VASCULAR MECHANISMS OF MONODESMOSIDIC TRITERPENE SAPONINS ISOLATED FROM *Passiflora quadrangularis* L.

MECANISMOS VASCULARES DE SAPONINAS TRITERPÉNICAS MONODESMOSÍDICAS AISLADAS DE *Passiflora quadrangularis L.*

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ABSTRACT

Background: *Passiflora quadrangularis* L. has antihypertensive and anxiolytic properties observed in experimental models. **Objectives:** The aim of this work was to establish the vascular effects exerted by two known monodesmosidic triterpene saponins, 3-*O*-β-D-glucopyranosyloleanolic acid (Compound **1**) (not previously described for this plant) and, 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl] oleanolic acid (Compound **2**), isolated from the ethanolic extract of *Passiflora quadrangularis* L. leaves. **Methods:** The structural elucidation was achieved by Nuclear Magnetic Resonance (NMR) experiments and High-Resolution Mass Spectrometry (HRMS). Aortic rings from Wistar rats, previously stimulated with phenylephrine (PE, 1μM) and washed, were exposed to cumulatively concentrations of compound **1** and compound **2** (10 to 400 μM). Ethanolic extract from leaves of *P. quadrangularis* L. (10 to 320 μg/mL) and clonidine (1nM to 100μM) were also used for comparison. Concentration response curves of compounds **1** and **2** were examined in presence and absence of: endothelium, the alpha-2 antagonist yohimbine (1 and 100μM), the alpha non selective antagonist phentolamine (1μM), the alpha-1 antagonist prazosin (1μM) and the calcium channel blocker verapamil (10 and 100 μM). In addition, cumulatively response curve of acetylcholine (ACh, 10nM to 10μM) and sodium nitroprusside (SNP, 1nM to 100μM) were assayed in rings precontracted with compounds **1** and **2** (400 μM). **Results:** Compounds **1** and **2** elicited a vasoconstriction response in intact aorta rings in similar way (pEC$_{50}$: 3.92±0.01 and 4.09±0.01, respectively), effect that did not change in denuded rings (pEC$_{50}$: 3.90±0.01 and 4.11±0.01). The potency order (pEC$_{50}$) of compounds **1** and **2** decreased according to the following: verapamil (3.53±0.01 and 3.90±0.02; p<0.05) < yohimbine (3.65±0.01 and 3.94±0.02; p<0.05) < prazosin (3.86±0.01 and 4.30±0.02) < phentolamine (4.05±0.02 and 4.05±0.01). SNP but not ACh, was able to decrease the vasopressor effect of compounds **1** and **2** (pIC$_{50}$: 8.61±0.01 and 8.24 ± 0.15, respectively). **Conclusions:** Compounds **1** and **2** are key metabolites responsible for the *ex vivo* vasoconstrictor response induced by *P. quadrangularis* L. Activation of voltage-dependent calcium channels and/or α_2-adrenergic receptors stimulation could be mechanisms implicated.

Keywords: *Passiflora*, oleanane triterpenes, saponins, aortic rings, vasoconstriction, antihypertensive.

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RESUMEN

Antecedentes: *Passiflora quadrangularis* L. tiene propiedades antihipertensivas y ansiolíticas observadas en modelos animales. **Objetivos:** El objetivo de este trabajo fue establecer los efectos vasculares ejercidos por dos conocidas saponinas triterpénicas monodesmosídicas: el ácido 3-*O*-β-D-glucopiranosiloleanólico (Compuesto **1**) (no descrito previamente para esta especie vegetal) y el ácido 3-*O*-[β-D-glucopiranosil- (1→2) -β-D-glucopiranosil]oleanólico (Compuesto **2**), aisladas del extracto etanólico de las hojas de *Passiflora quadrangularis* L. **Métodos:** La elucidación estructural se llevó a cabo mediante experimentos de Resonancia Magnética Nuclear (NMR) y determinaciones de Espectrometría de Masas de Alta Resolución (HRMS). Los anillos aórticos de ratas Wistar, previamente estimulados con fenilefrina (PE, 1 μM) y lavados, fueron expuestos a concentraciones acumulativas del compuesto **1** y compuesto **2** (10 a 400 μM). El extracto etanólico de las hojas de *P. quadrangularis* L. (10 a 320 μg / ml) y clonidina (1 nM a 100 μM) se utilizaron para la comparación. Las curvas de concentración respuesta de los compuestos **1** y **2** se examinaron en presencia y ausencia de: endotelio, el antagonista alfa-2 yohimbina (1 y 100 μM), el antagonista alfa no selectivo fentolamina (1 μM), el antagonista alfa-1 prazosina (1 μM) y el bloqueador de canales de calcio verapamilo (10 y 100 μM). Además, la curva de concentraciones acumulativas de acetilcolina (ACh, 10 nM a 10 μM) y nitroprusiato de sodio (SNP, 1 nM a 100 μM) se ensayó en anillos pre-contraídos con los compuestos **1** y **2** (400 μM). **Resultados:** Los compuestos **1** y **2** provocaron una respuesta de vasoconstricción en los anillos de aorta intactos de manera similar (pEC$_{50}$: 3.92 ± 0.01 y 4.09 ± 0.01, respectivamente), este efecto no cambió en los anillos denudados (pEC$_{50}$: 3.90 ± 0.01 y 4.11 ± 0.01). El orden de potencia (pEC$_{50}$) de los compuestos **1** y **2** disminuyó de la siguiente manera: verapamilo (3.53 ± 0.01 y 3.90 ± 0.02; p <0.05) < yohimbina (3.65 ± 0.01 y 3.94 ± 0.02; p <0.05) < prazosina (3.86 ± 0.01 y 4.30 ± 0.02) <fentolamina (4.05 ± 0.02 y 4.05 ± 0.01). SNP pero no ACh, fue capaz de disminuir el efecto vasopresor de los compuestos **1** y **2** (pIC$_{50}$: 8.61 ± 0.01 y 8.24 ± 0.15, respectivamente). **Conclusiones:** Los compuestos **1** y **2** son metabolitos clave responsables de la respuesta vasoconstrictora *ex vivo* inducida por *P. quadrangularis* L. La activación de los canales de calcio dependientes de voltaje y / o la estimulación de los receptores adrenérgicos α_2 podrían ser los mecanismos implicados.

Palabras clave: *Passiflora*, triterpenos oleanano, saponinas, anillos aórticos, vasoconstricción.

INTRODUCTION

Infusion of leaves from *Passiflora quadrangularis* L (known as Badea) is used in Colombian folk medicine for hypertension, anxiety and diabetes. This plant belongs to the *Passifloraceae* family, with 167 species found in Colombia, from the *Ancistrothyrsus*, *Dilkea* and *Passiflora* genera (1). In previous studies, the ethanolic extract of leaves administered orally was able to decrease blood pressure in Wistar rats with L-NAME-induced hypertension, as well as prevent against vascular remodeling (2). Aqueous and ethanolic extracts have also been shown to have anxiolytic-like properties (3).

The main constituents of *P. quadrangularis* L. are flavonoids (4,5), β-carboline harmane alkaloids (6) and triterpene saponins (4,5,7,8).

Triterpene saponins are natural products present in many organisms, especially in plants, and exhibit interesting biological and pharmacological activities, including anti-inflammatory, antimicrobial, antiviral (9,10) and antitumor effects (11).

Hypertension is a major modifiable risk factor for cardiovascular disease and premature mortality worldwide (12). Natural products have been important sources of new drugs, and the treatment of hypertension with plant extracts or plant derived products is well documented (13).

This work shows the isolation and structural elucidation of two known monodesmosidic triterpene saponins from *P. quadrangularis* L. with vasoconstrictor effects: 3-*O*-β-D-glucopyranosyloleanolic acid (compound **1**) (14) and 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]oleanolic acid (compound **2**) (8).

MATERIAL AND METHODS

Plant material and extraction

Leaves of *P. quadrangularis* L. were collected in Bella Vista – Algeciras – Huila, Colombia (latitude 2.54750 – longitude -75.28727), with an elevation 1100 MASL, and an average temperature of 24 °C. Voucher specimens were deposited at the Colombian National Herbarium (COL N° 602879). The leaves were dried at 40 °C in a circulating air oven. Then, the dried material was pulverized and macerated with 96% ethanol for 72 hours, then filtered and concentrated with a rotary evaporator.

Extraction and isolation

The ethanolic extract of *P. quadrangularis* L. (39 g) was dissolved in methanol and stored at 18 °C for 14 hours until the wax precipitated. The extract without wax (37 g) was separated into two fractions, with dichloromethane (CH_2Cl_2) and methanol (MeOH), in different proportions, obtaining a soluble (22 g) and insoluble (15 g) CH_2Cl_2 fraction. The soluble CH_2Cl_2 fraction was further fractionated by flash column chromatography. The elution system was based on previous thin layer chromatographic (TLC) analysis using hexane, ethyl acetate (EtOAc) and MeOH. Silica gel 60 (0.040-0.063 mm) (Merck®) was used as the stationary phase and the mobile phase increased in polarity. Then, 15 mL fractions 180-237 (1.1 g) and 254-270 (1.5 g) were collected according to the TLC profile.

Fractions 180-237 were mixed and flash chromatographed on silica gel using CH_2Cl_2/ MeOH (9:1) as the mobile phase. This process led to sub-fractions 5-6, which yielded 331 mg of pure compound **1** (Figure 1). Fractions 254-270 were also separated by flash column chromatography. The system of elution was CH_2Cl_2/MeOH (8:2) as the mobile phase and silica gel as the stationary phase; sub-fractions 35-43 were obtained, yielding 1g of pure compound **2** (Figure 1).

HRMS analysis was performed using an Applied Biosystems QSTAR XL spectrometer, UV spectra were recorded on a Perkin-Elmer FT-IR, System spectrum BX spectrophotometer, the melting point was measured on Gallen Kamp apparatus and NMR (^{1}H and ^{13}C) spectra were recorded on a Bruker DRX-

400 spectrometers. Chemical shifts (δ) were given in ppm downfield from tetramethylsilane (TMS) as an internal standard and coupling constants (*J* values) were in Hertz.

Compound 1: 3-*O*-β-D-glucopyranosyloleanolic acid

MP: 230-233°C (EtOAc)

HRMS: *m/z* [M+Na$^+$] calcd for $C_{36}H_{58}O_8$: 641.4030

IR (KBr): 3417, 1693, 1463, 1386, 1077 cm^{-1}.

^{1}H NMR (400 MHz, CD_3OD) δ: 3.25 (1H, m, H-3), 5.23 (1H, bs, H-12), 3.40 (1H, dd, *J* = 2.0, 12.0 Hz, H-18), 1.04 (3H, s, H-23), 0.90 (3H, s, H-24), 0.80 (3H, s, H-25), 0.93 (3H, s, H-26), 1.15 (3H, s, H-27), 0.94 (3H, s, H-29), 0.84 (3H,

s, H-30), 4.30 (1H, d, *J* =7.2 Hz, glc-H1), 3.83 (1H,dd, *J*=2.0, 9.8 Hz, glc-H6a), 3.65 (1H,dd, *J*=5.2, 9.8 Hz, glc-H6b)

^{13}C NMR (400 MHz, CD_3OD) δ: 39.8 (CH_2, C-1), 27.0 (CH_2, C-2), 90.8 (CH, C-3), 40.1 (C, C-4), 57.0 (CH, C-5), 19.4 (CH_2, C-6), 33.7 (CH_2, C-7), 40.1 (C, C-8), 48.3 (CH, C-9), 37.9 (C, C-10), 24.1 (CH_2, C-11), 123.6 (CH, C-12), 145.2 (C, C-13), 42.7 (C, C-14), 28.8 (CH_2, C-15), 24.0 (CH_2, C-16), 47.6 (C, C-17), 42.9 (CH, C-18), 47.7 (CH_2, C-19), 31.6 (C, C-20), 33.8 (CH_2, C-21), 34.0 (CH2, C-22), 28.5 (CH_3, C-23), 16.0 (CH_3, C-24), 17.0 (CH_3, C-25), 17.7 (CH_3, C-26), 26.4 (CH_3, C-27), 180.8 (C, C-28), 33.8 (CH_3, C-29), 24.0 (CH_3, C-30), 106.7 (CH, C-1'), 77.6 (CH, C-2'), 78.2 (CH, C-3'), 71.6 (CH, C-4'), 77.9 (CH, C-5'), 62.8 (CH_2, C-6')

Compound 2: 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]oleanolic acid

MP: 248°C (EtOAc/Ether)

HRMS: *m/z* [M+Na$^+$] calcd for $C_{42}H_{68}O_{13}$: 803.4588

IR (KBr): 3400, 1645, 1458, 1365 cm^{-1}.

^{1}H NMR (400 MHz, CD_3OD) δ: 3.20-3.40 (1H, m, H-3), 5.23 (1H, bs, H-12), 1.07 (3H, s, H-23), 0.90 (3H, s, H-24), 0.80 (3H, s, H-25), 0.93 (3H, s, H-26), 1.15 (3H, s, H-27), 0.94 (3H, s, H-29), 0.86 (3H, s, H-30), 4.42 (1H, d, *J*=7.2 Hz, glc-H-1'), 4.66 (1H,d, *J*=7.6 Hz, glc-H-1"), 3.54 (2H, m, H-6'), 3.82 (2H, m, H-6")

^{13}C NMR (400 MHz, CD_3OD) δ: 39.8 (CH_2, C-1), 27.1 (CH_2, C-2), 91.5 (CH, C-3), 40.4 (C, C-4), 57.0 (CH, C-5), 19.3 (CH_2, C-6), 33.6 (CH_2, C-7), 40.5 (C, C-8), 48.3 (CH, C-9), 37.9 (C, C-10), 24.0 (CH_2, C-11), 123.6 (CH, C-12), 145.2 (C, C-13), 42.7 (C, C-14), 28.5 (CH_2, C-15), 24.5 (CH_2, C-16), 47.2 (C, C-17), 42.9 (CH, C-18), 47.6 (CH_2, C-19), 31.6 (C, C-20), 33.6 (CH_2, C-21), 33.8 (CH_2, C-22), 28.5 (CH_3, C-23), 16.0 (CH_3, C-24), 17.0 (CH_3, C-25), 17.7 (CH_3, C-26), 26.4 (CH_3, C-27), 181.2 (C, C-28), 33.6 (CH_3, C-29), 24.0 (CH_3, C-30), 106.4 (CH, C-1'), 77.6 (CH, C-2'), 78.3 (CH, C-3'), 71.9 (CH, C-4'), 78.7 (CH, C-5'), 62.8 (CH_2, C-6'), 105.4 (CH, C-1"), 81.0 (CH, C-2"), 77.9 (CH, C-3"), 71.5 (CH, C-4"), 78.4 (CH, C-5"), 63.0 (CH_2, C-6")

Aortic rings preparation

Wistar male rats (200–300 g) were euthanized using a CO_2 chamber. The descending thoracic aorta

was dissected by thoracotomy and immersed in Krebs solution for its preservation. The surrounding tissue of aorta was removed, and fragments were cut into 3 mm lengths. Thoracic aortic rings were connected using an isometric transducer (fort 10/WPI) and submerged in Allhin organ chambers containing 10 mL of Krebs solution, with bathing medium maintained at 37 °C, pH 7.38-7.42 and continuously exposed to a carbogen mixture of 95% O_2 and 5% CO_2.

A basal tension of 2 g was applied to each aortic ring with a stabilization period of 60 min, during which time the Krebs solution was periodically changed, and tension was reset. Once equilibrium was reached, the aortic rings were exposed to phenylephrine (PE 1μM) until the contractile response reached a steady tension and the subsequent concentration of acetylcholine (ACh 1μM) was added to verify the functionality of the endothelium. PE contractile response was used as a reference for normalization of the test compounds response.

Compounds **1** and **2** and ethanolic extract were added cumulatively between 10 and 400 μM and 10 and 320 μg/mL respectively, and clonidine (as reference drug) was added between 1 nM and 100 μM every 15 min using a logarithmic scale. In the other experiments, aortic rings without endothelium, test agents such as alpha-antagonist (yohimbine 1 and 100 μM, prazosin 1μM and phentolamine 1μM) and a blocker of L-type calcium channels (verapamil, 10 and 100 μM) were added to the organ bath 5–10 min before the cumulative addition of compounds **1** and **2**, extract or clonidine.

Independent and endothelium dependent mechanisms were examined with SNP (1nM to 100μM) and ACh (10nM to 10μM), respectively, in rings pre-contracted with saponins (400 μM, E_{max}) and the extract (320 μg/mL, E_{max}). When necessary, the endothelium was removed by rubbing the intimal surface with a thread, after which 1μM ACh was added to check for absence of the endothelium in the aortic rings; relaxation up to 10% was accepted. After this, they were washed with Krebs solution until reaching basal tension again and compounds **1** and **2**, ethanolic extract and clonidine were added cumulatively.

The experimental procedure was approved by the institutional ethics committee (Act 14, November 2017, Faculty of Sciences, at the Universidad Nacional de Colombia). The animals were supplied by the Bioterium of the Pharmacy Department at the Universidad Nacional de Colombia.

Data analysis and statistics

The response of aortic rings was expressed as a percentage of the initial contraction to 1μM PE. Dose-response curves were analyzed to give the negative log of the concentration of compounds **1** and **2**, ethanolic extract and clonidine, producing a 50% half maximal effective concentration (pEC_{50}) by sigmoidal curve-fitting analysis. All results are expressed as means $\pm$ the standard error of the mean (S.E.M.); n=5-14, where n indicates the number of animals or number of aortic rings. Differences in concentration response curves between the extract and isolated compounds with antagonists were analyzed by the unpaired Student´s *t*-test or Mann- Whitney as appropriate, with a criterion set for statistical significance at p<0.05. The responses to ACh and SNP are expressed as a percentage of PE contraction. Dose-response curves were analyzed to give the negative log the concentration of ACh or SNP, producing a 50% half maximal effective concentration (pIC_{50}) by sigmoidal curve-fitting analysis. Excel (Microsoft® Office Excel 2013) and GraphPad Prism 6 software were used for data analysis.

Drugs and solutions

The following salts and drugs were used: NaCl, $CaCl_2$, $NaHPO_4$, $NaHCO_3$, glucose, KCl, $MgCl_2$, KH_2PO_4, $MgSO_4$, ascorbic acid (Panreac), acetylcholine, phenylephrine, sodium nitroprusside (Sigma - Aldrich), dimethylsulfoxide (DMSO) (Loba Chemie), clonidine, verapamil, yohimbine, prazosin and phentolamine (Cayman Chemical). All substances were dissolved in DMSO. This vehicle was used at a final concentration of 0.1%.

RESULTS

Structural elucidation of saponins isolated from *Passiflora quadrangularis* L.

For compound **1**, the molecular formula $C_{36}H_{58}O_8$ was deduced based on the high-resolution mass spectrometry (HRMS) spectrum which displayed a pseudo-molecular ion peak at *m/z* 641.4030 [M+Na$^+$]$^+$. The structure idenfication of compound **1** was attained through the analysis of its ^{1}H and ^{13}C NMR spectral data resumed as follows. Carbon signals were observed for a free

carboxyl group at position C-28 (δ_C 180.8), and for one anomeric carbon of a β-D-glucopyranoside (δ_C 106.7, associated to a methine proton signal at δH 4.30, 1H, d, *J*=7.2), which along with the oxygenated methine signal at δ_C 90.8 (assigned to C-3 of the aglycone) and to the remaining ^{13}C and ^{1}H signals in the NMR spectra, supported the structure of an unsaturated pentacyclic triterpene acid, suggesting the proposal of an oleanolic acid derivative (14,15) for compound **1**. As the result, compound **1,** being isolated for the first time from this plant, was finally identified as 3-*O*-β-D-glucopyranosyloleanolic acid (Figure 1). As a proof of the glucoside location at C-3, in the HMBC 2D-NMR correlation spectrum for compound **1** (see suppl. mat. fig S6), the C-3 signal was correlated with those of the anomeric H-1′ and with those of protons H-23 and H-24 of the geminal methyl groups attached to C-4.

The striking similarity of the ^{13}C NMR spectra of compounds **1** and **2** readily suggested that compound **2** was also a derivative of oleanolic acid, though with two glucose (Glc) residues attached to C-3, as proved by the presence of two signals of anomeric carbons (δ_C 106.4 and δ_C 105.4) in its spectrum. In the HRMS, compound **2** exhibited a peak at *m/z* 803.4588 [M+Na+]+ that was consistent with the presence of two Glc residues. The inter-glycosidic linkage of this disaccharide was determined to be Glc (1→2) Glc by the chemical shift of a CH signal of the ^{13}C NMR spectrum (δ_C 81.0, C-2′) (14). Also, in the HMBC spectrum, the C-3 signal showed correlations with H-1′, and with those methyl protons H-23 and H-24 (see suppl. mat. fig S13). Additionally, The C-1" anomeric carbon correlations with protons H-2′ and H-2", thus establishing C-2' as the linking position of the second sugar unit. Consequently, compound **2** was identified as the monodesmosidic 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl] oleanolic acid ($C_{42}H_{68}O_{13}$), previously reported (8) (Figure 1).
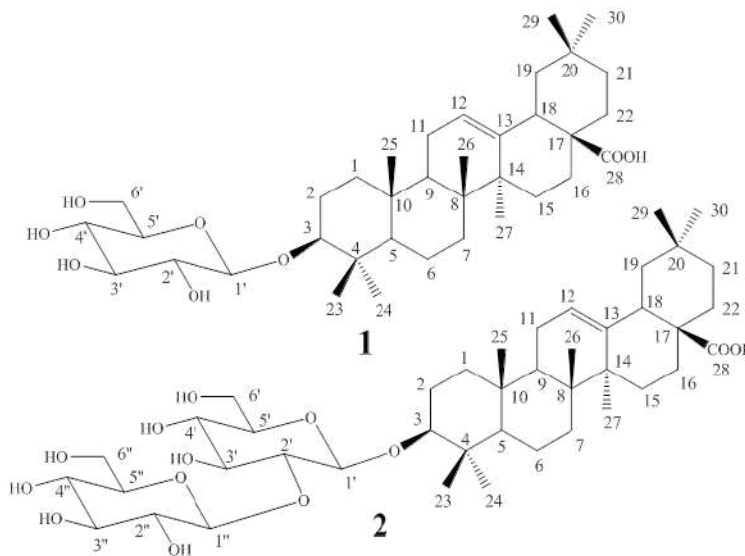

Figure 1. Structure of compounds isolated from *P. quadrangularis* L. (**1**) 3-*O*-β-D-glucopyranosyloleanolic acid. (**2**) 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]oleanolic acid.

Effect of yohimbine, phentolamine, prazosin and verapamil in aorta contracted with compound 1, compound 2 and ethanolic extract without endothelium

The α_1-adrenergic agonist PE, 1μM, used as reference agent in all assays, induced a maximal vasoconstrictor response of 1613 ± 55mg.

The ethanolic extract of *Passiflora quadrangularis* L. induced concentration-dependent contraction in intact and denuded rings without significant differences p>0.05 (maximum effect-E_{max}): 98±1.66%, pEC_{50}: 4.08±0.009 and E_{max}: 105±1.92%, pEC_{50} 4.0±0.007 respectively (Table 1). Pre-treatment with yohimbine (1μM), phentolamine (1μM) and prazosin (1μM) attenuated the ethanolic

extract induced contraction in a concentration-dependent manner in denuded rings (p<0.05 in all cases) (Figure 2-A, Table 2). Pre-treatment with verapamil (10μM) attenuated the ethanolic extract induced contraction in a concentration-dependent manner in denuded rings (p<0.05) (Figure 2-A, Table 2). SNP but not ACh, was able to decrease the vasopressor effect of compounds **1** and **2** (Figure 3-A; 3-B, Table 3).

Table 1: E_{max} and pEC_{50} of compound **1**, compound **2** and ethanolic extract in aortic rings with endothelium or denuded.

Fraction /Compound	Rings with endothelium		Rings without endothelium	
	pEC_{50}	Emax (%)	pEC_{50}	Emax (%)
Extract	4.08±0.009	98.00±1.66	4.0±0.008	105.00±1.92
Compound 1	3.92±0.011	109.00±1.96	3.90±0.007	115.00±2.26
Compound 2	4.09±0.011	109.00±1.14	4.11±0.008	112.00±1.69

The results are expressed as mean ± S.E.M of the number of dates. pEC_{50} (-logCE_{50}) and Emax (percent of 1μM, PE maximal contraction). Units for pEC_{50} are expressed as g/mL for whole crude ethanolic extract and M for Compound **1** and **2**. There are not statically differences for compound **1** and **2**, p>0.05.

Table 2. Effect of yohimbine, phentolamine, prazosin and verapamil in aorta contracted with compound **1**, compound **2** and ethanolic extract without endothelium.

	Ethanolic extract		Compound 1		Compound 2	
	pEC_{50}	Emax (%)	pEC_{50}	Emax (%)	pEC_{50}	Emax (%)
No drug	4.00±0.008	105.00±1.92	3.90±0.007	115.00±2.26	4.11±0.008	112.00±1.69
Yohimbine (1 μM)	3.94±0.010*	97.00±2.43*	3.88±0.028**	96.51±4.91**	4.02±0.012*	109.20±2.92*
Yohimbine (100 μM)	---	---	3.65±0.012**++	92.00±2.67**++	3.94±0.016**++	100.00±4.40**++
Phentolamine (1 μM)	3.88±0.030**	80.40±7.35**	4.05±0.020	111.00±2.37	4,05±0.005	117.00±1,54
Prazosin (1 μM)	3.92±0.012*	91.08±2.85*	3.86±0.010	121.00±2.36	4.30±0.022	114.50±1.93
Verapamil (10 μM)	3.84±0.011*	39.41±1.28*	3.77±0.009**	86.43±3.51**	3.94±0.023	92.04±5.05**
Verapamil (100 μM)	---	---	3.53±0.019**#	67.00±20.5**#	3.90±0.017**##	43.00±1.72**##

The results are expressed as mean ± S.E.M of the number of dates. pEC_{50} (-logCE_{50}) and Emax (percent of 1 μM, PE maximal contraction). Units for pEC_{50} are expressed as g/mL for whole crude ethanolic extract and M for Compound **1** and **2**. *p<0.05 vs. No drug; +p<0.05 vs. Y (1 μM); #p<0.05 vs. V (10 μM).

Compound **1** induced a vasoconstrictor response in intact rings (E_{max} 109±1.96%; pEC_{50}: 3.92±0,011) and denuded rings (Emax: 115±2.26%; pEC_{50}:3.90±0.007) (Table 1). Pre-treatment with yohimbine 1 μM and Verapamil 10 μM attenuated the compound **1**-induced contraction in a concentration-dependent manner in denuded rings (p<0.05) (Figure 2-B, Table 2), whereas pre-treatment with prazosin (1 μM) and phentolamine (1 μM) did not show significant differences (p>0.05) (Table 2). Yohimbine and verapamil 100 μM were also evaluated (p<0.05) (Figure 2-B). Concentration-response curves ACh and NPS showed the same behavior as the extract (Figure 3-A; 3-B, Table 3).

Compound **2** induced a vasoconstrictor response in intact rings (E_{max}: 109±1.14%; pEC_{50}: 4.09 ± 0.011) and denuded rings (E_{max}: 112 ± 1.69%; pEC_{50}: 4.11 ± 0.008) (Table 1). Pre-treatment with yohimbine (1 μM and 100 μM) and verapamil (10 μM and 100 μM), attenuated the compound **2** induced contraction in a concentration-dependent way in denuded rings (p<0.05) (Figure 2-C, Table 2). In contrast, pre-treatment with prazosin (1 μM) and phentolamine (1 μM) did not show any significant differences (p>0.05) (Table 2). Concentration-response curves ACh and NPS presented the same behavior as the extract (Figure 3-A; 3-B, Table 3).
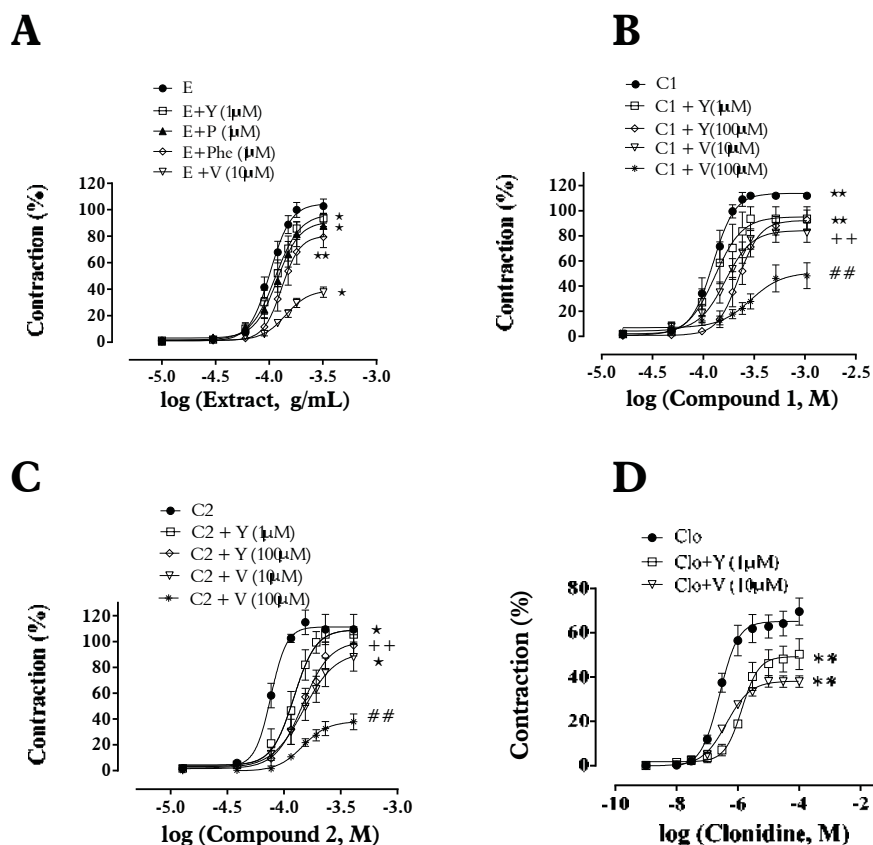
Figure 2. Effect of yohimbine (Y), α_2-adrenoceptor antagonist; phentolamine (Phe), α_1- and α_2-adrenoceptor antagonist, prazosin (P), α_1-adrenoceptor antagonist; and of verapamil (V), blocker of L-type calcium channels on the contraction effect induced by ethanolic extract (E) - **A**, compound **1** (C1) – **B**, compound **2** (C2) – **C** and clonidine (Clo) - **D** in endothelium-denuded the aortic rings of Wistar rats. E (10–320 μg/ml), C1, C2 (10–400 μM) and Clo (1nM- 100 μM) induced contraction was performed in arteries contracted with phenylephrine (1 μM) in the absence or presence of Y (1 μM), P (1 μM) or V (10 μM). Y (100 μM) and V (100 μM) only for C1 and C2. Data are reported as the mean ± S.E.M. (n = 5-9). ★p<0.05 vs. E, C1, C2 or Clo); +p<0.05 vs. Y (1 μM); #p<0.05 vs. V (10 μM). Student's *t*-test – Paired.

Table 3. Effect of ACh and SNP in aortic rings with endothelium contracted with C1, C2 (400μM) and E (320 μg/ml).

	Ach		SNP	
	pIC_{50}	E_{max} (%)	pIC_{50}	E_{max} (%)
Control	7.07±0.05	63.56±1.34	8.20±0.008	99.43±0.35
E	NC	0.75±0.75★	9.72±0.166	84.89±0.84
C1	NC	1.0±0.0★	8.61±0.096★	53.68±1.29★
C2	NC	0.53±1.21★	8.24±0.150★	81.02±1.53★

The results are expressed as mean ± S.E.M of the number of dates show in Figure 3. pIC_{50} (-logCE$_{50}$, M) and Emax (percent of 1 μM, PE maximal contraction).NC: Not calculated because the relaxation is too small. ★p<0.05 vs. Control
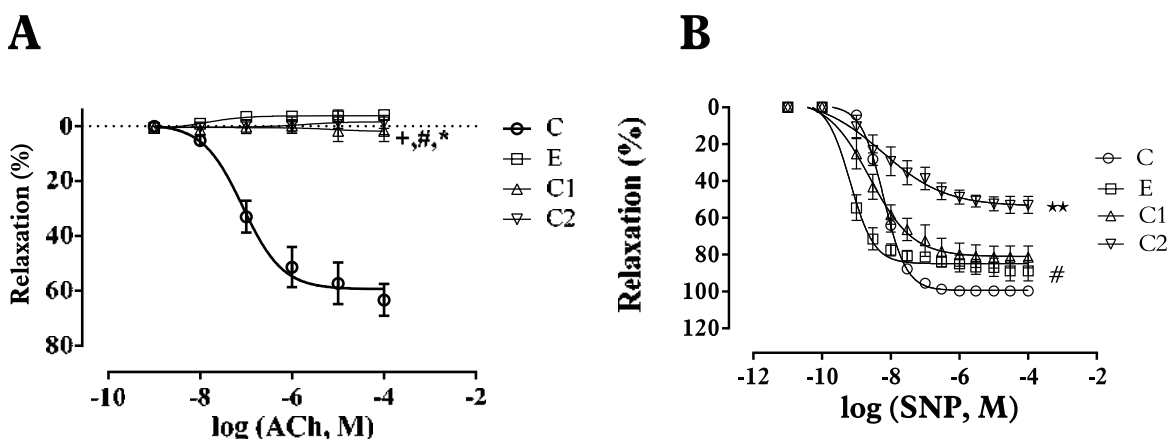
A

B

Figure 3. Effect of extract (E), compound **1** (C1) and compound **2** (C2) on the relaxation effect induced by ACh – **A** and SNP – **B** in endothelium-intact aortic rings of Wistar rats. Arteries contracted with E (320 μg/ml), C1, C2 (400 μM) induced contraction. Data are reported as the mean $\pm$ S.E.M. (n = 5-9). [+]p<0.05 (E vs ACh or SNP); [#]p<0.05 (C1 vs. Ach or SNP); [*]p<0.05 (C2 vs. ACh or SNP). Student's *t*-test – Paired.

Pre-treatment with yohimbine 1 μM and Verapamil 10 μM decreased the clonidine induced contraction in a concentration-dependent manner in denuded rings (p<0.05) (Figure 2-D, Table 4). Clonidine was used as a comparative α_2-adrenergic agonist with respect to compounds **1** and **2**.

Table 4. Effect of yohimbine and verapamil in aorta contracted with clonidine with endothelium denuded.

	Clonidine	
	pEC$_{50}$	**E$_{max}$ (%)**
No drug	6.60$\pm$0.038	65.00$\pm$1.15
Yohimbine (1μM)	5.88$\pm$0.038**	49.00$\pm$1,10**
Verapamil (10μM)	6.37$\pm$0.033**	38.00$\pm$057**

The results are expressed as mean $\pm$ S.E.M of the number of dates. pEC$_{50}$ (-logCE$_{50}$, M) and Emax (percent of 1 μM, PE maximal contraction). Units for pEC$_{50}$ are expressed as M.*p<0.05 vs. No drug.

Discussion

This work shows that compounds **1** and **2**, saponins isolated from *Passiflora quadrangularis* L., induced vasoconstrictor effects in isolated aortic rings. This response could be related to Ca^{2+} influx via voltage operated Ca^{2+} channels, and/or on α_2-adrenergic receptor activation.

However, other compounds could be implicated in the cardiovascular response induced by *P. quadrangularis* L. Among them: harmane-type or β-carboline-type alkaloids, vitexine-type flavonoids and cycloartenol saponins, that could add to the oleanane saponins response decrypted in this study.

In previous studies, the extract of leaves of *P. quadrangularis* L. and other species of this genera, showed sedative and anxiolytic effects and triterpene saponins could be responsible for these effects (3, 16), as well as for preventing the hypertension induced by nitric oxide deficit in rats (2).

It is known that stimulation of post-junctional α_2-adrenergic receptors on arterial smooth muscle mediates a pressor response, whereas their presynaptic activation on central nervous system induces a reduction in sympathetic outflow, manifested as a reduction in the arterial blood pressure, bradycardia and sedative effects (17). Thus, an agent with alpha-2 agonism properties elicits vasoconstriction in isolated aortic rings. That could be the case of compounds **1** and **2**.

Saponins show therapeutic interest for targeting several diseases, with reports of antitumor, hepatoprotective, neuroprotective, anti-hyperglycemic, osteogenic (18), anti-inflammatory, antifungal, antibacterial (9) and antihypertensive effects (19)14-week-old SHRs were randomly divided into three groups; the first was administered low-dose saponin, the second with high-dose saponin and the third with a placebo as the control group. An additional eight healthy male Wistar

rats were used as the normal group. The blood pressures (BP$_s$. The triterpenoids, specially from plants, also display biological and pharmacological properties like anticancer, antimicrobial and anti-inflammatory activities, and cardiovascular effects (10, 21).

For saponin structure-activity relationships, both the aglycone and the number of sugar units seem to play an important role in their biological activity. In addition, 1→2 (present in compound **2**) and 1→4 linkages give higher activity than the 1→3 linkage (11). Related to the present work, 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl] oleanolic acid was previously reported by Orsini (8); while 3-*O*-β-D-glucopyranosyloleanolic acid was previously described in *Passiflora alata* C. (14), but not in *P. quadrangularis* L. The vasoconstrictor effects of the two compounds are reported for the first time.

Compound **1**, compound **2** and the ethanolic extract, elicited maximal vasoconstrictor effect in aortic rings with intact endothelium, response that not changed after de-endothelization. This leaves to suppose that site of action of those metabolites places at vasculature media layer. Given that alpha adrenergic receptors are located in smooth muscle (α_1, α_2) (21,22), and they are connected to calcium signalling (23). compounds **1** and **2** could mediate calcium currents activation on smooth muscle via α stimulation, being key metabolites responsible for the global effect seen with *P. quadrangularis* L.

The idea of a possible mechanism of compounds **1** and **2** related to activation of α_2-adrenoceptor/calcium currents pathway is supported observing that their potency (pEC$_{50}$) and efficacy (E$_{max}$) of contraction were reduced more against yohimbine and verapamil than against prazosin and phentolamine (Figure 2, Table 2). Ethanolic extracts also contain other compounds with possible effects on α_1-adrenoceptor or unspecific receptors, while compounds **1** and **2** have effects on α_2-adrenoceptors or calcium channels.

Previous studies have described the influence of calcium channel blockers on the stimulation of postsynaptic α_1- and α_2-receptors in resistance vessels in pithed rats. The vasoconstriction induced by postsynaptic α_2-adrenoceptor stimulation is extremely sensitive to Ca^{2+} entry blockers evoked by Ca^{2+} antagonists, whereas the vasoconstriction produced by α_1-receptor stimulation is much less likely to be affected by Ca^{2+} antagonistic drugs.

For example, the effect of methoxamine, a selective α_1-adrenoceptor agonist, is unchanged by Ca^{2+} entry blockers. Also, the effect of α_1-agonists is not dependent on the extracellular current of Ca^{2+}, but on its intracellular stimulation from the sarcoplasmic reticulum. These *in vivo* findings have been confirmed in other studies (24).

It has been suggested that the response of vascular smooth muscle to α-adrenergic agonists like norepinephrine and PE depends on both, extracellular Ca^{2+} influx and the liberation of Ca^{2+} from intracellular stores. The effect of selective α_2-adrenergic agonist seems to depend exclusively on extracellular Ca^{2+} and via G$_i$-mediated inhibition of adenylyl clyclase (17, 25).

According to radioligand studies, calcium entry blockers have low affinity for adrenoceptors α_2. Therefore, the decrease in vasoconstriction induced by compounds **1** and **2** stimulation of the α_2-adrenoceptor cannot be attributed to the direct action of Ca^{2+} antagonistic drugs on the receptor. It is known that the postsynaptic stimulation of α_2-receptors by a suitable agonist is accompanied by an influx of Ca^{2+}, which allows the activation of contractile proteins; calcium antagonists inhibit this internal flow of Ca^{2+} and therefore impair the contractile process (24).

The α_2-adrenoceptors agonist clonidine and related compounds are recognized as clinically efficacious antihypertensive drugs (26); in the present study, cumulative concentration response curves of clonidine were constructed in denuded arteries. Yohimbine and verapamil attenuated clonidine-induced contractions in accordance with the above; compounds **1** and **2** showed similar behaviors, suggesting that these compounds induced contraction which involves α_2-adrenoceptor activation. In other studies of contracted aortic rings without endothelium with clonidine, this response decreased following the removal of extracellular Ca^{2+} and pre-treatment with nifedipine (25).

This study suggests that these substances induce contraction mainly dependent on Ca^{2+} influx from the extracellular space via voltage-operated calcium channels. But this effect could be, at least a partially, due to α_2-adrenergic receptors stimulation. Dexmedetomidine for example, an α_2-adrenoceptors agonists, induces contraction through the of Ca^{2+} influx (27). However, contraction mediated throw α_1-adrenergic cannot be totally discarded.

Considering previous reports that suggest the usefulness of *P. quadrangularis* L. extract in lowering blood pressure in vivo (2, 29), as well as its sedative effect (3), the monodesmosidic triterpene saponins identified in this work would induce moderately selective α_2-adrenoceptor activation of vascular smooth muscle and a$_2$ presynaptic stimulation on central nervous system(29). Notwithstanding, *P. quadrangularis* L. could contain other compounds which cardiovascular effect have yet to be studied.

Assays with ACh and SNP support the idea that the vasoconstrictor mechanisms seen on this work do not depend on endothelial-linked pathways, since ACh was unable to affect the vasoconstrictor response induced by compounds **1** and **2**. Therefore, SNP would reduce, via cGMP(30), the vasoconstrictor effect elicited throw α_2-adrenoceptor stimulation, extracellular calcium mobilization and adenylyl cyclase inhibition (17, 31) (Figure 3, Table 3).

According to these results, cGMP cascade induced by NO released by SNP would be more efficiently inhibited by compound **2** (32) and therefore, compound **2** would blockade the influx of Ca^{2+} into the cell, while compound **1** would stimulate the activation of α_2-adrenergic receptors (Figure 3, Table 3), but further studies regarding the signal transduction pathway associated with the calcium release induced by compounds **1** and **2** are needed to determine the detailed mechanism.

P. quadrangularis L. has several types of saponins (7, 8). Two known monodesmosidic triterpene saponins with vasoconstrictor activity in isolated rings of rat aorta were identified: 3-*O*-β-D-glucopyranosyloleanolic acid and 3-*O*-[β-D-glucopyranosyl–(1→2)-β-D-glucopyranosyl] oleanolic acid. 3-*O*-β-D-glucopyranosyl-oleanolic acid is reported here for first time for this plant. Further studies are required to determine the underlying mechanism of action of these compounds.

CONCLUSIONS

In conclusion, 3-*O*-β-D-glucopyranosyloleanolic acid and 3-*O*-[β-D-glucopyranosyl–(1→2)-β-D-glucopyranosyl]oleanolic acid are key metabolites responsible for the vasoconstrictor response elicited from *P. quadrangularis* L. Ca^{2+} influx via voltage-operated channels activation and/or α_2-adrenoceptor stimulation mechanisms could be implicated.

CONFLICTS OF INTEREST

No potential conflicts of interest were reported by the authors.

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AUTHOR CONTRIBUTIONS

Lesly L. Bareño (PhD student) performed the phytochemical and pharmacological experiments of this work, Pilar Puebla (PhD) directed the phytochemical studies, Arturo San Feliciano (PhD) supervised the chemical work and its writing, and Mario F. Guerrero (PhD) directed the pharmacological assays and analysis.

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