Med Chem Res
DOI 10.1007/s00044-017-1934-6

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Beneficial effects of hydroalcoholic extract and flavonoids from *Zuccagnia punctata* in a rabbit model of vascular dysfunction induced by high cholesterol diet

Julieta Roco[1] · Gabriela Alarcón[1] · Liliana Sierra[2] · Iris Catiana Zampini[2,3] · María Ines Isla[2,3] · Susana Jerez[1,2]

Received: 20 December 2016 / Accepted: 22 May 2017

Abstract This study evaluated the effects of a *Zuccagnia punctata* standardized hydroalcoholic extract (ZpE) and three of its major flavonoids [2′,4′-dihydroxychalcone (DHC), 7-hydroxyflavanone (HF) and 3,7-dihydroxyflavone (DHF)] on the vascular reactivity of aortic rings with endothelial dysfunction induced by feeding rabbits on a high cholesterol diet. Rabbits were fed with either normal chow or a diet containing 1% cholesterol for 5–6 weeks. Isometric contractions were measured. Concentration response curves to ZpE (range from 4×10^{-2} to 4×10 µg gallic acid equivalent/ml), DHC, DHF or HF (range from 10^{-9} to 10^{-4} M) showed concentration-dependent relaxation of arteries pre-contracted with phenylephrine. ZpE (4×10^{-2}, 4×10^{-1} and 4 µg gallic acid equivalent/ml), HF (10^{-9}, 10^{-7}, 10^{-5} M), DHC (10^{-9} M) and DHF (10^{-9} M) added to the bath improved acetylcholine affinity. Pre-treatment of arteries with ZpE (4×10^{-2} µg gallic acid equivalent/ml) and DHC (10^{-9} M) reduced phenylephrine-induced contraction. Incubation with the higher dose of ZpE (4 µg gallic acid equivalent/ml) reduced the angiotensin II-maximal contraction (C_{max}) acting as a non-competitive antagonist, while DHC and DHF (10^{-5} M) caused a non-parallel rightward of the angiotensin II-concentration response curves and reduced the C_{max} acting as mixed antagonists. ZpE (4×10^{-2} µg gallic acid equivalent/ml), DHC and DHF (10^{-9} M) caused a rightward displacement of angiotensin II-concentration response curves acting as competitive antagonists. In conclusion, the present study demonstrated that a ZpE and its major flavonoids had beneficial effects in arteries with vascular dysfunction induced by hypercholesterolemia. Therefore its use as herbal medicine to prevent cardiovascular risks factors may be promising.

Keywords *Zuccagnia punctata* · Hypercholesterolemia · Rabbit aorta · Angiotensin II · Vascular dysfunction · Flavonoids

Introduction

In the last decade, growing evidence on the beneficial effects of diets rich in fruits and vegetables for cardiovascular health have frequently been attributed to flavonoids (Tapas et al. 2008). In several experimental studies, the plant extracts rich in polyphenolic compounds and flavonoids are shown to induce beneficial effects on vascular function thought its vasorelaxant, antioxidant, and hypotensive properties (Ghosh and Scheepens 2009). The flavones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species (Heim et al. 2002).They also have long been recognized to possess anti-inflammatory (Serafini et al. 2010), antidiabetic (Vessal et al. 2003), and anti-atherosclerotic (Grassi et al. 2010) activities. Several studies have shown that flavonoids reduce vascular tone and agonist-induced contraction in isolated rat arteries by nitric oxide-soluble guanylate cyclase-cyclic guanosine monophosphate

✉ Susana Jerez
sjerez@herrera.unt.edu.ar

[1] Instituto Superior de Investigaciones Biológicas (UNT-CONICET), Av Independencia 1800-San Miguel de Tucumán, Tucumán 4000, Argentina

[2] Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán (UNT), Miguel Lillo 205-San Miguel de Tucumán, Tucumán 4000, Argentina

[3] Instituto de Bioprospección y Fisiología Vegetal (CONICET-UNT), San Lorenzo 1469-San Miguel de Tucumán, Tucumán 4000, Argentina

Published online: 14 June 2017

relaxant pathway coupled to increase of NO release from the endothelium (Duarte et al. 1993; Chan et al. 2000; Ajay et al. 2003). Chronic treatment with antioxidant flavonoids such as quercetin reduces blood pressure as well as to improve endothelium dependent relaxation in several animal models of hypertension (Duarte et al. 2001; Ajay and Mustafa 2005).

Zuccagnia punctata Cav. belongs to the family of Fabaceae. It is a monotypic species broadly distributed in western Argentina from the Jujuy to Mendoza Provinces (Ulibarri 2005). *Z. punctata*, known under the common name of "jarilla macho, pus pus, lata", is reported to have antioxidant, antibacterial, antifungal, and anti-inflammatory properties (Morán Vieyra et al. 2009; Zampini et al. 2012; Butassi et al. 2015; Moreno et al. 2015a). The *Z. punctata* components include several phytochemicals such as flavonoids (flavanones, flavones, chalcones) and caffeoyl esters (Pederiva and Giordano 1984; Svetaz et al. 2004; Agüero et al. 2010). The ethanolic extract of aerial and flower from *Z. punctata* was standardized in previous reports and the major identified bioactive marker compounds were 2′,4′-dihydroxychalcone (DHC) and two flavonoids derived from cyclization of DHC, 7-hydroxyflavanone (HF) and 3,7-dihydroxyflavone (DHF) (Svetaz et al. 2004; Zampini et al. 2012; Moreno et al. 2015b).

One of the main causes of endothelial dysfunction is the lipid accumulation in blood vessel walls during hypercholesterolemia. This condition is characterized by an impairment of endothelium-dependent vasodilation and reduced NO availability. Several studies have demonstrated that hypercholesterolemia and atherosclerosis are associated with increased angiotensin II (Ang II) production through the enhancement of both angiotensin converting enzyme (ACE) and chymase activities as well as the up regulation of angiotensin type I receptor gene expression (Yang et al. 1998). Jerez et al. (2008) demonstrate that high cholesterol diet reduces acetylcholine (ACh)-relaxation and improves Ang II-contractile response. The mechanism involves cyclooxygenase-1 dependent endothelial dysfunction.

The present study intended to demonstrate, by taking into account its anti-inflammatory and antioxidant properties, that a *Z. punctata* standardized hydroalcoholic extract (ZpE) as well as three of its major flavonoids with analogous substitution pattern and differences in ring C (DHC, HF, and DHF) have beneficial effects on the vascular dysfunction induced by a high cholesterol diet.

Materials and methods

Plant material

Z. punctata-aerial parts (leaves and stems) were collected (January–February, 2012) from the locality of Amaicha del Valle at 2000 m.a.s. level (masl), Tucumán, Argentina. The samples were dried at room temperature in the dark. Voucher specimens (IML 605935) were kept at Miguel Lillo Foundation-Herbarium, Tucumán, Argentina. Dr Soledad Cuello authenticated the samples from *Z. punctata*.

Preparation of *Z. punctata* hydroalcoholic extract

Ground air-dried plant material was macerated in ethanol 80% (1 g/5 ml) with stirring (40 cycles/min) for 7 days at room temperature. The extract was then filtered through Whatman No. 4 paper. Total amount of phenolic compounds was determined by using Folin-Ciocalteu reagent. Results were expressed as mg gallic acid equivalent/ml extract (mg gallic acid equivalent (GAE)/ml). To obtain the dry extract (DE) the solvent was removed in a rotatory evaporator under reduced pressure. Before using, stock solution (40 mg GAE/ml) was prepared by dissolving the DE in dimethyl sulphoxide (Sigma Chemical, St Louis, USA). Working solutions were prepared each day from this stock solution by diluting accordingly in water (range from 4×10^{-2} to 4×10 µg GAE/ml).

High-performance liquid chromatography (HPLC)-diode array detector (DAD) analyses

The ZpE was analyzed by HPLC attached to a DAD. The HPLC system used for DAD analysis was a Waters equipment (Water Corporation, Milford, Massachusetts) consisting of a binary pump 1525, a ultraviolet (UV) diode array detector 2998, Water X-bridge C18 column (150×4.6 mm i.d.; 4,6 µm). The HPLC-DAD analyses were performed using a linear gradient solvent system consisting of 9% acetic acid in water (A) and methanol (B) as follow: 25–45% B over 10 min, followed by 45% B 10–20 min, 45–70% B 20–40 min, 70–75% B 40–50 min, 75–100% B 50–55 min. The detection was in UV at 280 nm. The flow rate was 0.8 ml/min and the volume injected was 20 µl. The compounds were monitored at 280, 320, and 350 nm and UV spectra from 200 to 600 nm were recorded for peak characterization. The Empower 2TM software was used. The UV spectra and co-injection with standards were used to identify and quantify three of the major flavonoids previously obtained from ZpE: DHC, HF, and DHF (Zampini et al. 2012). Calibration curves of pure compounds were prepared by using five dilutions of stock solutions from authentic samples commercially obtained.

The DHC, HF, and DHF were purchased from Indofine Chemical Company (New Jersey, USA).

Animals

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee (Bioethics Committee of the Medicine School from the National University of Tucumán, Argentina). Male hybrid rabbits were purchased from two renowned local breeders (Cabaña "Los Prieto", Villa Mariano Moreno and Cabaña "Paz", San Miguel de Tucumán). Animals (850–1000 g) were housed individually in gridded cages under controlled temperature and conditions at constant 12-h light/dark cycle. After about a week acclimation period, they were randomly divided into two groups. The group of control rabbits ($n = 12$) was fed with a standard chow diet (CD) which is an suitable maintenance diet for a normal adult rabbit. The group of hypercholesterolemic rabbits ($n = 12$) were fed with a diet prepared by addition of 1% cholesterol (Sigma chemical, St Louis, USA) to standard rabbit diet (HD). The feeding period was 5–6 weeks. Just male rabbits were used to avoid secondary variability associated to sex differences in this experimental model.

Biochemical parameters

By the end of the dietary intervention period, food was removed overnight and the next morning animals were anesthetized with Ketamin (75 mg/kg) and diazepam (0.5 mg/kg). Blood samples were obtained by direct cardiac puncture. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and glucemia were measured in serum by using commercial kits (Wiener, Rosario, Argentina).

Rabbit aortic ring preparation

The descending thoracic aorta was exposed through a midline incision and excised. It was dissected with care and all perivascular tissues were removed. Aortic rings (about five millimeters wide) were cut and mounted between force transducers (Grass Technologies, West Warwinck, USA) and a rigid support for measurement of isometric force (Jespersen et al. 2015) and incubated in organ baths (10 ml) containing warmed (37 °C), oxygenated (95% O_2/5% CO_2) Krebs solution (pH 7.2) with the following composition (in mM): NaCl 128, KCl 4.7, $NaHCO_3$ 14.4, NaH_2PO_4 1.2, Na_2-EDTA 0.1, $CaCl_2$ 2.5, glucose 11.1.

Passive tension was adjusted over a 90 min equilibration period to an initial tension of 2 g, which had previously been found to be the optimal tension for KCl-induced contraction (96 mM). During equilibration period all preparations were washed with Krebs solution every 15 min changes in isometric tension were continuously monitored by a data acquisition system (BIOPAC MP 100, Aero Camino Goleta, California, USA).

After washing and equilibration, pre-contraction of the aortic rings from both diet groups was induced by a submaximal dose of phenylephrine (5×10^{-6} M). Once the contractile response to agonist reached a plateau value, the ZpE (range from 10^{-2} to 4×10 µg GAE/ml) or one of the major flavonoids previously identified from it (Zampini et al. 2012) DHC, HF, or DHF (range from 10^{-9} to 10^{-4} M) were added to the organ bath in progressively increasing cumulative concentrations at the time intervals necessary to reach a concentration-response curve (CRC) (Fig. 1).

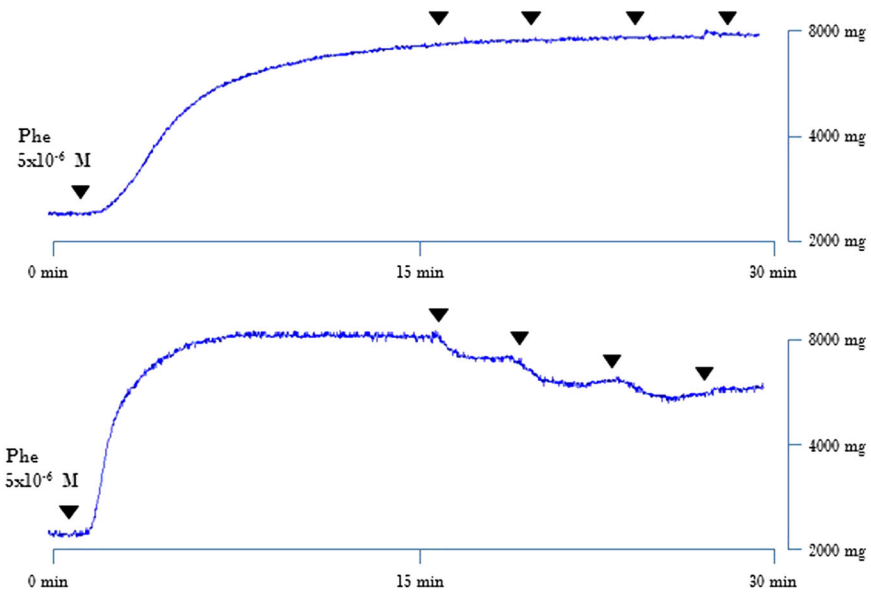

Fig. 1 Example traces of responses to cumulative doses (indicated by *arrows*) of vehicle (*upper panel*) and hydroalcoholic extract of *Zuccagnia puncata* (range from 4×10^{-2} to 4×10 µg GAE/ml), DHC, HF, or DHF (range from 10^{-9} to 10^{-5} M) (*lower panel*) added once the response to 5×10^{-6} M phenylephrine reached a stable contraction

The effect of the ZpE, DHC, HF, or DHF on the relaxation to the endothelium-dependent vasorelaxant ACh was checked. Aortic segments from rabbits fed a CD or a HD were incubated for 30 min with either the ZpE (4×10^{-2}, 4×10^{-1}, or 4 µg GAE/ml) or its flavonoid compounds (10^{-9}, 10^{-7}, or 10^{-5} M). Then, a sub-maximal dose of phenylephrine (5×10^{-6} M) was added to the bath and when contraction reached steady-state, cumulative concentrations of Ach (range from 10^{-8} to 10^{-5} M) were added to obtain a CRC.

The effect of the ZpE, DHC, HF, and DHF on the response to vasocontractile agonists was study in aortic rings from both diet groups by incubating for 30 min with either the vehicle or ZpE (4×10^{-2}, 4×10^{-1}, or 4 µg GAE/ml), DHC, HF, or DHF (10^{-9}, 10^{-7}, or 10^{-5} M). After this treatment, arteries were separated into two groups, one of them were stimulated with a single dose of phenylephrine 5×10^{-6} M and the other one with cumulative concentrations of Ang II (range from 10^{-10} to 10^{-6} M).

The contraction of aortic rings to phenylephrine or Ang II was expressed in mg, and the relaxations to ACh were expressed as percentage of the contraction to phenylephrine stimulation.

Statistical analysis

All concentrations found in the text or in the figures indicated the final tissue-bath concentrations of each drug. The responses were shown as mean ± standard error of the mean (SEM) for the number of experiments. The CRC was plotted for each experimental condition and the values of maximal contraction (C_{max}) or maximal relaxation (R_{max}) and the concentration of the agonist (expressed as negative log molar) that produce 50% of maximum contraction or relaxation (pEC_{50}) were deduced from the fit. Prism Version 3.0 (GraphPad Software, USA) was used. Student's *t*-test for paired or unpaired data was used to compare pEC_{50} values or maximal response. $P < 0.05$ was considered statistically significant (two-tail test). The significance of the differences between and within the groups was examined with an analysis of variance (ANOVA) for repeated measures followed by a Duncan's test.

Results

Standardization of *Z. punctata* hydroalcoholic extract

ZpE was standardized. The extract contained 55 mg of dry weight of soluble principles/g dry plant material and 40 mg GAE/ml. The phenolic compound profile was realized and the major bioactive compound, DHC and two flavonoids derived from them were identified by UV spectra and co-chromatography with the pure compounds obtained commercially, and quantified by HPLC-DAD. The quantity of DHC (105.74 ± 4.79 mg/g of dry weight of soluble principle, 5.81 mg/g of dry plant material) was ten-fold higher than HF (13.04 ± 0.66 mg/g of dry weight of soluble principle; 0.71 mg/g of dry plant material) and 12-fold higher than DHF (8.18 ± 0.26 mg/g of dry weight of soluble principle; 0.44 mg/g of dry plant material).

Biochemical parameters

Plasma levels of TC, HDL-C, LDL-C, and TG were higher in rabbits fed the HD than animals fed the CD. At the end of the experiment, no differences were found in the body weights between both diet groups (Table 1).

Vasodilator effects of *Z. puntacta* hydroalcholic extract, DHC, HF, and DHF

The cumulative addition of ZpE (range from 4×10^{-2} to 4×10 µg GAE/ml), DHC, HF or DHF (range from 10^{-9} to 10^{-4} M) to aortic rings pre-contracted with phenylephrine resulted in concentration-dependent relaxation. HF-maximal relaxation (R_{max}) was higher than ZpE-R_{max} on arteries from both diets groups (Table 2). Furthermore, HF-R_{max} was higher than DHC-R_{max} on CD group. Actually, the relaxation pattern was different in arteries from hypercholesterolemic with respect to normocholesterolemic rabbits: ZpE was more potent (pEC_{50}-HD: 4.4 ± 2.2 µg GAE/ml vs. pEC_{50}-CD: 1.8 ± 0.2 µg GAE/ml; $p < 0.05$) and DHC was more efficient (R_{max}-HD: 17.6 ± 2.1% vs. R_{max}-CD: 6.2 ± 1.2%; $p < 0.05$).

Effects of *Z. puntacta* hydroalcoholic extract, DHC, HF, and DHF on endothelium-dependent relaxation to ACh

Incubation with ZpE or its major flavonoids did not change the resting tone (data not shown). The pre-treatment of the tissues with ZpE, DHC, HF, or DHF at any doses did not modify ACh-responses in aorta from rabbits fed the CD (Table 3). R_{max} and potency to Ach were reduced in arteries

Table 1 Values of lipid levels and weight from rabbits fed a control diet (CD) and diet enriched with 1% cholesterol (HD)

	CD	HD
Total cholesterol (mg/dl)	66 ± 6.8	928 ± 99[a]
HDL-cholesterol (mg/dl)	57.8 ± 3.6	130 ± 28[a]
LDL-cholesterol (mg/dl)	36.6 ± 2.9	740 ± 120[a]
Triglyceride (mg/dl)	97.4 ± 14.9	215 ± 27[a]
Weight (g)	2088 ± 99	2188 ± 64

Data were expressed as mean ± SEM of 12 rabbits

[a] $P < 0.05$ indicated statistically significant differences between both diet groups

Table 2 Maximal relaxation (R_{max}) and pEC_{50} from standardized hydroalcoholic extract (ZpE) and flavonoids from *Zuccagnia punctata*

	CD		HD	
	R_{max} (%)	pEC_{50}	R_{max} (%)	pEC_{50}
ZpE (μg GAE/ml)	8.9 ± 2.3^{a}	1.8 ± 0.2	9.6 ± 1.5^{a}	4.4 ± 2.2^{b}
DHC (M)	6.2 ± 1.2^{c}	4.4 ± 0.6	17.6 ± 2.1^{b}	4.9 ± 0.2
HF (M)	20.6 ± 6.7	5.8 ± 0.7c	27.1 ± 8.1	5.3 ± 0.6
DHF (M)	16 ± 3.1	4.0 ± 0.6	17.4 ± 2.7	4.8 ± 0.2

CD control diet, *HD* high cholesterol diet. Values were expressed as means ± SEM

[a] $p < 0.05$ indicate statistically significant differences of ZpE with respect to HF-induced relaxation

[b] $p < 0.05$ indicate statistically significant differences between arteries from rabbits fed a CD and rabbits fed a HD

[c] $p < 0.05$ indicate statistically significant differences between DHC and HF or DHF-induced relaxation

Table 3 pEC_{50} to acetylcholine from arteries pre-incubated or not with standardized hydroalcoholic extract (ZpE) and major flavonoids from *Zuccagnia punctata*

	CD	HD
Control	7.04 ± 0.09	6.67 ± 0.11^{a}
ZpE 4×10^{-2} μg GAE/ml	7.21 ± 0.09	7.02 ± 0.09^{b}
ZpE 4×10^{-1} μg GAE/ml	7.22 ± 0.06	7.48 ± 0.13^{b}
ZpE 4 μg GAE/ml	7.13 ± 0.18	7.09 ± 0.04^{b}
DHC 10^{-9} M	7.07 ± 0.09	$7.31 \pm 0.13^{a,b}$
DHC 10^{-7} M	7.37 ± 0.10	$7.81 \pm 0.07^{a,b}$
DHC 10^{-5} M	6.91 ± 0.13	6.87 ± 0.05
HF 10^{-9} M	7.13 ± 0.08	7.28 ± 0.08^{b}
HF 10^{-7} M	7.27 ± 0.07	7.14 ± 0.12^{b}
HF 10^{-5} M	6.94 ± 0.16	7.06 ± 0.08^{b}
DHF 10^{-9} M	7.04 ± 0.05	$7.42 \pm 0.21^{a,b}$
DHF 10^{-7} M	7.31 ± 0.08	7.23 ± 0.19^{b}
DHF 10^{-5} M	7.05 ± 0.23	7.01 ± 0.10

CD control diet, (*HD*) high cholesterol diet. Values were expressed as means ± SEM

[a] $p < 0.05$ indicate statistically significant differences between arteries from rabbits fed the CD and rabbits fed the (HD)

[b] $p < 0.05$ indicate statistically significant differences between arteries incubated with ZpE or major flavonoids from *Z. punctata* and untreated arteries (control)

from rabbits fed the HD with respect those from rabbits fed the CD. The incubation of arteries from rabbits fed the HD resulted in different effects according to the compound checked: (a) DHC 10^{-5} M improved but did not normalize Ach-R_{max} (CD: $55 \pm 9\%$ vs. HD: $27 \pm 4\%$, vs. HD-DHC: $41 \pm 2\%$; $p < 0.05$, one-way ANOVA); (b) all doses of ZpE and HF and the lowest doses of DHC and DHF improved ACh-potency with respect to untreated arteries; (c) ZpE 4×10^{-1} μg GAE/ml, DHC 10^{-7} and 10^{-9} M and DHF 10^{-9} M improved Ach-potency even with respect to arteries from rabbits fed the CD (Table 3).

Effect of *Z. puntacta* hydroalcoholic extract, DHC, HF, and DHF on phenylephrine contraction

Pre-incubation of the arteries from rabbits fed the CD with ZpE (4×10^{-2} or 4×10^{-1} μg GAE/ml), DHC, HF, or DHF (10^{-9} or 10^{-7} M) significantly reduced phenylephrine contractile response (5×10^{-6} M). In contrast, only the lowest dose of ZpE (4×10^{-2} μg GAE/ml) and DHC (10^{-9} M) significantly decreased the phenylephrine contractile response in arteries from hypercholesterolemic rabbits (Fig. 2a–d).

Effect of *Z. puntacta* hydroalcoholic extract, DHC, HF, and DHF on Ang II contractile response

Cumulative addition of Ang II (range from 10^{-10} to 10^{-6} M) to rabbit aortic rings resulted in concentration-dependent contractile responses (Fig. 3). Incubation of arteries from rabbits fed the CD with ZpE, DHC, and DHF at any doses studied did not change Ang II-contractile response. With respect to HF, only at 10^{-5} M shifted to the right the Ang II-CRC (Fig. 4a–d).

The pre-treatment of rabbit aortic rings from hypercholesterolemic rabbits caused different effects according to the doses of the compound checked: (a) the highest dose of ZpE (4 μg GAE/ml), DHC and DHF (10^{-5} M) reduced the maximal contraction (C_{max}) to Ang II; (b) the lowest doses of ZpE (4×10^{-2} and 4×10^{-1} μg GAE/ml) and all doses of DHC and DHF caused a rightward displacement of the CRC to Ang II (Fig. 5a–d).

Discussion

This study characterized the effect of *Z. punctata* standardized hydroalcoholic extract and derived flavonoids from it on the vascular reactivity in normal and hypercholesterolemic rabbit isolated aorta. Results showed that the ZpE induced a weak but significant relaxation of pre-contracted aorta. ZpE had similar efficiency in both diet groups but was more potent in arteries from hypercholesterolemic rabbits. As was stated in introduction, regarding its chemical composition, DHC, and flavonoids (DHF and HF) derived from it have been previously isolated from the aerial parts of *Z. punctata* (Pederiva and Giordano 1984; Svetaz et al. 2004; Agüero et al. 2010; Moreno et al. 2015b). Considering the widely demonstrated vasorelaxant properties of flavonoids (Duarte et al. 1993; Chan et al. 2000) the three compounds isolated from the *Z. punctata* extract were studied to check its role on the ZpE-relaxant response. HF

was the most efficient to induce relaxation in both diet groups. DHF induced similar relaxation in both diet groups and DHC was more efficient in arteries from hypercholesterolemic rabbits than arteries from rabbits fed a CD. These results indicated that flavonoids present in the ZpE may be responsible for its vasorelaxant properties. Considering that vascular dysfunction was demonstrated in hypercholesterolemic rabbits (Jerez et al. 2008), chalcones may be more efficient in conditions of endothelial dysfunction caused by hypercholesterolemia (Figs. 4 and 5).

Flavonoids may also prevent endothelial dysfunction by enhancing the vasorelaxant process (Ghosh and Scheepens 2009). Thus, the effect of the ZpE and its major flavonoids on the endothelium-dependent relaxant response to Ach was studied. Pre-incubation of arteries from rabbits fed the CD or the HD with ZpE, DHC, HF, or DHF caused no effect on R_{max} to Ach. However, pre-incubation of arteries from hypercholesterolemic rabbits either with ZpE and HF or with low doses of DHC and DHF (10^{-9} M and 10^{-7} M) improved the Ach-affinity. Even DHC and DHF improved the Ach-affinity with respect to arteries from rabbits fed the CD. These results evidenced that flavonoids present in the ZpE from *Z. punctata* had beneficial effects on endothelial dysfunction induced by high cholesterol diet. According to this result, Ajay et al. (2007) found that a non-antioxidant flavone enhanced endothelium dependent vascular relaxation induced by Ach in Wistar Kyoto, spontaneously hypertensive and diabetic rat isolated aorta.

The effect of the ZpE and its flavonoids on the response to vasocontractile agonists was also checked. Low doses of the ZpE and DHC attenuated phenylephrine induced contraction in arteries from both diet groups. These results agreed with data from the bibliography reporting that flavonoids reduce contractile response to phenylephrine (Herrera et al. 1996; Dong et al. 2011).

Another significant finding of the present study was the fact that flavonoids present in the ZpE induced a marked rightward displacement of the Ang II CRC in the arteries from rabbits fed the HD. These results suggested that flavonoids from this plant may interact at the Ang II-receptor level. Furthermore, this effect was dose dependent. High doses of ZpE reduced the C_{max} to Ang II acting as a non-

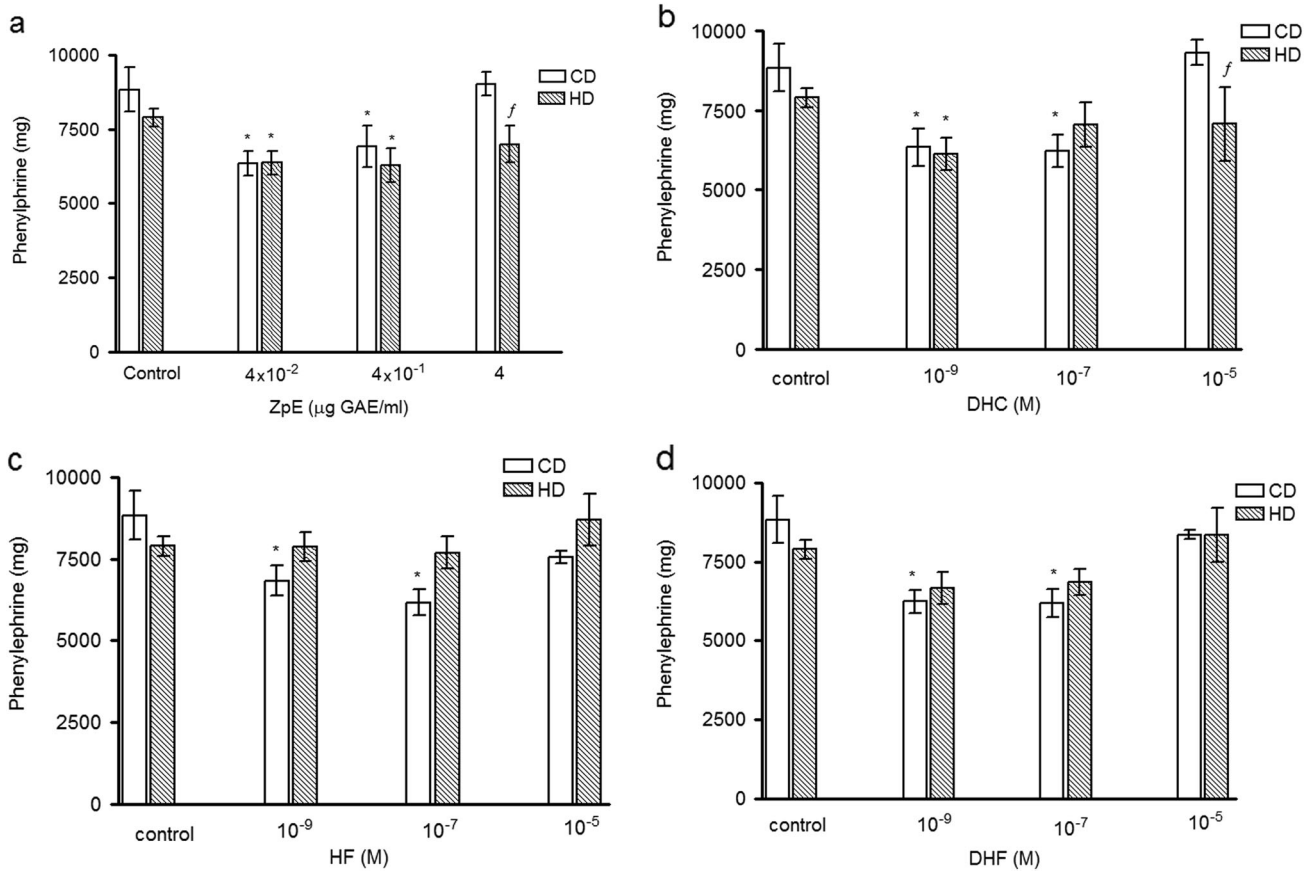

Fig. 2 Effect of: **a** ZpE (4×10^{-2}, 4×10^{-1}, or 4 µg GAE/ml), **b** DHC, **c** DHF, **d** HF (10^{-9}, 10^{-7}, or 10^{-5} M) on phenylephrine 5×10^{-6} M-induced contraction in aortic rings from rabbits fed the control diet (CD) and the high cholesterol diet (HD). * $p < 0.05$ statistically significant differences between untreated arteries (control) and arteries incubated with the ZpE, DHC, HF or DHF at different doses. ϝ $p < 0.05$ statistically significant differences between arteries from rabbits fed the CD and rabbits fed the HD

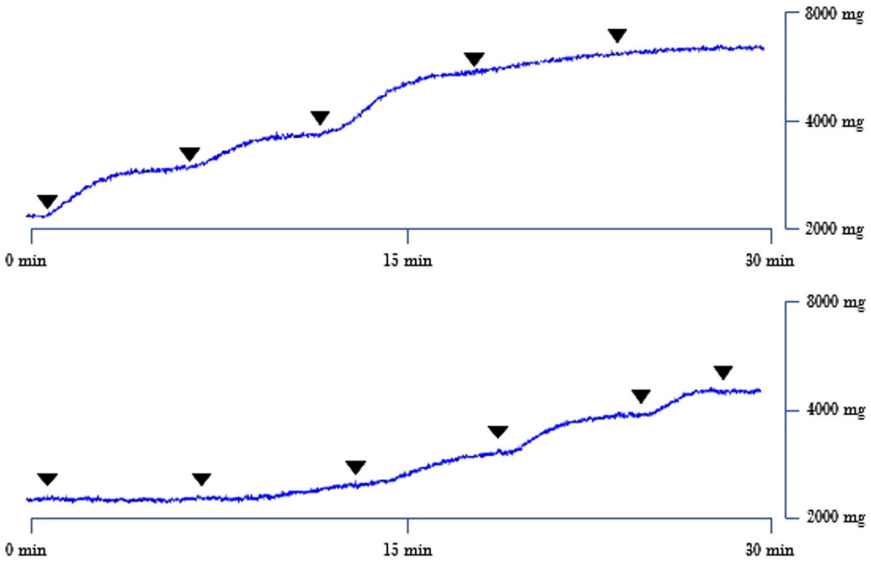

Fig. 3 Example traces of responses to cumulative doses (indicated by *arrows*) of angiotensin II in rabbit aortic rings incubated with either vehicle (*upper panel*) or with hydroalcoholic extract of *Zuccagnia puncata* (4, 4×10^{-1} or 4×10^{-2} μg GAE/ml) or DHC or HF or DHF (10^{-5}, 10^{-7}, or 10^{-5} M) (*lower panel*)

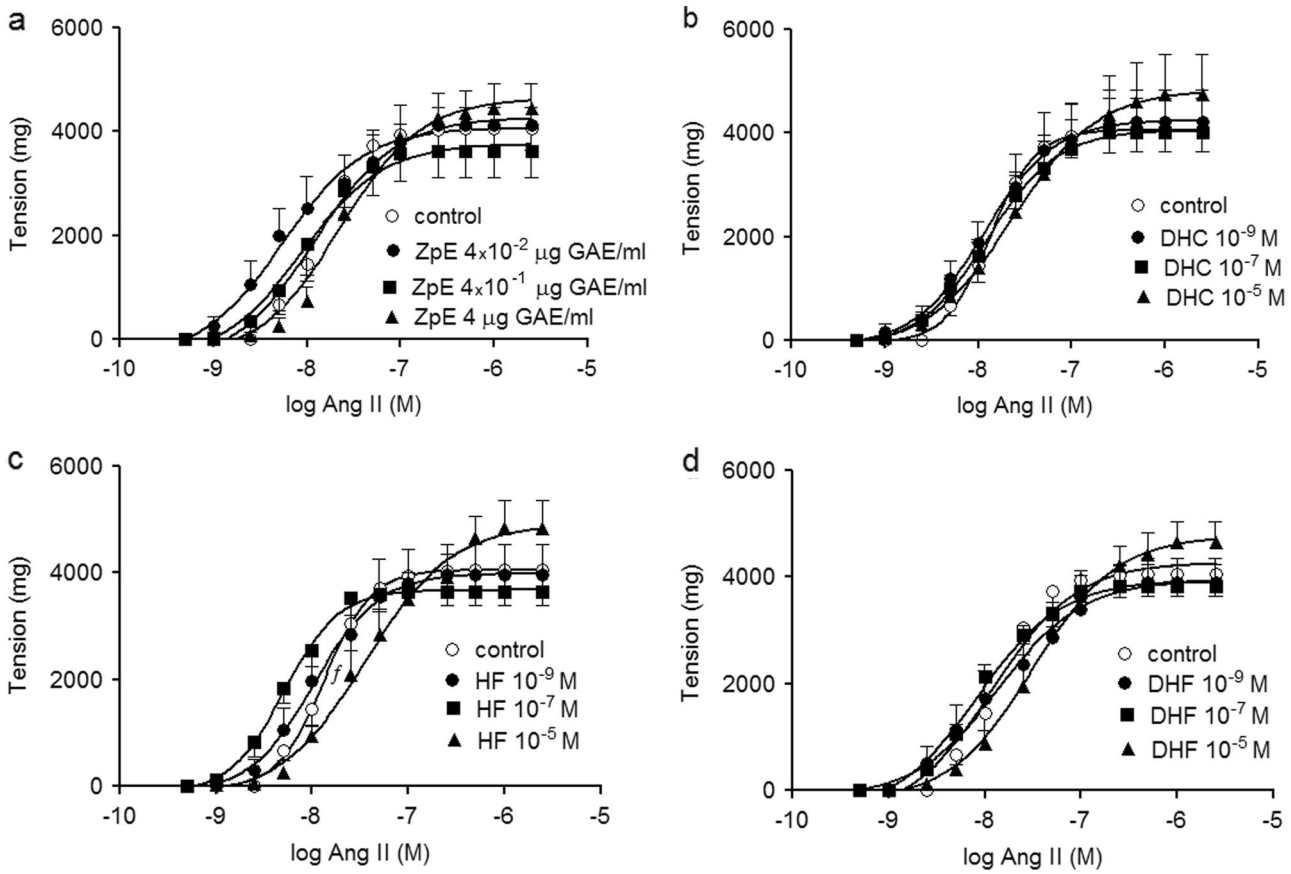

Fig. 4 Effect of: **a** ZpE (4×10^{-2}, 4×10^{-1}, or 4 μg GAE/ml), **b** DHC, **c** DHF, **d** HF (10^{-9}, 10^{-7}, or 10^{-5} M) on angiotensin II-induced contraction in aortic rings from rabbits fed the control diet (CD). F $p < 0.05$ statistically significant differences between pEC_{50} from untreated arteries (control) and pEC_{50} from arteries incubated with HF 10^{-5} M

competitive antagonist, while DHC and DHF reduced the C_{max} and caused a non-parallel shift to the right of the Ang II-CRC acting as a mixed antagonist. Low doses (10^{-9} and 10^{-7} M) of DE, DHC, and DHF caused a rightward displacement of Ang II-CRC acting as competitive antagonists. Hypercholesterolemia can modify the interaction between

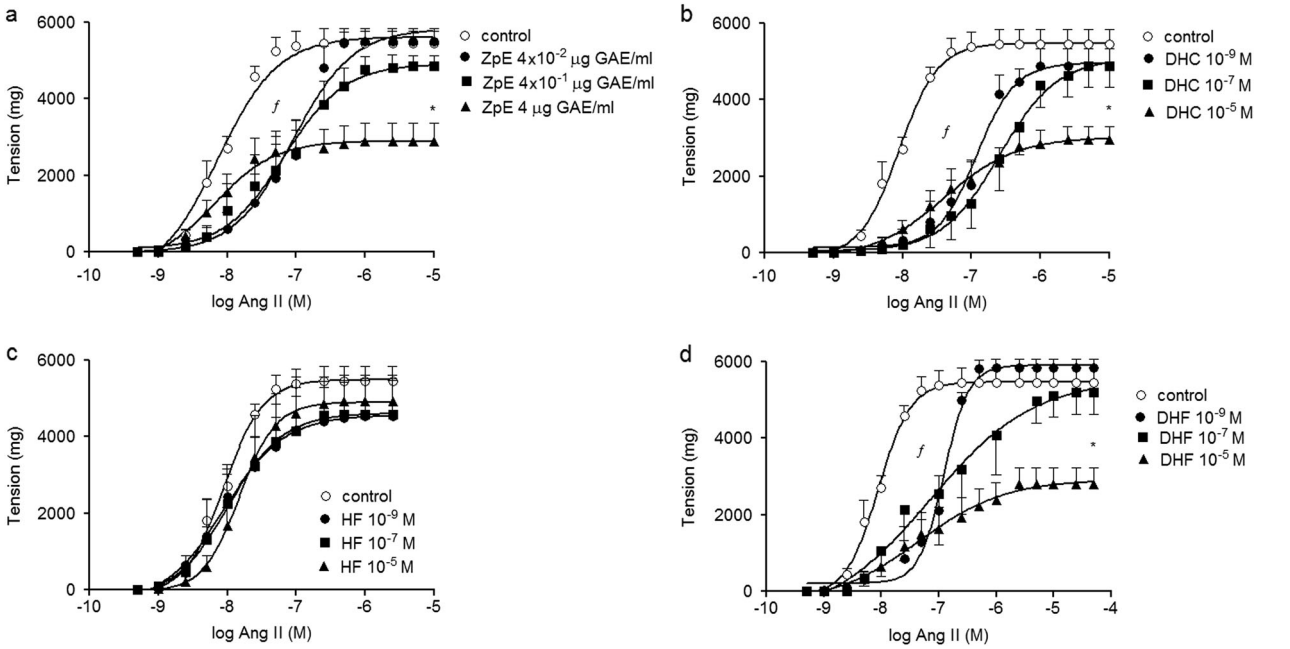

Fig. 5 Effect of: **a** ZpE(4×10^{-2}, 4×10^{-1}, or 4 µg GAE/ml), **b** DHC, **c** DHF, **d** HF (10^{-9}, 10^{-7}, or 10^{-5} M) on angiotensin II-induced contraction in aortic rings from rabbits fed the high cholesterol diet (HD). * $p < 0.05$ statistically significant differences between C_{max} from untreated arteries (control) and arteries incubated with the highest doses of the ZpE, DHC or DHF. ƒ $p < 0.05$ statistically significant differences between pEC_{50} from untreated arteries (control) and arteries incubated with DHC or DHF10^{-5} M and the lowest doses of ZpE (4×10^{-1} or 4×10^{-2} µg GAE/ml), DHC or DHF (10^{-9} or 10^{-7} M)

Ang II and adrenergic receptors (cross talk) (Jerez et al. 2010). This phenomenon may account for the dual inhibition of the Ang II- and phenylephrine-contractile response induced by ZpE and DHC at low doses in rabbits fed the HD.

On the other hand, given the structure activity relationship of flavonoids (Duarte et al. 1993; Herrera et al. 1996; Dong et al. 2011), no effect of HF on the Ang II-contractile response further suggests that the presence of two –OH substitution may be necessary for the Ang II-antagonism. Consistent with this view Huai et al. (2014) recently report vasorelaxant and antihypertensive effects of 7,8-DHF.

The study of flavonoid-effects on the renin-angiotensin system has been limited to characterize its properties as inhibitors of the ACE (Balasuriya and Vasantha Rupasinghe 2011). At present, no data from the literature refers specific effects of flavonoids as antagonists of Ang II. The reasons for the differential antagonistic effect of ZpE and flavonoids on the Ang II-contractile response between hypercholesterolemic and normocholesterolemic rabbits are unknown. Plasmatic membranes from smooth muscle cells (SMCs) are highly sensitive to cholesterol enrichment with LDL-cholesterol. Furthermore, increases in membrane cholesterol content improve cytosolic calcium levels in SMCs and are associated with increase of vascular reactivity. In addition, a decrease in membrane fluidity and alterations of lipid rafts composition may be involved in altered agonist receptor responsiveness that has been found during hypercholesterolemic conditions (Gleason et al. 1991). Thus, altered Ang II-receptor conformation during hypercholesterolemia may account for the ZpE, DHC, and DHF effect. More studies are necessary to prove this hypothesis.

Conclusion

The present study clearly demonstrates that a standardized hydroalcoholic extract from *Z. punctata* and its major flavonoids had vasorelaxant effect, sensitized Ach-response, reduced phenylephrine vasoconstriction and antagonized Ang II-contractile response in arteries from hypercholesterolemic rabbits. All these properties give a rationale for the use of the ZpE from *Z. punctata* or its major flavonoids as herbal treatment to prevent cardiovascular diseases accompanied by vascular dysfunction.

Acknowledgements This work was supported by grants from the Consejo de Investigaciones de la Universidad Nacional de Tucumán (PIUNT I521/1), Consejo de Investigaciones Científicas y Técnicas de la República Argentina (CONICET PIP 11-232), and Institutional funds from INSIBIO (Instituto Superior de Investigaciones Biológicas). We thank veterinary Rosa Alejandra Molina for bioterio management and Ms Erika Georgieff for her help in data collection.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Agüero MB, González M, Lima B, Svetaz L, Sánchez M, Zacchino S, Egly Feresin G, Schmeda-Hirschmann G, Palermo J, Wunderlin D, Tapia A (2010) Argentinean propolis from *Zuccagnia punctata* Cav. (Caesalpinieae) exudates: phytochemical characterization and antifungal activity. J Agric Food Chem 58:194–201

Ajay M, Achike FI, Mustafa MR (2007) Modulation of vascular reactivity in normal, hypertensive and diabetic rat aortae by a non-antioxidant flavonoid. Pharmacol Res 55:385–391

Ajay M, Gilani AH, Mustafa MR (2003) Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. Life Sci 74:603–612

Ajay M, Mustafa MR (2005) Chronic treatment with flavonoids prevents endothelial dysfunction in spontaneously hypertensive rat aorta. J Cardiovasc Pharmacol 46:36–40

Balasuriya NBW, Vasantha Rupasinghe HP (2011) Plant flavonoids as angiotensin converting enzyme inhibitors in regulation of hypertension. Funct Foods Health Dis 5:172–188

Butassi E, Svetaz LA, Ivancovich JJ, Feresin GE, Tapia A, Zacchino SA (2015) Synergistic mutual potentiation of antifungal activity of *Zuccagnia punctata* Cav. and *Larrea nitida* Cav. extracts in clinical isolates of *Candida albicans* and *Candida glabrata*. Phytomedicine 22:666–678

Chan ECH, Pannangpetch P, Woodman OL (2000) Relaxation of flavones and flavonols in rat isolated thoracic aorta: mechanism of action and structure–activity relationships. J Cardiovasc Pharmacol 5:326–333

Dong X, Wang Y, Liu T, Wu P, Gao J, Xu J, Yang B, Hu Y (2011) Flavonoids as vasorelaxant agents: synthesis, biological evaluation and quantitative structure activities relationship (QSAR) studies. Molecules 16:8257–8272

Duarte J, Pérez Palencia R, Vargas F, Ocete MA, Perez-Vizcaino F, Zarzuelo A, Tamargo J (2001) Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. Br J Pharmacol 133:117–124

Duarte J, Pérez Vizcaíno F, Utrilla P, Jiménez J, Tamargo J, Zarzuelo A (1993) Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure–activity relationships. Gen Pharmacol 24:857–862

Ghosh D, Scheepens A (2009) Vascular action of polyphenols. Mol Nutr Food Res 53:322–331

Gleason MM, Medow MS, Tulenko TN (1991) Excess membrane cholesterol alters calcium movements, cytosolic calcium levels, and membrane fluidity in arterial smooth muscle cells. Circ Res 69:216–227

Grassi D, Desideri G, Ferri C (2010) Flavonoids: antioxidants against atherosclerosis. Nutrients 2:889–902

Heim KE, Tagliaferro AR, Bobilya DJ (2002) Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. J Nutr Biochem 13:572–584

Herrera DH, Zarzuelo A, Jimenez J, Marhuenda E, Duarte J (1996) Effects of flavonoids on rat aortic smooth muscle contractility: structure-activity relationships. Gen Pharmacol 27:273–277

Huai R, Han X, Wang B, Li C, Niu Y, Li R, Qu Z (2014) Vasorelaxing and antihypertensive effects of 7,8-dihydroxyflavone. Am J Hypertens 27:750–760

Jerez S, Sierra L, Coviello A, Peral de Bruno M (2008) Endothelial dysfunction and improvement of the angiotensin II-reactivity in hypercholesterolemic rabbits: role of cyclooxygenase metabolites. Eur J Pharmacol 580:182–189

Jerez S, Sierra L, Scacchi F, Peral de Bruno M (2010) Hypercholesterolemia modifies angiotensin II desensitization and cross talk between alpha 1-adrenoceptor and angiotensin AT1 receptor in rabbit aorta. Eur J Pharmacol 635:149–155

Jespersen B., Tykocki NR, Watts SW, Cobbett PJ (2015). Measurement of smooth muscle function in the isolated tissue bath-applications to pharmacology research. J Vis Exp. (95), e52324, doi:10.3791/52324

Morán Vieyra F, Boggetti H, Zampini IC, Ordoñez RM, Isla MI, Alvarez RMS, De Rosso V, Mercadante AZ, Borsarelli CD (2009) Singlet oxygen quenching and radical scavenging capacities of structurally related flavonoids present in *Zuccagnia punctata* Cav. Free Radic Res 43:553–564

Moreno A, Nuño G, Cuello S, Sayago JE, Alberto MR, Zampini C, Isla MI (2015a) Anti-inflammatory, antioxidant and antimicrobial activity characterization and toxicity studies of flowers of "jarilla", a medicinal shrub from Argentina. Nat Prod Commun 6:991–994

Moreno MA, Mercado MI, Nuño G, Zampini IC, Cuello AS, Ponessa GI, Sayago JE, Isla MI (2015b) Histochemical localization and characterization of chalcones in foliar surface of *Zuccagnia punctata* Cav. Insight into their physiological role. Phytochem Lett 13:134–140

Pederiva R, Giordano OS (1984) 3,7-Dihydroxy-8-methoxyflavone from *Zuccagnia punctata*. Phytochemistry 23:1340–1341

Serafini M, Peluso I, Raguzzini A (2010) Flavonoids as anti-inflammatory agents. Proc Nutr Soc 69:273–278

Svetaz L, Tapia A, López SN, Furlán RLE, Petenatti E, Pioli R, Schmeda-Hirschmann G, Zacchino SA (2004) Antifungal chalcones and new caffeic acid esters from *Zuccagnia punctata* acting against soybean infecting fungi. J Agric Food Chem 52:3297–3300

Tapas AR, Sakarkar DM, Kakde RB (2008) Flavonoids as nutraceuticals: a review. Trop J Pharm Res 7:1089–1099

Ulibarri EA (2005) *Zuccagnia punctata* (Leguminosae) ¿Nuevo o viejo endemismo argentino? Darwiniana 43:212–215

Vessal M, Hemmati M, Vasei M (2003) Antidiabetic effects of quercetin in streptozocin-induced diabetic rats. Comp Biochem Physiol C 135C:357–364

Yang BC, Phillips MI, Mohuczy D, Meng H, Shen L, Mehta P, Mehta JL (1998) Increased angiotensin II type 1 receptor expression in hypercholesterolemic atherosclerosis in rabbits. Arterioscler Thromb Vasc Biol 18:1433–1439

Zampini IC, Villena J, Salva S, Herrera M, Isla MI, Alvarez S (2012) Potentiality of standardized extract and isolated flavonoids from *Zuccagnia punctata* for the treatment of respiratory infections by *Streptococcus pneumoniae*: in vitro and in vivo studies. J Ethnopharmacol 140:287–292