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Original Article

Vasorelaxant effects in aortic rings of eight diterpenoids isolated from three Venezuelan plants†

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ABSTRACT

Vasorelaxant effects of eight diterpenoids isolated from three Venezuelan plants [(+)-manool [(+)-labda-8(17),14-dien-13-ol], (+)-manoyl oxide, (+)-2-oxomanoyl oxide, sandaracopimara-8(14), 15-dien-3β, 19-diol, jhanidiol acetate (18-acetoxy-1β-hydroxymanoyl oxide), jhanidiol (1β,18-dihydroxymanoyl oxide), *ent*-kaur-16-en-19-ol and grandiflorenic acid (*ent*-kaur-9(11),16-dien-19-oic acid)] aortic rings were assessed in intact endothelium and endothelium-denuded isolated rat. Thw cumulative addition (10^{-6} to 10^{-4} M) of each product were carried out after contraction with phenylephrine (10^{-6} M). Jhanidiol acetate and *ent*-kaur-9,16-en-19-oic acid at 10^{-4} M dose concentration, exhibit the maximal vasorelaxant effect in endothelium-intact rings (51.61 ± 7.62% and 79.27 ± 7.41%, respectively). In endothelium-denuded aortic rings, the maximum vascular response exerted by both compounds was not abolished (64.14 ± 5.64% and 84.84 ± 3.62%, respectively). In denuded aortic rings, the half-maximal inhibitory concentration (IC_{50}) Jhanidiol was obtained by the ethyl less than those obtained in rings endothelium (1.09×10^{-4} vs 7.29×10^{-5} M, respectively), although this difference was not significant. These results suggested that the mechanism behind the vasorelaxant effect of the two diterpene is mediated by endothelium-independent pathways.

Introduction

Currently, the phytochemical compounds are playing an important role in health. A considerable number of bioactive compounds derived from plant material, in addition to possessing cardioprotective effect, have been shown to reduce the risk of cardiovascular disease which is a leading cause of death worldwide (Vasanthi et al., 2012). In some cases these beneficial effects have been associated with their vasorelaxant activity, and for this reason there has

† Supporting Information available (^{1}H NMR and ^{13}C NMR spectra data of compounds **1-8**) in Supplementary Material.

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been a great deal of interest in vasoactive phytochemical compounds (Tirapelli et al., 2010). Terpenoids compound, the monoterpenoids and diterpenoids, have proved to produce effects on the cardiovascular system (Awang et al., 2012; Hipolito et al., 2011; Santos et al., 2011). Diterpenoids, in particular, are among the main that compounds have been linked with cardiovascular properties such as vasorelaxant, inotropic, diuretic, and hypotensive activity (Alfieri et al., 2007; El Bardai et al., 2003; Pieta et al., 1995; Tirapelli et al., 2008b). Despite the fact that plants provide a rich source of novel biologically active compounds, only a small percentage of phytochemicals have been investigated and studied for their medical potential (Tirapelli et al., 2008a). The vascular action exerted by these products seems to involve multiple mechanisms as endothelium-dependent and endothelium-independent actions, increase of prostacyclins, and blockade of voltage-gated calcium channels (Tirapelli et al., 2008a).

In this study we have investigated the vascular effects of eight already known diterpenoids which will be described in the materials and methods part. These eight compounds isolated from three plants collected from different regions of Venezuela [*Pinus caribaea* var. *hondurensis* (Sénécl.) W.H.G. Barrett & Golfari, Pinaceae, *Lourteigia stoechadifolia* (L. f.) R.M. King & H. Rob., Asteraceae, and *Espeletia schultzii* Wedd., Asteraceae] belong to different subgroups of this class of natural compounds: five labdanes [(+)-manool (**1**), (+)-manoyl oxide (**2**), (+)-2-oxomanoyl oxide (**3**), jhanidiol acetate (**5**) and jhanidiol (**6**)], one isopimarane [sandaracopimara-8 (14), 15-dien-3β, 19-diol (**4**)] and two *ent*-kauranes [*ent*-kaur-16-en-19-ol (**7**) and grandiflorenic acid (**8**)], isolated from *P. caribaea, L. stoechadifolia,* and *E. schultzii,* were not found medicinal uses for these plants described.

Materials and methods

General

Melting points are uncorrected. The optical rotation and IR spectra were measured in CHC_{l3}. ^1H NMR (200.13 MHz) and ^{13}C NMR (50.3 MHz) spectra were measured in $CDCl_3$, with TMS as standard. Iterative searche of ^{13}C NMR chemical shift performed within our NAPROC-13 spectroscopic NMR database (López-Pérez et al., 2007) were used for the identification of compounds.

Plant materias and characterization of diterpenoids

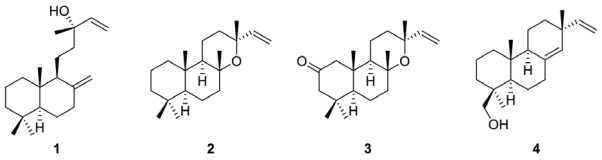
The source of the plants assayed compounds were collected at different regions of Venezuela. The selection of these plants was based on preliminary phytochemical study showed that a high content of diterpenoid compounds. *Pinus caribaea* var. *hondurensis* (Sénécl.) W.H.G. Barrett & Golfari, Pinaceae, collected in Uverito, State of Monagas (December, 2010; voucher specimen, J. M. Amaro-Luis, Nº 2526); *Lourteigia stoechadifolia* (L. f.) R.M. King & H. Rob., Asteraceae, collected in Páramo El Delgadito, State of Mérida (November, 2010; voucher specimen, J. M. Amaro-Luis, Nº 2522), and *Espeletia schultzii* Wedd., Asteraceae, collected in the high plateau of Gavidia, Distrito Rangel, State of Mérida (July, 2011; voucher specimen, J. M. Amaro, Nº 2535). The taxonomic identification of plants were confirmed by Engineer Juan Carmona, curator of Herbarium MERF (Facultad de Farmacia, Universidad de los Andes). Voucher specimens of each species were deposited in this herbarium.

Isolation, purification and characterization of diterpene

Pinus caribaea

The residual non-volatile resin (45 g) was chromatographed on a silica gel column, which was eluted with mixtures of *n*-hexane-CH_2Cl_2 increasing polarity. Ten fractions (A-J), of 500 ml each, were collected. Fraction B (1.50 g), eluted with 5% CH_2Cl_2 in *n*-hexane, was further fractionated to afford 345 mg of (+)-manool (**1**), m.p. 52-54 ºC, $[\alpha]^{25}_D$: +26º (*c*, 0.026, MeOH). IR, ^1H, and ^{13}C NMR spectra are in agreement with the data published for this product (Buckwalter et al., 1975). Original ^1H, and ^{13}C NMR spectral data of all the compounds described in this paper are accessible in Supporting Information. Fraction C (3.22 g), eluted with 10% CH_2C_{12} in *n*-hexane, afforded a colorless oil which was purified by preparative TLC, yielding 95 mg of (+)-manoyl oxide (**2**), m.p. 29-31 ºC, $[\alpha]^{25}_D$: +20º (*c*, 0.050, MeOH). IR, ^1H, and ^{13}C NMR spectra are in agreement with the published for this product (Demetzos et al., 2002; Wu and Asakawa, 1987). Fraction E (1.88 g), eluted with 20% CH_2Cl_2 in *n*-hexane, afforded, after recrystallization, 78 mg of (+)-2-oxomanoyl oxide (**3**), m.p. 72-74 ºC, $[\alpha]^{25}_D$: +29º (*c*, 0.049, MeOH). IR, ^1H, and ^{13}C NMR spectra are in agreement with the data published for this product (Barton et al., 1987); (Buckwalter et al., 1975). Fraction G (2.45 g), eluted with *n*-hexane - CH_2Cl_2 4:1, gave 85 mg of sandaracopimara-8(14),15-dien-3β,19-diol (**4**); m.p. 164-166 ºC, $[\alpha]^{25}_D$: -9º (*c*, 0.035, $CHCl_3$). IR, ^1H, and ^{13}C NMR spectra are in agreement with the data published for this product (Grant and Munro, 1965).

Lourteigia stoechadifolia

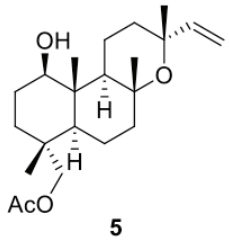


1 2 3 4

The aerial parts of the plant (5.7 kg) were dried in the dark and at room temperature for two weeks. The dried plant materials was pulverized into a fine powder, extracted with MeOH at room temperat ure for three days, with occasional stirring, and then filtered. The extract was evaporated in a vacuum to give 800 g of an oily material, 300 g of which was adsorbed onto silica gel and chromatographed on a silica gel column eluted with mixtures of CH_2Cl_2-acetone and acetone-MeOH of increasing polarities. Fraction 1, eluted with CH_2Cl_2-acetone 9:1, afforded jhanidiol acetate (**5**) (175 mg), an oil; $[\alpha]^{25}_D$ +75°. IR, ^1H, and ^{13}C NMR spectra are in agreement with the data published for this product (González et al., 1977). Fraction 6 (22.3 g) was subjected to crystallization from acetone-CH_2Cl_2

to afford a large quantity (10 g) of jhanidiol (**6**), m.p 189 °C - 190 °C; $[\alpha]^{25}_{D}$ +90°. IR, ^{1}H, and ^{13}C NMR spectra are in agreement with the data published for this product (González et al., 1977).

Espeletia schultzii



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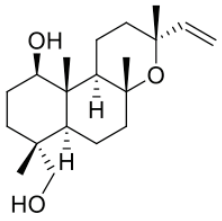
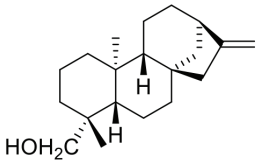


6

Woody rosette stems of the plant were air-dried under shade at room temperature for two weeks. The dried vegetable materials was ground to a fine powder (3.7 g) and extracted with acetone in a Soxhlet apparatus for 24 h to give a crude extract (135 g). This extract was adsorbed onto silica gel and chromatographed on a silica gel column eluted with mixtures of hexane-CH_2Cl_2 of increasing polarities. Fraction C-2, eluted with 20% CH_2Cl_2 in hexane, provided the semi-pure compound, which was rechromatographed over silica gel to afford 200 mg of ent-kaur-16-en-19-ol (**7**), m.p. 140-141 °C (MeOH), $[\alpha]^{25}_{D}$: -79° (c, 0.45, $CHCl_3$). IR, ^{1}H, and ^{13}C NMR spectra are in agreement with the data published for this product (Patra et al., 1980). Fraction C-4, eluted with 30% CH_2Cl_2 in hexane, yielded a semi-pure compound which was purified by preparative TLC to afford 90 mg of grandiflorenic acid (**8**), m.p. 159 °C - 161 °C (MeOH), $[\alpha]^{25}_{D}$: -34° (c, 1.22, $CHCl_3$). IR, ^{1}H, and ^{13}C NMR spectra are in agreement with the data published for this product (Enriquez et al., 1997).

Animals



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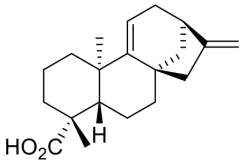

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Healthy male Sprague-Dawley rats, weighing 200 to 220 g, and bred in the Animal House of the School of Veterinary Medicine, Universidad de Panamá, were used. All animals were kept under standard animal room conditions (temperature 21 ± 1 °C; humidity 55 to 60%) with food and water ad *libitum*. The experimental procedures followed the Guide for the Care and Use of Laboratory Animals (1996), after protocol approval by the Bioethics Committee of the Pharmacology Department (CBF-02dec11).

Drugs and reagents

Phenylephrine hydrochloride (PE), acetylcholine chloride (Ach), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All diterpenes were dissolved in DMSO prior totesting. The other drugs and Krebs-Henseleit (KH) were prepared in double distilled water.

Preparation of rat aortic rings

Rats were anesthetized with sodium pentobarbital (40 mg/kg, *i.p.*) and euthanized by exsanguination. The thoracic aorta was mm excised and immediately placed in KH solution at 4 °C with the following composition: 115.5 mM NaCl, 4.6 mM KCl, 1.3 mM NaH_2PO_4, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 11.1 mM glucose, at pH 7.4. After removing adherent fat and connective tissue, the aorta was transversally cut into rings of about 3 to 4 mm width. For some of the aorta rings, the endothelium was mechanically removed by gently rubbing the lumen with a cotton ball. Vascular rings were suspended in an organ bath filled with 10 ml of KH solution, continuously gassed with 95% O_2 and 5% CO_2 at 37 °C, and connected to a force transducer (model TRI201; Letica Corporation, Rochester, MI). Changes in tension were recorded continuously by a biosignal recording system (PowerLab/400; ADInstruments, Sydney, Australia). The basal tension of each ring was 2 g, and the tissue was allowed to equilibrate for 60 min. The bath solution was replaced every 20 min with 10 ml of previously warmed and oxygenated KH solution.

Measurement of isometric vascular tone

Relaxation responses to cumulative concentrations of each of the compounds assayed in this study (10^{-6} to 10^{-4} M) were obtained in endothelium-intact and endothelium-denuded aortic rings following precontraction with PE (10^{-6} M). The presence of functional endothelium was verified by the ability of acethylcholine (ACh) (10^{-6} M) to induce more than 80% relaxation in that rings were precontracted by PE (10^{-6} M). In endothelium-denuded rings, ACh caused less than 10% relaxation. The previous set of experiments indicated that the contractile response produced by PE reached its maximum value during the first 15 min, and was relatively well sustained over the subsequent 60 min. In order to avoid fatigue of the arterial preparation, each ring was used only once with a single compound or positive and negative control vehicle and Ach, respectively).

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Within-group comparisons were performed by an analysis of variance (ANOVA) test for repeated measurements followed by the Bonferoni t-test and the difference was statistically significant considered when p was less than 0.05.

Results

Eight diterpenoid isolated from three plant species from Venezuela were tested for vascular screening.

In the course of our pharmacological screening it was found that the compounds evaluated had various percentages of vascular-relaxing activity (Table 1, Figs. 1, 2 and 3). Among diterpenoids tested, compounds **5** and **8** had significantly more vascular effects (51.61 ± 7.62% and 79.27 ± 7.41%, respectively). Other diterpene, compounds **3**, **6**, and **7**, displayed vasorelaxing effect only partial and compounds **1**, **2**, and **4** presented no significant effect vasorelaxing .

Compounds **5** and **8** were tested in mechanically denuded aortic rings in order to determine if the vasorelaxation was an endothelial-dependent mechanism. Significantly endothelial removal did not modify the maximal relaxant response to either compound compared with endothelium-intact preparations (Fig. 4). However, we did observe a no significative reduction in the half-maximal inhibitory concentration (IC_{50}) for compound **5** in denuded aortic rings compared to the results obtained in endothelial-intact tissue (1.09×10^{-4} vs 7.29×10^{-5} M, respectively), while the IC_{50} of compound 8 did not exhibit significant changes (1.71×10^{-5} vs 2.84×10^{-5} M, respectively) (Table 2).

Table 1
Vasodilator effect induced by diterpenoid compounds isolated from Venezuelan plants on the contraction induced by phenylephrine in endothelium-intact thoracic aorta rings.

Compound	Maximal relaxation (%)
1	5.33 ± 6.85
2	6.12 ± 5.24
3	26.88 ± 2.78*
4	9.36 ± 6.92
5	51.61 ± 7.62*
6	28.15 ± 7.62*
7	26.36 ± 7.89*
8	79.27 ±7.41*
Acethylcholine	86.81 ± 3.05*
Vehicle	7.69 ± 2.93

Results are presented as mean ± SEM of n = 6.
[a]$p < 0.05$ compared with vehicle (DMSO).

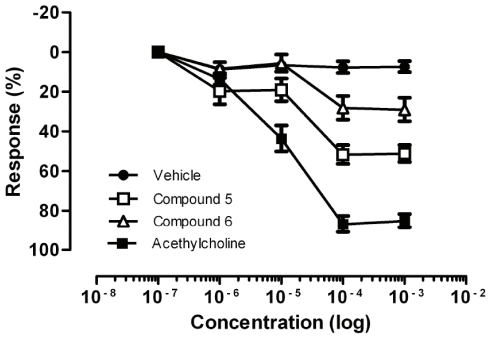

Fig. 2 - Vasorelaxant response induced by the compounds **5** and **6** evaluated on endothelium-intact aortic rings pre-contracted with phenylephrine. Each point represents the mean ± SEM, n = 6. $p < 0.05$ compared with vehicle (DMSO).

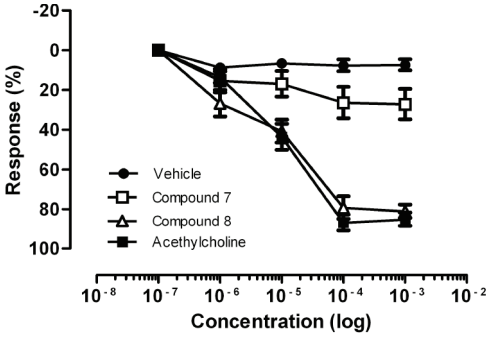

Fig. 3 - Vasorelaxant response induced by the compounds **7** and **8** evaluated on endothelium-intact aortic rings pre-contracted with phenylephrine. Each point represents the mean ± SEM, n = 6. $p < 0.05$ compared with vehicle (DMSO).

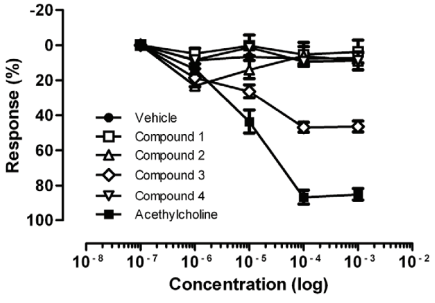

Fig. 1 - Vasorelaxant response induced by the compounds **1**-**4** evaluated on endothelium-intact aortic rings pre-contracted with phenylephrine. Each point represents the mean ± SEM, n = 6. $p < 0.05$ compared with vehicle (DMSO).

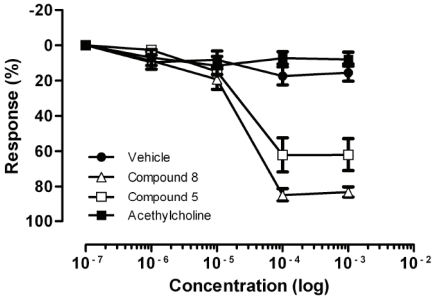

Fig. 4 - Vasorelaxant response induced by the compounds **5** and **8** evaluated on endothelium-denuded aortic rings pre-contracted with phenylephrine. Each point represents the mean ± SEM, n = 6. $p < 0.05$ compared with vehicle (DMSO).

Table 2
Maximal relaxation (E_{max}) and half-maximal inhibitory concentration values of compounds **5** and **8** on endothelium-intact (E+) and endothelium-denuded (E-) thoracic aorta rings.

E+			E-	
Compound	**Maximal relaxation (%)**	**Half-maximal inhibitory concentration (M)**	**Maximal relaxation (%)**	**Half-maximal inhibitory concentration (M)**
Vehicle	7.69 ± 2.93	ND	17.40±5.13	ND
5	51.61 ± 7.62*	1.09 × 10-4	62.14 ± 9.64*	7.29 × 10-5
8	79.27 ± 7.41*	1.71 × 10-5	84.84 ± 3.62*	2.84 × 10-5
Acethylcholine	93.75 ± 3.05*	8.33 × 10-5	5.24 ± 4.13	ND
6	28.15 ± 7.62*			
7	26.36 ± 7.89*			
8	79.27 ±7.41*			
Acethylcholine	86.81 ± 3.05*			
Vehicle	7.69 ± 2.93			

D, not determined.
Results are expressed as mean ± SEM of n = 6.
[a]$p < 0.05$ compared with vehicle (DMSO).

Discussion

The vasorelaxant activity of several naturally occurring diterpenoids has been investigated. Many studies point to diterpenoids as a promising source of new prototypes for the discovery and development of novel cardiovascular therapeutic agents (Baccelli et al., 2005; Guerrero et al., 2004; Tirapelli et al., 2004; Tirapelli et al., 2008b). For this reason, the aim of the present study is to obtain new evidence on the vascular properties of those that diterpenoids present a different framework.

In this vascular screening, different kinds of diterpenoids from different plants exerted a vasorelaxant effect. We evaluated five labdane-type diterpenoids in isolated rat aortic rings. Among those labdanes, jhanidiol acetate (**5**) showed the greatest relaxant effect in endothelium-intact rat aortic rings, and this effect was preserved in endothelium-denuded arteries contracted with PE, suggesting an endothelium-independent effect. Other labdane-type diterpenoids have been linked to endothelium-independent vasorelaxant action (de Oliveira et al., 2006; Lahlou et al., 2007). Several mechanisms of action have been proposed to explain the vascular activity exhibited by these labdane-type diterpenes: (1) blocking extracellular Ca2+ influx (Silva et al., 2005), (2) blocking intracellular Ca^{2+} influx (Ambrosio et al., 2002; El Bardai et al., 2003) and (3) increasing cyclic nucleotides (Insel and Ostrom, 2003). It is well known that these mechanisms play an important role in the regulation of vascular tone, and consequently they have been used to develop drugs to treat cardiovascular diseases.

Regarding the *ent*-kaurane–type diterpenoids assayed, both compounds (**7** and **8**) showed a significant vasorelaxant effect. Compound 8 exhibited a more potent effect than compound **7**, and its effect seems to be endothelium-independent since the removal of the endothelium did not abolish the response. These date are in agreement with the previous endothelium-independent vasorelaxant effect described for other *ent*-kaurane–type diterpenoids (Ambrosio et al., 2004; Tirapelli et al., 2004). Reported in other assays (Ambrosio et al., 2006), it has been shown that also some *ent*-kaurane–type diterpenoids are able to inhibit vascular contractility, mainly by blocking extracellular Ca^{2+} influx.

While *ent*-kaur-16-en-19-ol (7), with a C-19 hydroxy group, a discreet showed vasorelaxant effect, grandiflorenic acid (**8**), with a carboxyl group at this position, the presented vasoleraxant significant effect. This finding is in agreement with the results published by Ambrossio et al. (2004). These authors studied the influence of the C-19 carboxyl group in *ent*-kaur-16-en-19-oic acid and found that this fraction methylation produced a decrease in vasorelaxant effect. This suggests that the C-19 has a free carboxyl group great vasorelaxant effect independent of the endothelium.

Our study provides information about the vascular actions of several diterpenoids, and describes the endothelium-independent vasorelaxant effect of jhanidiol acetate (**5**) and grandiflorenic acid (**8**). The effects observed support our idea that these products are a promising source of new drugs for prototypes for the development of novel cardiovascular therapeutic agents. However, it is important to emphasize that more biological, toxicological, and *in vivo* studies of these diterpenes should be undertaken, and structure-activity relationships should be established.

Conclusions

The *in vitro* pharmacological findings of this article are the first reported information on the vasorelaxant effect of grandiflorenic acid and jhanidiol acetate. Our results demonstrate that these two diterpenoids produce an endothelum-independent vasorelaxant effect and could have

beneficial effects on the cardiovascular system. Nevertheless, the need to which mechanisms by grandiflorenic acid and jhanidiol acetate exert their endothelum-independent vasorelaxant effect, and the signaling mechanisms, remain to be established.

Authorship

The work presented here was carried out in collaboration between all authors. EG and JMA-L defined object of research. FR-M and AA performed phytochemical work. JLL-P, which developed ^{13}C NMR spectroscopic database NAPROC-13 (http://c13.usal.es), held the spectroscopic analyses natural products. EM took bioassays. JM-P and EG interpreted the results and wrote the publication.

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