VASORELAXANT CONSTITUENTS OF THE LEAVES OF *PRUNUS SEROTINA* "CAPULÍN"

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This paper is dedicated to Professor Doctor Rachel Mata for her 60th birthday

ABSTRACT

Phytochemical study of the vasorelaxant ethyl acetate fraction obtained from the methanolic extract of the leaves of *Prunus serotina* resulted in the isolation of three known natural products, hyperoside (**1**), prunin (**2**), and ursolic acid (**3**). Compounds **1** (EC_{50} = 91.3 ± 14.1 µg/ml), **2** (EC_{50} = 66.0 ± 19.4 µg/ml) and **3** (EC_{50} = 154.4 ± 7.5 µg/ml) displayed a concentration-dependent relaxation of vascular smooth muscle. Compound **1** was approximately ten fold less potent than acetylcholine (ACh) (EC_{50} = 8.7 ± 0.8 µg/ml), which was employed as a positive control. However, this compound induced a maximum vasodilator effect (E_{max} = 92.3 ± 7.7 %) that was higher than that of ACh (E_{max} = 69.5 ± 5.7 %). In addition, it was found that the essential oil obtained from the leaves of *P. serotina* promoted vascular smooth muscle relaxation. The oil was analyzed by gas chromatography coupled to mass spectrometry and fifty seven compounds were detected. Four of the major constituents, benzyl alcohol (**4**) (20.3 %), benzaldehyde (**5**) (12.1 %), cinnamyl alcohol (**6**) (4.7 %), and cinnamaldehyde (**7**) (1.1 %) also induced a concentration-dependent relaxation of rat aorta. The greatest vasorelaxant effect was observed for compound **6** (EC_{50} = 42.2 ± 5.7 µg/ml). The results derived from this study provide a scientific basis for the traditional use of the leaves of this plant for the treatment of hypertension.

Key words: Essential oil, hyperoside, ursolic acid, isolated rat aorta, *Prunus serotina*, vasorelaxation

RESUMEN

La fracción de acetato de etilo obtenida a partir del extracto metanólico de las hojas de *Prunus serotina* indujo una relajación significativa del músculo liso arterial. El estudio fitoquímico de dicha fracción condujo a la purificación de tres compuestos conocidos, el hiperósido (**1**), la prunina (**2**) y el ácido ursólico (**3**). Los compuestos

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1 (EC_{50} = 91.3 ± 14.1 μg/ml), **2** (EC_{50} = 66.0 ± 19.4 μg/ml) y **3** (EC_{50} = 154.4 ± 7.5 μg/ml) indujeron una relajación, dependiente de la concentración, de la aorta aislada de rata. El compuesto **1** resultó ser sólo 10 veces menos potente que la acetilcolina (ACh) (EC_{50} = 8.7 ± 0.8 μg/ml), la cual se empleó como control positivo. Sin embargo, este flavonoide (E_{max} = 92.3 ± 7.7 %) fue más eficaz que la ACh (E_{max} = 69.5 ± 5.7 %) para inducir vasodilatación. De manera adicional, el aceite esencial de las hojas produjo relajación del músculo liso arterial. El análisis, mediante cromatografía de gases acoplada a espectrometría de masas, mostró que la esencia contiene cincuenta y siete constituyentes. La evaluación farmacológica de cuatro de estos componentes, el alcohol bencílico (**4**) (20.3 %), el benzaldehído (**5**) (12.1 %), el alcohol cinámico (**6**) (4.7 %) y el cinamaldehído (**7**) (1.1 %), indicó que estos compuestos inducen una relajación, dependiente de la concentración, de la aorta aislada de rata. El compuesto **6** produjo el efecto vasodilatador más potente (EC_{50} = 42.2 ± 5.7 μg/ml).

Palabras clave: Aceite esencial, hiperósido, ácido ursólico, aorta aislada de rata, *Prunus serotina*, vasodilatación.

INTRODUCTION

A recent documental and ethnomedical survey of medicinal plants grown in México and widely used by the Mexican population revealed that teas prepared from the leaves of *Prunus serotina* (Cav. ex Spreng) McVaugh (Rosaceae) are highly valued for treating hypertension, stomach upsets, mouth infections, diarrhea, paludism, bronchitis and cough; and the fruits are used, alone or prepared in syrups and liquors, for the treatment of cough and diarrhea (Martínez, 1991; Argueta, 1994). *P. serotina* is a 60 to 90 foot-tall native North American tree, which has an oval silhouette. Its low branches normally droop and touch the ground, and the finely-toothed, deciduous leaves are dark green and shiny. This species is widely distributed in Mexico, commonly called "capulín", "taunday", "t-munduya", "tzuúri", "chencua", "chengua", American black cherry, virginian prune and bird cherry (Martínez, 1991; Olszewska, 2005a). Besides their use in traditional medicine, the fruits of this plant are also part of Mexican diet, and are consumed fresh, dried or prepared in jam (Martínez, 1991). The wood is prized by wood workers and it has been used since colonial days for furniture. *P. serotina* has been the subject of few chemical studies. Kaempferol, quercetin and isorhamnetin glycosides, ursolic acid derivatives and prunasin have been detected in the leaves (Biessels *et al.*, 1974; Olszewska, 2005a; 2005b). Cyanogenic glycosides such as amygdalin and prunasin have been isolated from the seeds (Santamour *et al.*, 1998), and anthocyanidins have been detected in the fruit skin (Ordaz-Galindo *et al.*, 1999). Previous studies reported in the bibliography, concerning the pharmacological effects of this plant indicated that the methanolic extract prepared from the bark exhibited anti-proliferative activity in cancer cells of human colon (Yamaguchi *et al.*, 2006). As a part of a study directed towards the screening of plants used in Mexican traditional medicine for the treatment of cardiovascular diseases, our research group found that the lyophilized aqueous (EC_{50} = 190.4 ± 37.1 μg/ml) (Ibarra-Alvarado *et al.*, 2009) and the methanolic (EC_{50} = 211.0 ± 39.9 μg/ml) extracts prepared from the aerial parts of *P. serotina* induced a concentration-dependent relaxation of rat aorta. This finding, which supported the ethnomedical use of *P. serotina* as an anti-hypertensive

agent, led us to carry out a phytochemical study of the leaves of this species in order to isolate and identify the smooth muscle relaxing compounds. In addition, the essential oil from the leaves was analyzed, and four of the major chemical components were tested for their ability to modify the tone of arterial smooth muscle.

MATERIAL AND METHODS

Plant material

Leaves of *P. serotina* (1.7 Kg) were collected in the localities of Huimilpan and Pedro Escobedo, State of Querétaro (México) in August 2004. Specimens were identified and authenticated by Ph.D. Mahinda Martínez, Faculty of Natural Sciences, University of Querétaro. Voucher specimens are deposited in the Ethnobotanical Collection of the Herbarium of Querétaro "Dr. Jerzy Rzedowski" QMEX, located at the Faculty of Natural Sciences, University of Querétaro under the reference number 6375a.

Chemicals

Benzaldehyde, benzyl alcohol, cinnamyl alcohol, cinnamaldehyde, acetylcholine, and L-phenylephrine were obtained from Sigma (St. Louis, MO, USA). Methanol, isopropyl alcohol, ethyl acetate, dichloromethane, hexane, all salts and other reagents were obtained from J.T. Baker (Phillipsburg, NJ, USA) or Sigma.

Experimental animals

All pharmacological experiments were performed in accordance with The Mexican Official Standard NOM-062-ZOO-1999 for the production, care, and use of laboratory animals (Norma Oficial Mexicana, 2001). Male Wistar rats (275-325 g) were housed in a room maintained at 23 ± 1 °C with a 12 h light/dark cycle. Food and water were available *ad libitum*.

Isolated rat aortic rings assay

Rats were anesthetized with chloroform and sacrificed by decapitation. The descending thoracic aorta was removed and placed in ice-cold oxygenated Krebs-Henseleit solution of the following composition: 126.8 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 30 mM $NaHCO_3$, and 5 mM D-glucose (pH 7.4). Then, the aorta was immediately flushed with Krebs-Henseleit solution to prevent intravascular clot formation. The aorta was dissected free of adipose and connective tissue and cut at 4- to 5-mm intervals into rings. The aortic rings were mounted between stainless steel hooks and suspended in water-jacked, 7-ml organ baths containing oxygenated (95 % O_2 and 5 % CO_2) Krebs-Henseleit solution at 37°C. The tissues were allowed to equilibrate for 60 min under a resting tension of 1.5 g. During this period, the bathing medium was exchanged every 15 min. After final adjustment of the passive resting tension to 1.5 g, aortic segments were contracted with 100 mM KCl. Once a stable contractile tone was reached, the bathing medium was exchanged to restore a resting tension of 1.5 g. After that, the tissues were contracted with 1 µM L-phenylephrine; the developed force of contraction was recorded, and this contraction was defined as 100 %. Then, the fractions, isolated compounds, and the essential oil were added to the organ bath at final concentrations ranging from 1 µg/ml to 3000 µg/ml. The fractions, the flavonoids, the triterpenoid, and the essential oil constituents were initially prepared as stock solutions in dimethyl sulfoxide (DMSO). The essential oil was initially dissolved in Tween 80. All stock solutions were further diluted in deionized water. The highest concentrations of DMSO and Tween 80 used in the bioassay experiments were 0.2 % v/v. The isometric tension was measured by a Grass FT03 force-displacement transducer attached to a Grass 7D polygraph. The responses were expressed

as a percentage of the initial contraction achieved with phenylephrine.

Data analysis

Results of the experiments are expressed as the mean ± S.E.M. from n = 4 to 6 experiments. Concentration-response curves for the tested substances were plotted and fitted to the sigmoidal concentration-response equation using the data analysis and graphics program Prism 4.0 (GraphPad Software, San Diego, CA, USA). The values of the mean effective concentrations (EC_{50}) and maximum vasorelaxant effects (E_{max}) were obtained from the concentration-response curves.

General experimental procedures

Thin layer chromatography (TLC) for monitoring the fractions obtained by column chromatography (CC) was performed on Merck silica gel 60 F_{254} aluminum sheets. The TLC plates were sprayed with 1 % vanillin/sulphuric acid and heated (110°C). TLC spots were visualized by inspection of the plates under UV light (254 and 366 nm) on a Cole-Parmer 9818 darkroom series UV viewing system. All CC were performed with Kieselgel 60 Merck 70-230 mesh, 0.063-0.200 mm; Sephadex LH-20 (Fluka Chemie GmbH), and Diaion HP-20 (Supelco). Melting points of the isolates were determined using a Fisher-Johns melting point apparatus and are uncorrected. IR spectra were obtained with an ATI Mattson Genesis Series ST-IR spectrophotometer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded at room temperature on a Varian VNMRS instrument. APT, DEPT, COSY, HMQC, and HMBC NMR experiments were run using the manufacturer's software. Compounds were analyzed in $CDCI_3$, MeOH-d_4 or DMSO-d_6 with tetramethylsilane (TMS) as internal standard. EIMS were recorded on a Thermo-electron DFS mass spectrometer.

Extraction, fractionation, and isolation of pure compounds

The air dried leaves of *P. serotina* were pulverized through a 2-mm screen using a Wiley mill. The powder (0.942 Kg) was extracted with methanol (MeOH; 3 L). Evaporation of the solvent under reduced pressure afforded a solid residue (38.9 g), which was dissolved in MeOH and partitioned with dichloromethane and ethyl acetate. Water was added to the MeOH extract to afford a 50 % aqueous MeOH solution before partitioning with dichloromethane. The resulting fractions (dichloromethane, ethyl acetate, and aqueous MeOH) were evaluated in the isolated rat aorta model. The crude ethyl acetate soluble fraction (F2, 11.75 g) was found to exhibit a significant concentration-dependent relaxation of rat aorta. Hence, the ethyl acetate soluble fraction was subjected to silica gel column chromatography and eluted with a gradient of increasing polarity with ethyl acetate–MeOH to give 8 pooled fractions (F2-I – F2-VIII). Pooled fraction F2-II (9.02 g) was chromatographed over a silica gel column with ethyl acetate–MeOH of increasing polarity to yield 10 sub-fractions (F2-II-1 – F2-II-10). Compound **1** (300 mg) was obtained by reverse-phase silica gel column chromatography, eluted with MeOH–H_2O (7:3), from the pooled sub-fraction F2-II-4. Sub-fraction F2-II-5, eluted with ethyl acetate–MeOH (3:7), was subjected to Diaion HP-20 column chromatography, and eluted with a gradient mixture of H_2O–MeOH (1:0 to 0:1, 50 mL per fraction) to give five pooled fractions. Fraction F2-II-5-3, which was chromatographed over a Sephadex column, using MeOH as a solvent, afforded compound **2** (68 mg). On the other hand, fraction F2-I, eluted with ethyl acetate–MeOH (95:5) was chromatographed over a silica gel VLC column, using a gradient mixture of hexane–isopropyl alcohol (98:2 to 50:50), to afford compound **3** (42 mg).

Essential oil extraction

Air dried leaves (450 g) were subjected to hydrodistillation for 3 h. The obtained aqueous solution was partitioned with dichloromethane, and the dichloromethane soluble partition, which contained the essential oil, was dried with anhydrous sodium sulphate. After filtration, the solvent was evaporated under reduced pressure (yield: 0.27 g). The essential oil was stored at -4 °C until tested and chemically analyzed.

Gas chromatography-mass spectrometry

The essential oil was injected to an Agilent 6890N gas chromatograph with an automatic liquid sampler Agilent 7683B coupled to a LECO Pegasus 4D mass spectrometer. The column was an HP-5MS 10 m × 180 µm, film thickness 0.18 µm. Helium at 1 ml/min was used as the carrier gas. The column oven was temperature-programmed from 180 to 280 °C at 8 °C/min. The injector and detector temperatures were both 280 °C; electron energy 70 eV. Masses scanned 33–600 a. u. The constituents of the essential oil were identified by matching their 70 eV mass spectra with compound libraries.

RESULTS AND DISCUSSION

The vasorelaxant methanolic extract of the leaves of *P. serotina* was fractionated using the isolated rat aorta model to monitor vascular smooth muscle relaxing activity (Figure 1). Subsequent chromatography of the ethyl acetate soluble fraction (F2), which elicited a significant relaxation of rat aorta, resulted in the isolation of quercetin-3-O-β-D-galactopyranoside (hyperoside, **1**) (Calis *et al.*, 1999), naringenin-7-O-β-D-glucoside (prunin, **2**) (Erez and Lavee, 1969), and ursolic acid (**3**) (Sang *et al.,* 2002) (Figure 2). The structures of these known compounds were identified by spectroscopic data (^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC, HMBC; MS) measurement and by comparison with published values. Compounds **1** and **3** have been previously isolated from the leaves of *P. serotina* (Biessels *et al.*, 1974; Olszewska, 2005a). However, **2** is being reported from *P. serotina* for the first time.

Compounds **1**, **2**, and **3** elicited a concentration-dependent relaxation of aortic rings with functional endothelium. Figure

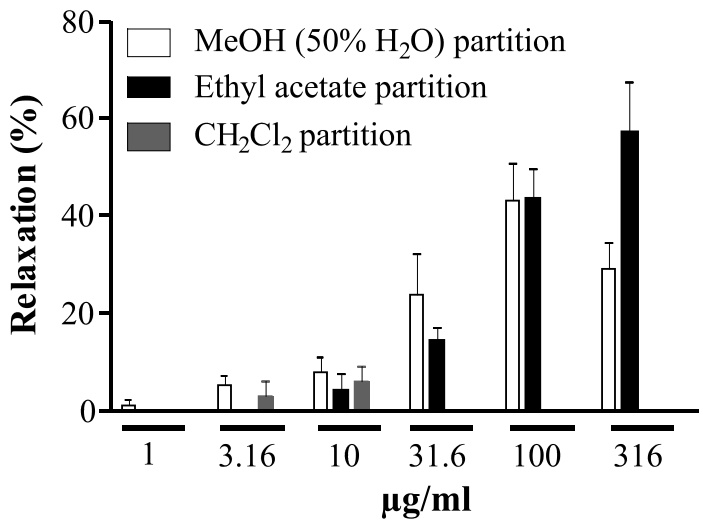

Figure 1. Vasorelaxant effect of the aqueous-MeOH, ethyl acetate, and dichloromethane fractions obtained from the MeOH extract of the leaves of *P. serotina*

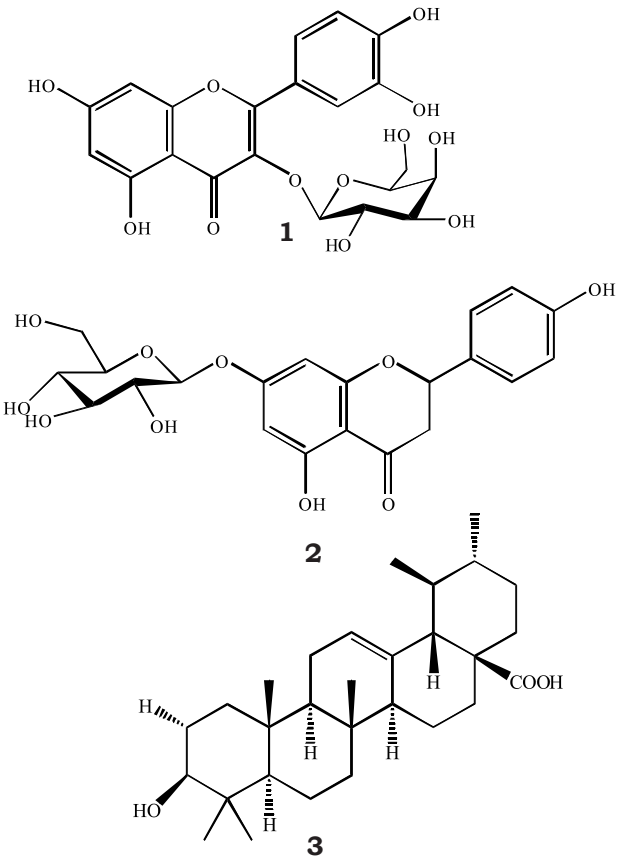

Figure 2. Structures of compounds 1-3

3 shows the concentration-response curves for the three compounds and acetylcholine (ACh), which was used as a positive control. Compounds **1** (EC_{50} = 91.3 ± 14.1 μg/ml; E_{max} = 92.3 ± 7.7 %) and **3** (EC_{50} = 154.4 ± 7.5 μg/ml; E_{max} = 94.1 ± 5.9 %) were approximately ten and eighteen fold less potent than ACh (EC_{50} = 8.7 ± 0.8 μg/ml; E_{max} = 69.5 ± 5.7 %), respectively. However, both compounds elicited a maximum vasodilator effect greater than that of the positive control. Compound **2** (EC_{50} = 66.0 ± 19.4 μg/ml; E_{max} = 44.2 ± 5.6 %) induced a maximum vasodilatory effect that was less than 45 %.

Although several research articles have been published concerning the pharmacological effects of flavonoids on the cardiovascular system, including antithrombotic, antioxidant, anti-ischaemic and vasorelaxing properties (Mullen *et al.*, 2002; Firuzi *et al.*, 2005; Halliwell *et al.*, 2005; Vita, 2005; Perez-Vizcaino *et al.,* 2006; *inter alia*), the vascular relaxation promoted by **1** and **2** have not been previously reported. A marked difference was observed in the maximum vasodilator effect elicited by **1** and **2**. This finding provides additional evidence for the important role of the degree of unsaturation on the relaxant effects of flavonoids, since it has been demonstrated that flavones more efficiently relax vascular smooth muscle than flavanones (Duarte *et al.*, 1993).

On the other hand, our results showed that compound **3** exhibited a significant vasodilation. This observation is consistent with that found by Aguirre-Crespo *et al.* (2006), who reported that this triterpenoid induced an endothelium-dependent vasodilation that seemed to be mediated

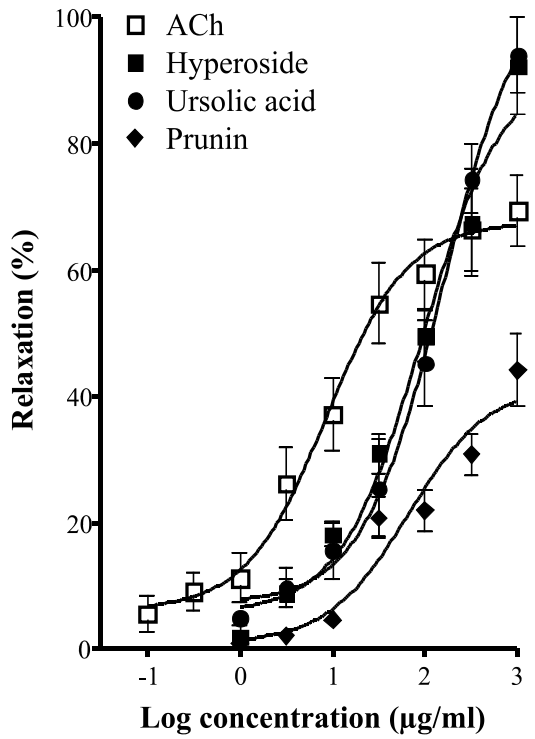

Figure 3. Concentration-response curves for the vascular relaxant effect of ACh, hyperoside (**1**), prunin (**2**), and ursolic acid (**3**)

by the release of nitric oxide from vascular endothelial cells.

In addition, the present study revealed that the essential oil prepared from the leaves of *P. serotina* caused a concentration-dependent relaxation of rat aorta (Figure 4). It is important to note that due to solubility problems, a clear maximal response was not obtained in the concentration-response curve. The essential oil was analyzed by gas chromatography-mass spectrometry and 57 compounds were detected. The major constituents were benzaldehyde (**4**, 12.1 %, t_R = 5.2 min), benzyl alcohol (**5**, 20.3 %, t_R = 6.0 min), α-methoxytoluene (21.2 %, t_R = 6.7 min), benzylethyl ether (5.8 %, t_R = 7.3 min), cinnamyl alcohol (**6**, 4.7 %, t_R = 7.6 min), cinnamaldehyde (**7**, 1.1 %, t_R = 8.0 min), and benzylbutyl ether (2.6 %, t_R = 8.1 min), which represent 67.8 % of the oil essence. The oil is classified as a mixed type because of the lack of a dominant compound (Chao *et al.*, 2005).

Compounds **4**, **5**, **6**, and **7** (Figure 5) were tested individually for their vasorelaxant effect. **4** (EC_{50} = 415.6 ± 56.1 µg/ml; E_{max} = 100.0 ± 0.1 %), **5** (EC_{50} = 470.9 ± 59.2 µg/ml; E_{max} = 88.1 ± 4.0 %), **6** (EC_{50} = 42.2 ± 5.7 µg/ml; E_{max} = 97.0 ± 1.5 %), and **7** (EC_{50} = 373.9 ± 38.7 µg/ml; E_{max} = 93.2 ± 5.4 %) induced a concentration-dependent relaxation of rat aorta. Figure 4 shows their respective concentration-response curves. All the isolates induced maximum vasodilator effects that were higher than 88%. The greatest vasorelaxant effect was observed for **6**, which was approximately ten fold more potent than the other tested oil com-

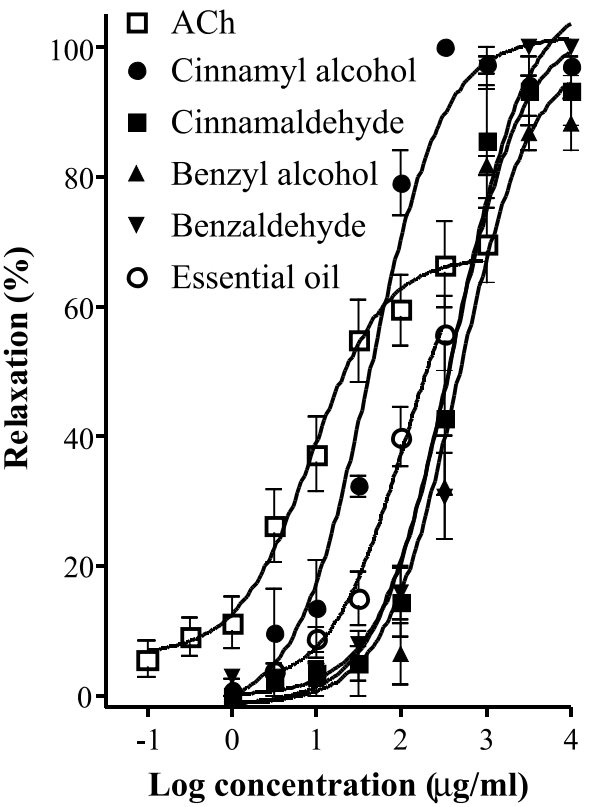

Figure 4. Concentration-response curves for the vascular relaxant effect of ACh, the essential oil obtained from the leaves of *P. serotina*, and four of its major components

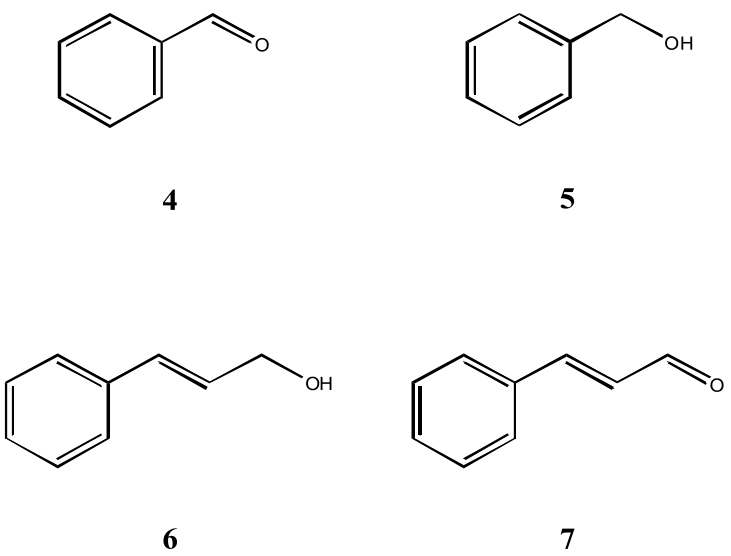

Figure 5. Structures of compounds **4**-**7**

pounds. In fact, **6** was more potent than **1** and **3**. The difference in the potency of the vasodilation elicited by **6** and **7** suggests that the presence of the hydroxyl group in the side chain plays a key role in the vasorelaxant activity. The present study evidenced that the essential oil of the leaves of *P. serotina* contain secondary metabolites, whose vascular relaxant properties have not been described before. The presence of these oil components may account, at least partly, for the health benefits attributed to the leaves of *P. serotina* for the treatment of hypertension.

CONCLUSIONS

The results derived from this study provide a scientific basis for the traditional use of the leaves *P. serotina* in the treatment of hypertension. Further investigations are currently in progress in order to characterize the mechanisms of action of the bioactive compounds and to identify the metabolites that are responsible for the vasorelaxant activity of the aqueous MeOH fraction obtained from the MeOH extract of the leaves.

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