6–17.1) μM, and 10.6 (4.6–24.4) μM, for HDS and podoandin, respectively. Regarding the effects of IGM-A, a less pronounced vascular relaxation was recorded when this sesquiterpene was incubated in PE-contracted aortic rings (Fig. 2C), which was able to induce a maximal relaxation of 51.1 ± 11.6% and 26.0 ± 5.1%, in endothelium-intact and endothelium-denuded vessels. Even though it has been observed in previous studies that different sesquiterpene lactones cause vascular relaxation (Campos et al., 2003; Hong et al., 2005), this is the first time report of this effect induced by a fraction prepared with *H. brasiliense*, as well as with these three sesquiterpene lactones (*i.e.* HDS, podoandin, and IGM-A).

Endothelium-dependent vascular relaxation has often been associated with the production of nitric oxide, mainly by the endothelial isoform of the enzyme nitric oxide synthase (NOS), despite the well known existence of other endothelial mediators able to relax the smooth muscle cells and their involvement in the control of vascular tone, such as cyclooxygenase derivatives and the endothelium-derived hyperpolarizing factor, that remains to be identified (Durand and Gutterman, 2013). To verify the role of nitric oxide in our findings, L-NAME, a non-selective NOS inhibitor, was incubated with endothelium-intact preparations before the assessment of the vasorelaxant effects of HDS, podoandin, and IGM-A. Both HDS and podoandin had their ability to cause relaxation partially avoided by L-NAME (Fig. 2A and B). Indeed, when incubated with L-NAME, the effects induced by these sesquiterpene lactones in endothelium-intact vessels were reduced to the same degree of relaxation observed in endothelium-denuded aortic rings. The incubation of L-NAME has been inconclusive on whether IGM-A-induced relaxation does or does not depend on nitric oxide production, perhaps because the window of difference in the responses for this compound between E+ and E− preparations was reduced, as well as due the wide variability of effects obtained with IGM-A in aortic rings (Fig. 2C). Nevertheless, our results clearly demonstrate that the higher effectiveness of both HDS and podoandin in endothelium-intact arteries, when compared with endothelium-denuded vessels, is dependent on stimulation of nitric oxide production. We did not perform further approaches to explore the downstream mechanisms involved in the vascular relaxation mediated by HDS and podoandin. However, nitric oxide is classically known as a biological activator of the soluble guanylate cyclase, including in vascular smooth muscle cells (Arnold et al., 1977). In addition, nitric oxide is also able to directly interact with and open calcium-activated potassium channels (Bolotina et al., 1994). We have previously demonstrated the involvement of both soluble guanylate cyclase and calcium activated potassium channels in the vasodilatory effects associated with other medicinal plants (Rattmann et al., 2006; Da Silva et al., 2012). Thus, it is reasonable to speculate that once these sesquiterpene lactones had their endothelium-dependent effects fully avoided by NOS inhibition, the activation of the classical nitric oxide/guanylate cyclase pathway accounts for the vascular relaxation described in this study. Importantly, a significant part of the vascular relaxation mediated by the three sesquiterpene lactones investigated in here persisted after endothelium damage or NOS inhibition (Fig. 2, closed circles and squares). If on one hand the mechanisms involved in such effect remains to be investigated, on the other hand it may confer on these compounds the ability to induce some degree of vascular relaxation even in those conditions characterized by endothelial dysfunction.

At least to our knowledge, *H. brasiliense* has not been indicated for the treatment of cardiovascular diseases in the traditional medicine. Nevertheless, according to folk medicine when 30 g of the fresh leaves of *H. brasiliense* is infused in 600 g of white wine, both tonic and aphrodisiac effects are obtained (Reitz, 1965). The search for aphrodisiac products is diffused around the world and it is frequently associated with sexual disorders, such as erectile dysfunction. Penile erection results from a complex interaction between spinal reflex, autonomic and somatic inputs, as well as locally produced mediators, which create and maintain the appropriate relaxation of the smooth muscle of the *corpus cavernosum* and local arteries (Andersson and Wagner, 1995; Dean and Lue, 2005). Importantly, impaired blood flow by penile artery has been ascribed as a main cause of erectile dysfunction and an early indicator of other cardiovascular diseases. Although we have not evaluated the behavior of the vasculature from male genitalia in this study, it is reasonable to suggest that the vasodilatory effect of HFHB may contribute to the popular use of *H. brasiliense* as an aphrodisiac plant. We did investigate the effects of both HFHB and its sesquiterpene lactones on *in vitro* preparations of rat *corpus cavernosum*. The addition of HFHB on phenylephrine-contracted *corpus cavernosum* strips did not result in any significant relaxation (Fig. 1C), when compared with the preparations exposed to vehicle

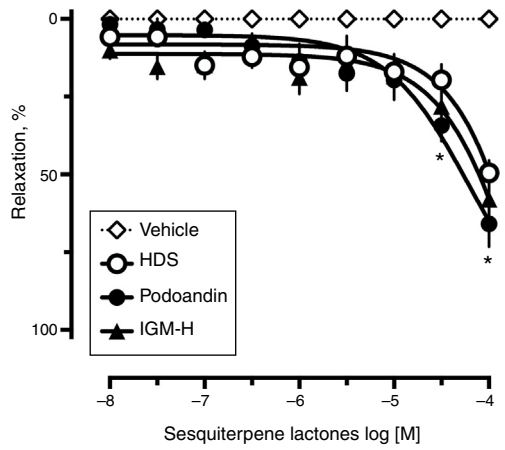

Fig. 3. Relaxation of strips of *corpus cavernosum* from rats by sesquiterpene lactones isolated from *Hedyosmum brasiliense*. Phenylephrine contracted preparations were exposed to cumulative concentrations of HDS (open circles), podoandin (closed circles) or IGM-A (triangles). The dashed line (diamond-shaped symbols) shows the lack of influence of the vehicle used to dissolve the compounds on the tone of cavernosal smooth muscle. Each point represents the mean ± S.E. of 5–6 preparations from different animals per group. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by *t*-test subjected to Bonferroni correction. * Indicates a value of $p < 0.05$ when the results obtained in all groups of compounds were compared with the vehicle group.

only. However, precontracted *corpus cavernosum* strips relaxed by 49.5 ± 3.9%, 65.9 ± 7.3%, and 57.9 ± 5.5% when incubated with 100 μM HDS, podoandin, and IGM-A, respectively (Fig. 3). Among the locally produced mediators, nitric oxide released by endothelial cells and nerve endings plays a major role in penile erection. Once produced, it diffuses to adjacent smooth muscle cells dilating the arteries and the smooth muscle of the *corpus cavernosum*. Indeed, the potentiation of the nitric oxide/guanylate cyclase pathway by agents such as sildenafil is the main pharmacological strategy used in the treatment of erectile dysfunction (Andersson, 2001; Dean and Lue, 2005). Our data does not allow us to state which intracellular mechanisms these sesquiterpene lactones could be using to cause the relaxation of the *corpus cavernosum*, but taking into account the results obtained in rat aortic rings, the role of the nitric oxide in this effect deserves further investigation.

Conclusions

This work discloses that a hexane fraction prepared with fresh leaves of *H. brasiliense*, as well as the sesquiterpene lactones HDS, podoandin, and IGM-A, isolated from this fraction, are able to induce both endothelium-independent and endothelium-dependent relaxation of rat aortic rings. Our results demonstrated that, at least for HDS and podoandin, the endothelium-dependent relaxation is fully dependent on nitric oxide production. In addition, although folk medicine has already pointed the use of *H. brasiliense* as an aphrodisiac (Reitz, 1965), this is the first time that a study reports some experimental evidence that support this effect, since we found that HDS, podoandin and IGM-A are able to induce *in vitro* relaxation of *corpus cavernosum*. Considering that the penile erection depends on both relaxation of cavernosal smooth muscle and inflow of blood for the cavernous bodies, additional studies should be developed to further investigate the effects of *H. brasiliense*, as well as its isolated sesquiterpene lactones, on both vascular function and penile erection using *in vivo* approaches.

Authorship

S.K.S.A. worked in the preparation of extracts and isolation of the sesquiterpene lactones during his Ph.D. studies, under supervision of MWB. A.L. performed the *in vitro* studies and analyzed the results obtained. A.L. and S.K.S.A. drafted the manuscript. JESS was responsible for the conception of the study, including protocols and interpretation of data while supervising A.L. during her graduate studies in Pharmacology, and worked on the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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