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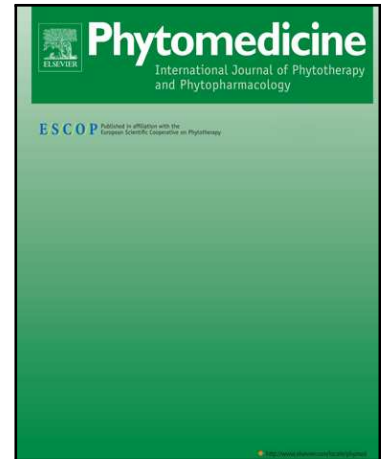
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Oral administration of *Zuccagnia punctata* extract improves lipid profile, reduces oxidative stress and prevents vascular dysfunction in hypercholesterolemic rabbits

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ABSTRACT

Background: The consumption of flavonoids has been shown to prevent cardiovascular diseases including atherosclerosis. In this sense, in a recent *in vitro* study we demonstrated that a rich in flavonoids extract from *Zuccagnia punctata* has beneficial effects on vascular function in aorta from hypercholesterolemic rabbits.

Purpose: The aim of this study was to evaluate the ability of a hydroalcoholic extract from *Z.puncata* (ZpE) to prevent alterations induced by high cholesterol diet in rabbits.

Methods: The major components of the ZpE, flavonoids, were analyzed by using a validated reversed phase HPLC method. Rabbits were separated in five groups: fed standard chow (CD); CD orally administrated 2.5 mg, 5 mg or 10 mg GAE/day ZpE (ZpE- CD); fed 1% cholesterol-enriched chow (HD); HD orally administrated 2.5 mg GAE/day ZpE (ZpE-HD); HD orally administrated 2.5 mg rosuvastatin/day (Ro-HD). All diets were administrated by 6 weeks. Body weights (BW), mean blood pressure (MAP), heart rate (HR), visceral abdominal fat (VAF), organ weight (heart, kidney, liver) and vascular morphology were determined. Total cholesterol (TC), triglycerides (TG), fasting glucose (FG), aspartate amino transferase (AST), alanine amino transferase (ALT), bilirubin, creatinine, thiobarbituric acids reactive substances (TBARS) and glutathione reduced/oxidized index were measured in serum. Abdominal aorta was excised and vascular function was assessed by acetylcholine and sodium nitroprusiate relaxation and contractile response to norepinephrine and angiotensin II.

Results: The major compounds of ZpE identified were chalcones: 2′,4′-dihydroxy-3′-methoxychalcone and 2′,4′-dihydroxychalcone. Oral treatment with ZpE reduced MAP, TC, TG, TBARS, aortic intima/media ratio and increased glutathione reduced/oxidized index in HD rabbits. No differences were found in AST, ALT, bilirubin or creatinine. Acetylcholine relaxation was normalized and contractile response to norepinephrine and angiotensin II was reduced in ZpE-HD.

Conclusion: Oral administration of ZpE as natural product in the prevention of cardiovascular disease related with hypercholesterolemia and endothelial dysfunction is very promising.

Introduction

Atherosclerosis, the principal contributor to the pathogenesis of myocardial and cerebral infarction, is known to be one of the main causes of morbidity and mortality worldwide (Singh et al. 2002). Elevated plasma concentration of cholesterol, especially low density lipoprotein (LDL-C), is recognized to have a crucial role in the development of atherosclerosis (Maiolino et al. 2013). High lipid levels, including triglycerides (TG), non-esterified fatty acids (NEFAs) and LDL-C, damage vascular tissues and produce endothelial dysfunction. This condition is known as lipotoxicity (Kin et al. 2012). Endothelial dysfunction caused by lipotoxicity is mediated through several mechanisms that include increased oxidative stress and pro-inflammatory responses. Impairment of endothelium-dependent vasodilation and reduction of nitric oxide (NO) availability are the main manifestations of such condition (Jerez et al. 2008).

The consumption of flavonoids can prevent a number of cardiovascular diseases including hypertension and atherosclerosis. Flavonoids from various sources have been reported to prevent LDL-C oxidation *in vitro* and show markedly hypolipidemic activity *in vivo*, suggesting the effectiveness of flavonoids for the prevention and treatment of atherosclerosis (Fuhrman and Aviram, 2001; Koshy et al. 2001). Several studies show protective effects of flavonoid-rich diets on vascular endothelial function (Hodgson and Craft, 2006). Flavonoids decrease vascular tone and agonist-induced contraction in isolated rat arteries through EDNO-sGC-cGMP relaxant pathway coupled to stimulation of endogenous NO production from the endothelium (Ajay et al. 2003). Chronic administration of antioxidant flavonoids such as quercetin reduces blood pressure as well as enhances endothelium dependent relaxation in various animal models of hypertension (Duarte et al. 2001; Ajay and Mustafa, 2005).

Zuccagnia punctata Cav., which belongs to the family of Fabaceae is a monotypic species widely distributed in western Argentina (Cabrera, 1971). *Z. punctata* is reported to have antioxidant (Moran Vieyra et al. 2009), anti-inflammatory effects attributed to cyclooxygenase-2 (COX-2) and LOX (lypooxygenase) inhibition (Moreno et al. 2015; Nuño et al. 2016), and antigenotoxic (Zampini et al. 2008) properties. In addition, phytochemical analysis of *Z. punctata* shows that it is a rich source of flavonoids (flavanones, flavones, chalcones) and caffeoyl esters (Svetaz et al. 2004; Moreno et al. 2015). The major constituents from the leaf resin of *Z. punctata* were of 2´, 4´dihydroxychalcone and 2´, 4´-dihydroxy-3´-methoxychalcone (Agüero et al. 2010; Moreno et al. 2015).

In previous works we characterized a model of hypercholesterolemia by feeding rabbits on a high cholesterol diet (HD) by 6 weeks. This model shows hyperlipidemia (Medina et al. 2014), increase of oxidative stress and inflammatory markers (Karbiner et al. 2013) and vascular dysfunction accompanied by reduction of acetylcholine relaxation and increase of angiotensin II reactivity (Jerez et al. 2008). Recently, we demonstrate *in vitro* beneficial effects of hydroalcoholic *Z. punctata* extract (ZpE) and its major flavonoids on vascular function in hypercholesterolemic rabbits (Roco et al. 2017). Thus, the aim of this work was to study the effects of oral treatment with a ZpE on our model of hypercholesterolemia. We hypothesized that flavonoid-rich ZpE would have beneficial effects on lipid profile, oxidative status and vascular function without any renal, hepatic or hematological toxicity.

Materials and methods

Plant material

Z. punctata aerial parts (leaves and stems) were collected (January to February, 2012) from the locality of Amaicha del Valle at 2000 meters above sea level (masl), Tucuman, 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 Zuccagnia punctata extract

20 g of ground air-dried plant material was macerated in 100 ml of ethanol: water (80:20) for 7 days with stirring (40 cycles/min) at room temperature.The extract was filtered through Whatman No. 4 filter paper. Total phenolic (TP) in the samples were determined according to Folin-Ciocalteu method. The non- flavonoid (NFP) and flavonoid (FP) phenolic compounds were determined according to Isla et al. (2014) and Popova et al. (2005) respectively. Then, the solvent was removed under reduced pressure in a rotary evaporator to obtain a dry ZpE (3.6 g) that was stored at -20 °C until further use.TP and NFP were expressed as mg gallic acid equivalent/g dry ZpE (mg GAE/g ZpE) and FP was expressed as mg quercetin equivalent/g dry ZpE.

HPLC-DAD analyses

The ZpE was analyzed by HPLC attached to a diode array detector (DAD). The HPLC system used for DAD analysis was a Waters equipment (Water Corporation, Milford, Massachusetts) consisting of a binary pump 1525, a 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 to 45% B over 10 min, followed by 45% B 10 - 20 min, 45% to 70% B 20 - 40 min, 70% to 75% B 40 - 50 min, 75% to 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 the flavonoid markers. The major compounds identified were: 2′,4′-dihydroxy-3′-methoxychalcone (DHMC) and 2′,4′-dihydroxychalcone (DHC). These compounds were also quantified. A calibration curve was prepared by using commercial standards to determine the relationship between the peak area and concentration. The compounds concentration was expressed as mg/g dry ZpE.

Animals

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 Tucuman, Argentina).All animal care and use programs were performed according to the Guide for the Care and Use of Laboratory Animals (NIH Publication 86 to 23, revised 1985). Male hybrid Flanders rabbits were acquired from two recognized local breeders (Cabaña "Los Prieto", Villa Mariano Moreno and Cabaña "Paz", San Miguel de Tucumán). Animals initially weighing 850-1,000 g were housed individually in gridded cages on a constant 12-hour light/dark cycle under controlled temperature and conditions. They were fed on rabbit chow 100 g/day.Water was given *ad libitum*. Only male rabbits were used to avoid secondary variability related to sex differences in this experimental model.The animals were weighed before experimental manipulationand every day throughout the period of the experiment.

Diets and treatment

Experiment I: toxicity and doses ranging study in rabbits fed a control diet

After acclimatization period, sixteen rabbits were divided into four groups of four rabbits. ZpE was re-dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) prior to use.Three groups were administered orally with ZpE suspended in DMSO/water (1:100).The doses of ZpE were 2.5 mg GAE/day (group 2), 5 mg GAE/day(group 3) or 10 mg GAE/day (group 4). Control rabbits (group

1) received the vehicle (DMSO/water, 1:100). The treatments were given once daily at 7 to 8 a.m. for 6 weeks.

The animals were observed continuously during the first hour, and then every hour for 6 h, then after12 and 24 h, and finally after every 24 h, up to 6 weeks for any physical signs of toxicity such as writhing, gasping, salivation, diarrhea, cyanosis, any nervous manifestations, or mortality.

Measurement of clinical, biochemical, hematological and morphological parameters

At the end of the 6weeks, food was withdrawn for 12 hours; rabbits were weighed and then anesthetized with ketamine (20 mg/Kg) and diazepam (0.5 mg/Kg). Mean arterial blood pressure (MAP) and heart rate (HR) were measured directly in the carotid artery through a catheter connected to a pressure transducer (Gould-Statham P23, California, USA) and recorded using a data acquisition system (Biopac MP100, Aero Camino Goleta, USA).

After MAP measurement, blood samples were collected through the catheter inserted in the carotid artery into non-heparinized and EDTA containing tubes for biochemical and hematological analyses. Serum was separated by allowing the blood clot at 37 °C and centrifuge at 3000 rpm for 10 min. Fasting glucose, total cholesterol (TC), triglycerides (TG), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin (total, direct and indirect) were measure by using colorimetric reactions with commercial kits (Wiener, Rosario, Argentina).The hematological study included white blood count and hematocrit estimation.

Immediately after the blood collection, the animals were sacrificed for tissue studies. The organs such as liver, heart and kidneys, were removed, blotted free of blood and weighed immediately.

Experiment II: effect of oral administration of ZpE in rabbits fed a high cholesterol diet

Results from experiment I (Table 1) showed that ZpE 2.5 mg GAE/day was the only dose that statistically significantly reduced TC (even in normocholesterolemic rabbits), and did no modify hepatic, renal or hematological parameters. For these reasons, 2.5 mg GAE/day was the selected dose to study the effect of ZpE in hypercholesterolemic rabbits. After one week acclimation period, thirty rabbits were randomly divided into five groups of six rabbits each. Animals in group 1 and 2 received a standard chow diet (CD) which is an appropriate maintenance diet for a normal adult rabbit (100 g/day). Animals in group 3, 4 and 5 were fed with a diet (100 g/day) prepared by adding 1% cholesterol (Sigma, St Louis, USA) to standard rabbit diet (HD). Groups 2 (ZpE-CD) and 4 (ZpE-HD) were administrated ZpE (2.5 mg GAE/day); Group 5 (positive control) received 2.5 mg/day rosuvastatin (Ro-HD). Both ZpE and rosuvastatin were administrated orally once daily at 7 to 8 a.m. Animals were fed with appropriate diet and received the treatments for 6 weeks.

Measurements of clinical, biochemical, hematological and morphological parameters were performed as was described in previous paragraph (Experiment I).

Effects of oral treatment with 2.5 mg GAE/day of ZpE on oxidative stress parameters

Lipid peroxidation was evaluated by measuring the thiobarbituric acid reactive substances (TBARS) and GSH/GSSG was calculated from total glutathione (TGL) and reduced glutathione (GSH) levels as was previously described (Karbiner et al. 2013). Briefly, 700 μl of trichloroacetic acid 10% was added to 500 μl of serum from rabbits fed a HD or ZpE-HD. The mixture was centrifuged (15 min; 10,000 rpm; 4 °C), and the supernatant was used to measurement of TBARS, TGL and GSH. The TBARS concentration was calculated using malondialdehyde as a standard. The results are expressed as μM.

To determinate GSH the supernatant was incubated with 0.3 mM NADPH and 6 mM of 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) by 2 min at room temperature. Next, glutathione

reductase 0.077 U was added and the mixture was incubated 10 min. To calculate TGL, the supernatant was incubated 10 min with DTNB 6mM and buffer phosphate pH 7.4. The absorption was measure at 412 nm. GSH and TGL were calculated by using GSH standard curve.

The oxidized glutathione (GSSG) was calculated as GSSG = TGL – GSH.

Results were expressed as µg/mg proteins determined by the Lowry´s method.

Effects of oral treatment with 2.5 mg GAE/day of ZpE on vascular morphology

Histological analysis of segments of the thoracic aortas (adjacent to the aortic arch) was performed. Aortic segments were rinsed with normal saline solution and preserved in 10% formaldehyde buffered solution (pH 7.4) for the next step. From each sample, serial sections were made (3-5 sections/aorta). The 7 µm sections were stained by haematoxylin-eosin method. Media and intima thickness were measured by image analysis with the software Media Cybernetics® Image-Pro Plus TM. The ratio between the tunica intima and the tunica media was calculated. A code number was assigned to each section observed.

Effects of oral treatment with 2.5 mg GAE/day of ZpE on vascular function

The descending thoracic aorta from the four experimental groups were exposed through a midline incision and excised. It was carefully dissected and all adherent fat and connective tissue were removed. Five-millimeter wide rings were cut and mounted in a 10 ml organ bath containing Krebs solution of the following composition (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, pH 7.2. Krebs solution was kept at 37° C and aerated with 95% O_2 and 5% CO_2.

Isometric contractions were measured by using force-displacement transducers (Grass Technologies, West Warwick, USA) and were recorded under an initial tension of 2 g, which had been found to be the optimal tension for KCl-induced contraction (96 mM). All preparations were allowed to equilibrate for 120 min and were washed with Krebs solution at 15 min intervals.

In order to check endothelial function after equilibration, aortic rings were pre-contracted with phenylephrine (5×10^{-6} M) and after they reached a stable and sustainable contraction, acetylcholine (10^{-8} to 10^{-5} M) was added cumulatively to the organ bath to construct a concentration-response curve (CRC). In other group, only one dose (5×10^{-6} M) of the endothelium independent vasodilator sodium nitroprusside was used in a similar protocol. The vasodilator effects were expressed as percent decrease of the peak phenylephrine contraction.

To evaluate vascular reactivity, contractile response to agonists was checked. Arteries were stimulated either with increasing doses of norepinephrine (10^{-8}–10^{-4} M) or angiotensin II (10^{-10}–10^{-6} M) to construct CRCs.

The contractile responses of aortic rings were expressed as mg of isometric contraction.

Statistical analysis

Kolmogorov–Smirnov goodness-of-fit test was used to test for normal distribution. Results were expressed as mean ± standard error of the mean (SEM) for the number of rabbits. The CRC for each experimental condition was plotted and from it were deduced the values of maximal response (R_{max}) and the concentration of the agonist (expressed as negative log molar) producing 50% of maximum contraction or relaxation (pEC_{50}) recorded (Prism Version 3.0, GraphPad Software, USA).The differences in the mean values between the four diet groupswere tested by two way ANOVA followed by a Duncan'stest.The differences in the mean values between ZpE-treated and untreated rabbits were tested by the unpaired Student's t-test.$p < 0.05$ was considered as statistically significant.

Results

Analysis of flavonoids markers in ZpE

ZpE contained 1215 ± 45 mg GAE/g dry extract of TP compounds. About 53% of TP were FP (644 ± 12 mg QE/g dry ZpE) and 47% were NFP (541 ± 15mg GAE/g dry ZpE). The phenolic compound profile was performed and the major bioactive compound MDHC and DHC derived from them were identified by UV spectra and co-chromatography with the pure compounds obtained commercially (Indofine Chemical Company, New Jersey, USA), and quantified by HPLC-DAD (Fig 1). The quantity of flavonoids was: MDHC: 152.6 ± 5.6 mg/g dry ZpE, DHC: 105.74 ± 4.79 mg/g dry ZpE.

MDHC and DHC were chosen as analytical markers of ZpE extracts based on previous reports about antioxidant and anti-inflammatory properties of these compounds (Moreno et al. 2015; Nuño et al. 2016) and recent evidences about the therapeutic potential of chalcones as cardiovascular agents (Mahapatra and Bharti, 2016). In addition, in a recent work we demonstrate beneficial vascular effect of ZpE and its major flavonoids in aorta from rabbits fed a CD and a HD (Roco et al. 2017).

Measurement of clinical, biochemical and hematological parameters

Experiment I: *toxicity and doses range studies*

No acute toxicity was found by oral administration of ZpE in the form of sedation, itching and mortality in experimental rabbits.The body weight (BW) increased in all groups throughout the treatment without significant differences among them (data not shown). At the end of the experiment BW was similar in all groups (Table 1).

Oral administration of ZpE (2.5, 5 or 10 mg GAE/day) did not modify PAM, HR, fasting glucose, creatinine or hematological parameters. TC level was statistically significantly reduced (about 47%) by the dose of 2.5 mg GAE/day (Table 1). Treatment with ZpE (5 or 10 mg GAE/day) reduced AST and direct bilirubin levels as compared with untreated rabbits (Table 1).

No differences were found in the liver/ BW ratio or the heart /BW ratio between ZpE-treated and untreated groups. However, the kidney/BW ratio was increased in rabbits receiving 10 mg GAE/day of ZpE (Table 1).

Experiment II: *effect of oral administration of 2.5 mg GAE/day of ZpE in rabbits fed a high cholesterol diet*

Addition of cholesterol to the diet significantly increased TC, TG, bilirubin (total, direct and indirect) and AST levels as well as the liver/BW ratio, reduced hematocrit and increased white cells count (Table 2). The oral administration of ZpE 2.5 mg GAE/day reduced TC levels, normalized TG, bilirubin (total, direct and indirect) and AST levels, hematocrit, white cells count and did not modify the liver/BW ratio. In addition, administration of ZpE significantly reduced MAP in rabbits fed on HD. Treatment with equivalent doses (2.5 mg/day) of rosuvastatin did not reduce TC levels, normalized TG, direct bilirubin, AST levels, hematocrit, white cells and also reduced MAP. Analysis of HDL-C and LDL-C showed that neither ZpE nor rosuvastatin at doses used in the present study modify lipoprotein plasma profiles.

Oxidative stress parameters

Serum lipid peroxidation was significantly reduced by oral administration of ZpE (2.5 mg GAE/day) in rabbits fed on HD (Fig 2a).The GSH/GSSH ratio was higher in rabbits fed on HD

treated with ZpE compared with rabbits treated with vehicle (Fig 2b). Overall, these results imply that ZpE enhanced the serum antioxidant capacities under hypercholesterolemic conditions.

Effects of oral treatment with 2.5 mg GAE/day of ZpE on vascular morphology

Representative photographs of the morphological changes in the aorta from the five groups stained with hematoxylin-eosin method are shown in Supplement 1. Histological examination showed moderate thickening of the tunica intima in arteries from rabbits fed on HD. The administration of ZpE or rosuvastatin to rabbits fed on HD normalized the intima/media ratio (CD: 0.05 ± 0.002 vs HD: 0.71 ± 0.04 vs ZpE-HD: 0.039 ± 0.003 vs Ro-HD: 0.04 ± 0.002; $P < 0.01$, one way ANOVA and Duncan´s post test).

Effects of oral treatment with 2.5 mg GAE/day of ZpE on vascular reactivity

Acetylcholine (10^{-8} to 10^{-5}M) caused an endothelium-dependent relaxation in a concentration-responsive manner in all of the studied groups. Vessels derived from rabbits fed on HD demonstrated significantly impaired responses to acetylcholine (Fig.3).Administration of ZpE did not change acetylcholine relaxation in CD group and normalized the maximal relaxing effect of acetylcholine in the hypercholesterolemic group (R_{max}CD: $66.2 \pm 5.2\%$ vs HD: $27.3 \pm 3.8\%$ vs ZpE-CD: $60.5 \pm 6.2\%$ vs ZpE-HD: $65.9 \pm 8\%$ vs Ro-HD: $42.5 \pm 4.2\%$; $p < 0.05$, two way ANOVA and Duncan´s post test). In addition, ZpE-treatment improved acetylcholine affinity in both diet groups (pEC_{50} CD: 7.01 ± 0.07 vs HD: 6.67 ± 0.11 vs ZpE-CD: 7.18 ± 0.05 vs ZpE-HD: 7.23 ± 0.08 vs Ro-HD: 7.06 ± 0.11; $p < 0.05$, two way ANOVA and Duncan´s post test). Treatment with rosuvastatin also improved R_{max} and pEC_{50} to acetylcholine in hypercholesterolemic rabbits, but differences were not statistically significant.

The endothelium-independent vasorelaxant response to sodium nitroprusside 5 x 10^{-6} M, a NO donor, was similar in all groups (CD: $94 \pm 5\%$ vs HD: $91.3 \pm 3\%$ vs ZpE-CD: $95 \pm 2\%$ vs ZpE-HD: $91 \pm 2\%$ vs Ro-HD: $97.5 \pm 0.8\%$).

R_{max} and pEC_{50} to norepinephrine was similar in rabbits fed on CD and rabbits fed on HD. The oral administration of ZpE reduced the affinity to norepinephrine in both diet groups and the R_{max} only in rabbits fed on CD. Rosuvastatin increased affinity to norepinephrine (Fig.4).

According to previous work (Jerez et al. 2008), results showed that HD increased R_{max} and affinity to angiotensin II with respect to CD. The effect of ZpE-administration on angiotensin II contractile response was diet- dependent: reduced affinity in rabbits fed on CD and R_{max} in rabbits fed on HD. Treatment with rosuvastatin also reduced significantly contractile response to angiotensin II in hypercholesterolemic rabbits (Fig.5).

Discussion

The antioxidant and anti-inflammatory activities of the ZpE and its major flavonoids have been demonstrated (Moran Vieyra et al. 2009). Recently, *in vitro* studies show that ZpE and its major flavonoids have vasorelaxant effect, sensitize acetylcholine-response, reduce phenylephrine vasoconstriction and antagonize angiotensin II-contractile response in arteries from hypercholesterolemic rabbits (Roco et al. 2017). However, at present there are no *in vivo* studies evaluating the effect of ZpE on hypercholesterolemia. Present study characterized the effect of the oral administration of a ZpE, rich in flavonoids, on a rabbit model of hypercholesterolemia induced by a HD. ZpE effectively lowered TC and TG levels by about 65% and 50% respectively, reduced oxidative stress parameters and PAM, normalized hematological parameters (hematocrit and white cells count) and improved vascular function. Even more, ZpE improved markers of liver function (AST, bilirubin) in rabbits fed a HD. Effects of ZpE and rosuvastatin were similar in almost all

clinical and biochemical parameters except for TC levels. Li et al. (2016) show that rosuvastatin (1.5 mg/kg/day) reduces by about 30% TC and LDL-C in rabbits fed a HD. These authors do not find differences in TG levels between CD and HD animals. Thus, this difference in the lipid profile may account for our unexpected results.

Oral administration of flavonoid-rich ZpE at dose of 2.5 mg GAE/ml was not toxic as there was no sedation, itching and mortality seen; no differences existed on weight gain, organ size (heart and kidney), liver and kidney function or hematological parameters in experimental rabbits. These results were in agreement with previous studies (Zampini et al. 2008).

Flavonoids from various sources have been reported to show markedly hypolipidemic activity *in vivo*, suggesting the effectiveness of flavonoids for the prevention and treatment of atherosclerosis (Salvamani et al. 2014). In agreement with data from the bibliography (Agüero et al. 2010; Moreno et al. 2015; Nuño et al. 2016), we found that the most dominants components of the aerial parts of *Z. punctata* were chalcones (DHC and MDHC). Various chemically diverse chalcone scaffolds have been reported to inhibit various cardiovascular targets such as angiotensin converting enzyme (ACE), calcium/potassium channels, TG synthesis, diacylglycerol acyltransferase (DGAT), cholesteryl ester transfer protein (CETP), pancreatic lipase (PL), acyl-coenzyme A: cholesterol acyltransferase (ACAT), and lipoprotein lipase (LPL) (Mahapatra and Bharti, 2016). Thus, the hypolipidemic activity of the ZpE may be attributed to its chalcones content. The mechanism of such effect will be the goal of further studies.

The oral administration of 2.5 mg GAE/day ZpE reduced oxidative stress, restored acetylcholine relaxation and reduced aortic intima/media ratio reverting endothelial dysfunction in rabbits fed a HD. An imbalance between the production of ROS and the antioxidant defense mechanisms leading to oxidative stress has been suggested to contribute to the etiology and progression of the atherosclerosis (Singh et al. 2015). In hypercholesterolemic rabbits increased production of ROS has been implicated in the impairment of endothelial function (Ohara et al. 1993; Mugge et al. 1991). Under oxidative stress, excess of superoxide reacts with NO to form peroxynitrites (ONOO), a reactive molecule that impairs NO-dependent vasodilation. Previous studies have reported beneficial effects of flavonoids on the endothelial function (Ajay and Mustafa 2005; Hodgson and Croft 2006; Roco et al. 2017) as well as improved endothelial function following antioxidant interventions (Mugge et al. 1991; Papageorgiou et al. 2013). The enhancement of acetylcholine relaxation in aorta from HD rabbits treated with ZpE was consistent with these data. In this sense, the major flavonoids presents in ZpE, DHC and MDHC, are potent antioxidants and scavengers of ROS (Moran Vieyra et al. 2009; Moreno et al. 2015). Indeed, such antioxidant actions could contribute to restoring endothelial function in hypercholesterolemic rabbits. No changes in the response to the endothelium-independent vasorelaxant sodium nitroprusside in aorta from both diet groups either treated or not with the ZpE supported this view. In addition, increased sensitivity to acetylcholine was found in both diets groups. Some flavonoids increase endothelial NO synthase activity (Olszanecki et al. 2002; Duarte et al. 2012). Therefore, in such conditions a lower dose of acetylcholine should be necessary to achieve the same relaxant response. In hypercholesterolemic rabbits this mechanism would be additional to the antioxidant effects previously discussed and both together may contribute to the increased efficiency observed in HD. One of the characteristic morphological changes of atherosclerosis is increased intima/media ratio. Epidemiologic data have shown a clear correlation between this feature and cardiovascular disease. Thus, this parameter would be an early marker of atherosclerosis (Campuzano et al. 2003). Previously we found an increased intima/media ratio in aorta from rabbits fed a HD. Treatment with ZpE, as well as rosuvastatin, normalized intima/media ratio in aorta from hypercholesterolemic rabbits. This effect on vascular morphology supports the hypothesis that ZpE treatment may improve endothelial function.

One of the objectives of the present work was to evaluate the ability of ZpE treatment to prevent the alterations on vascular responsiveness to vasoconstrictor agonists. Our results showed that oral administration of ZpE significantly desensitized the contractile response to norepinephrine both in normal and hypercholesterolemic rabbits. Consistent with these results, natural flavonoids were found to possess alpha1-adrenergic receptor antagonistic effects (Ajay et al. 2003; Duarte et al. 1993; Li et al.2011). Hypercholesterolemia induces alterations in vascular reactivity to angiotensin II (Jerez et al. 2008). Oral administration of ZpE had a diet-dependent effect on the angiotensin II-contractile response. Desensitization was found in rabbits fed on CD and reduction of the R_{max} was found in rabbits fed on HD. Flavonoids have been shown to play a pivotal role in the inhibition of angiotensin II induced superoxide via activation of NADPH oxidase, a major source of superoxide generator (Lau et al. 2012). Therefore, angiotensin II effect of ZpE administration could be related with the antioxidant constituents of the extract, predominantly flavonoids. Moreover, previous work has shown improvement of angiotensin II-contractile response in aorta from rabbits fed on high cholesterol diet. Endothelial dysfunction caused by increasing cyclooxygenase activity ROS-dependent and releasing of vasoconstrictor prostanoids may account for this phenomenon (Jerez et al. 2008). Flavonoids from *Z. punctata* have been demonstrated to inhibit cyclooxygenase activity *in vitro,* in addition to its antioxidant properties (Moreno et al. 2015; Nuño et al. 2016). Futhermore, flavonoid inhibition of cyclooxygenase activity together with endothelial function improvement may contribute to reduce contractile response to angiotensin II in ZpE-treated rabbits.

A low-order, positive relationship of dietary cholesterol intake to systolic blood pressure with control for multiple possible confounders has been reported (Sakurai et al. 2011). The improvements in endothelium-dependent relaxations to acetylcholine following chronic antioxidant treatments are associated with either reduced or no significant changes in MAP (Kitiyakara and Wilcox, 1998). Oral administration of ZpE significantly reduced mean MAP in rabbits fed on HD. Because the MAP measurements were recorded at the end of the treatment, reduction in the blood pressure was not caused by acute vasodilatory actions of the ZpE. Results from the present study demonstrated that the ZpE was an inhibitor of vasoconstriction induced by stimulation of physiologically important vascular receptors, such as adrenergic and angiotensin II receptors, which play a pivotal role in the regulation of blood pressure. Furthermore reduction of vasoconstrictor response together with its hypolipidemic properties may account for the MAP reduction induced by ZpE treatment in rabbits fed on HD.

Although rosuvastatin did not improve relaxation to acetylcholine and increased norepinephrine affinity, it reduced angiotensin II contractile response. These data, taken together with no effect of rosuvastatin on TC levels, may imply differential role of lipids in the vascular dysfunction. Further study needs to be taken to understand the underlying mechanisms of these effects.

In conclusion, the present study clearly demonstrated that oral administration of ZpE had no toxicity at doses used, reduced TC and TG levels, improved oxidative status preventing endothelial dysfunction and increase of contractile response to vasoconstrictors in rabbits fed on HD. Therefore, the use of ZpE as natural product in the prevention of cardiovascular disease related with hypercholesterolemia and endothelial dysfunction would be very promising.

Acknowledgments

Conflict of interest

The authors have no relevant conflicts of interest to disclose.

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Table 1. Mean arterial pressure (MAP), heart rate (HR), biochemical and hematological parameters and organ weights from rabbits treated with vehicle or 2.5, 5, 10 mg/day of *Zuccagnia punctata* extract

	Vehicle	2.5 mg	5 mg	10 mg
Weigth (g)	2088 ± 99	1920 ± 144	2200 ± 100	1957 ± 99
PAM (mmHg)	60.5 ± 3.5	58.3 ± 2.5	79 ± 3.4	64.5 ± 0.5
Fasting Glucose (mg/dl)	113.2 ± 2.7	119.2 ± 4.1	116.8 ± 6.7	125.8 ± 5.5
Total Cholesterol (mg/dl)	66 ± 6.8	35.1 ± 6.1[a]	40.2 ± 11.3	58.4 ± 14.7
Triglycerides (mg/dl)	97.4 ± 14.9	81.0 ± 20.6	90.1 ± 12	131.3 ± 15.4
Creatinine (mg/dl)	0.81 ± 0.04	0.82 ± 0.2	0.81 ± 0.25	0.81 ± 0.25
Aspartate amino transferase (U/l)	21.3 ± 3.9	15.6 ± 0.6	4.2 ± 1.9[a]	3.0 ± 1.9[a]
Alanine amino Transferase (U/l)	20.5 ± 4	18.6 ± 0.5	20.4 ± 1.5	19 ± 1.9
Total bilirubin (mg/dl)	0.32 ± 0.03	0.24 ± 0.01	0.32 ± 0.04	0.39 ± 0.11
Direct bilirubin (mg/dl)	0.086 ± 0.02	0.07 ± 0.01	0.03 ± 0.01[a]	0.05 ± 0.01[a]
Indirect bilirubin (mg/dl)	0.2 ± 0.03	0.17 ± 0.02	0.28 ± 0.04	0.30 ± 0.08
Liver (% BW)	3.19 ± 0.14	2.87 ± 0.26	3.12 ± 0.16	3.5 ± 0.09
Kidney (% BW)	0.34 ± 0.05	0.30 ± 0.01	0.32 ± 0.02	0.55 ± 0.16[a]
Heart (% BW)	0.27 ± 0.02	0.22 ± 0.02	0.24 ± 0.01	0.26 ± 0.02
Hematocrit (%)	41.1 ± 1.2	39 ± 1.6	41.9 ± 1.7	45 ± 1.2
White cells (x 10^3mm^3)	13.2 ± 2.1	10.6 ± 1.3	11.6 ± 2.5	13.0 ± 1.5

Data are expressed as mean ± SE of 4 rabbits. [a]$p < 0.05$ indicates statistically significant differences between ZpE-treated and vehicle-treated rabbits.

Table 2.Clinical and biochemical parameters from rabbits fed a control diet (CD) and a high cholesterol diet (HD)

	CD	ZpE-CD	HD	ZpE-HD	Ro-HD
BodyWeight (g)	2088 ± 99	2037 ± 121	2188 ± 64	2086 ± 41	1860 ± 42
PAM (mmHg)	60.5 ± 3.5	55.3 ± 3.7	73.2 ± 2.4	50 ± 2[b]	50.2 ± 4.6
HR	277.6 ± 29	252 ± 10	226 ± 11	225 ± 5	230 ± 8
Fasting Glucose (mg/dl)	113.2 ± 2.7	120.7 ± 2.9	115.3 ± 3.5	129 ± 2	130 ± 11
Total Cholesterol (mg/dl)	78.4 ± 6.4	34.6 ± 3.7[b]	928 ± 99[a]	369 ± 84[b]	816 ± 225
HDL-C	54.7 ± 2.8	12.2 ± 2[b]	115.5 ± 36[a]	16.5 ± 0.8[b]	16.5 ± 1.5[b]
LDL-C	35.1 ± 2.3	8.4 ± 1.1[b]	692 ± 112	308 ± 76[a]	784 ± 195
Triglycerides (mg/dl)	97.4 ± 14.9	85.3 ± 14.8	215 ± 27[a]	93.7 ± 15[b]	79 ± 13[b]
Creatinine (mg/dl)	0.81 ± 0.04	0.74 ± 0.14	0.97 ± 0.13	0.70 ± 0.2	0.94 ± 0.09
Aspartate amino transferase (U/l)	21.1 ± 1.5	23.3 ± 1.4	32.3 ± 3.3	20.9 ± 3.2[b]	16.7 ± 3.8[b]
Alanine amino transferase (U/l)	19.6 ± 3.9	24.6 ± 1.8	9.3 ± 1.9[a]	8.44 ± 1.2	7.4 ± 1.4
Total bilirubin (mg/dl)	0.32 ± 0.03	0.24 ± 0.01	0.65 ± 0.17[a]	0.24 ± 0.03[b]	0.43 ± 0.1
Direct bilirubin (mg/dl)	0.086 ± 0.02	0.07 ± 0.01	0.1 ± 0.05	0.06 ± 0.02[b]	0.03 ± 0.01[b]
Indirect bilirubin (mg/dl)	0.2 ± 0.03	0.17 ± 0.02	0.55 ± 0.15[a]	0.18 ± 0.008[b]	0.41 ± 0.1
Liver (% BW)	3.19 ± 0.14	2.96 ± 0.15	4.1 ± 0.2[a]	4.27 ± 0.2	4.9 ± 0.1
Kidney (% BW)	0.34 ± 0.05	0.29 ± 0.14	0.44 ± 0.1	0.30 ± 0.01	0.43 ± 0.03

Heart (% BW)	0.27 ± 0.02	0.24 ± 0.1	0.23 ± 0.03	0.23 ± 0.01	0.25 ± 0.1
Hematocrit (%)	41.0 ± 1.2	39.0 ± 1.6	34.3 ± 1.0^{a}	41.0 ± 2.9^{b}	41.8 ± 1^{b}
White cells (x10⁻³)	12.2 ± 2.1	10.6 ± 1.3	16.7 ± 1.5^{a}	12.3 ± 0.8^{b}	12.6 ± 2.4^{b}

Data are expressed as mean $\pm$ SE of 8 rabbits. ZpE: hydroalcoholic extract from *Z. Punctata*. Ro: rosuvastatin. [a] $p < 0.05$ indicates statistically significant differences between rabbits fed a CD and rabbits fed a HD. [b] $p < 0.05$ indicates statistically significant differences between ZpE or Ro-treated and untreated rabbits.

Figure legends

Fig. 1. RP-HPLC chromatogram of hydroalcoholic *Zuccagnia punctata* extract at 330 nm for identification and quantification of major flavonoids: 2′,4′-dihydroxy-3′-methoxychalcone (DHMC); 2′,4′-dihydroxychalcone (DHC).
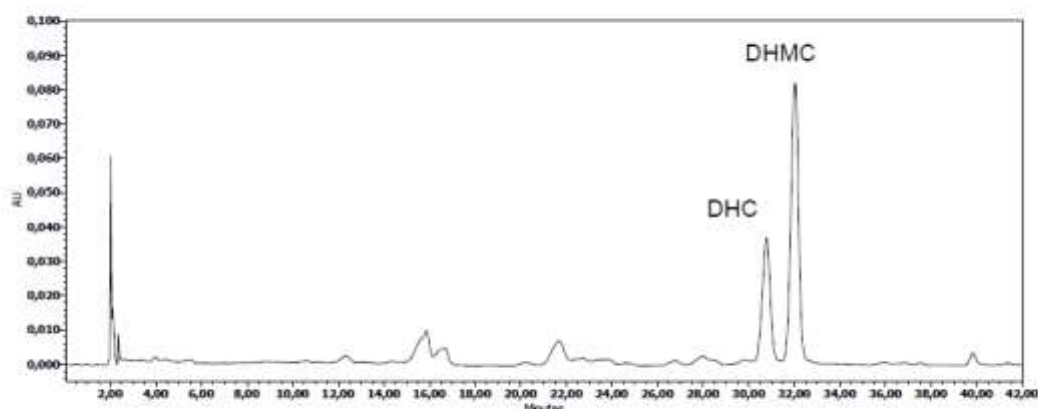
Figure 1

Fig. 2. Effects of 2.5 mg/day hydroalcoholic *Z. punctata* extract (ZpE) treatment on serum oxidative parameters: lipid peroxidation (TBARS) (a) and ratio of reduced glutathione to oxidized glutathione (GSH/GSSH) (b).
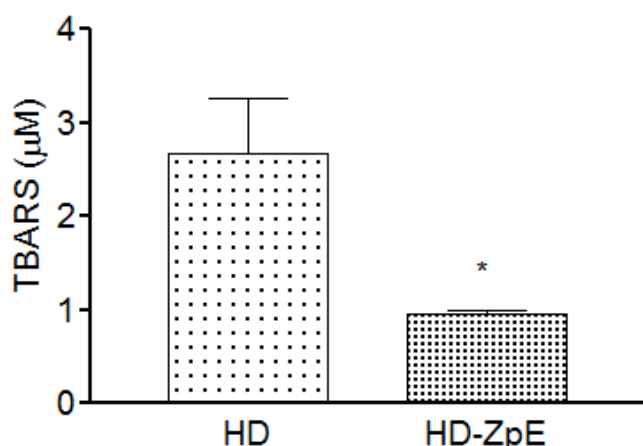
Figure 2a

Fig. 3. Concentration response curves to acetylcholine in aortic rings from: CD: rabbits fed a control diet; HD rabbits fed a high cholesterol diet (HD); ZpE-CD: rabbits fed a control diet and treated with 2.5 mg/day of hydroalcoholic *Z. punctata* extract; ZpE-HD: rabbits fed a high

cholesterol diet and treated with 2.5 mg/day of hydroalcoholic *Z. punctata* extract. *$p < 0.05$* indicates statistically significant differences in maximal relaxation between HD and ZpE-HD arteries (one way ANOVA).
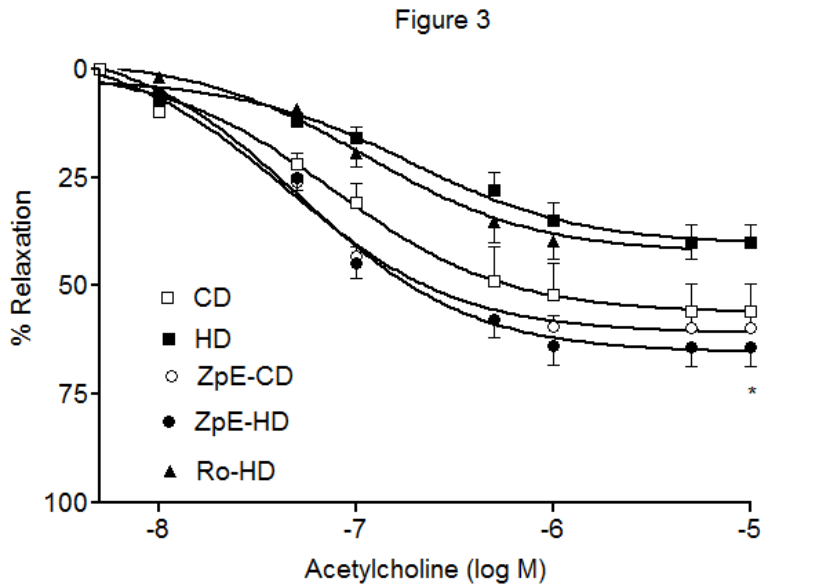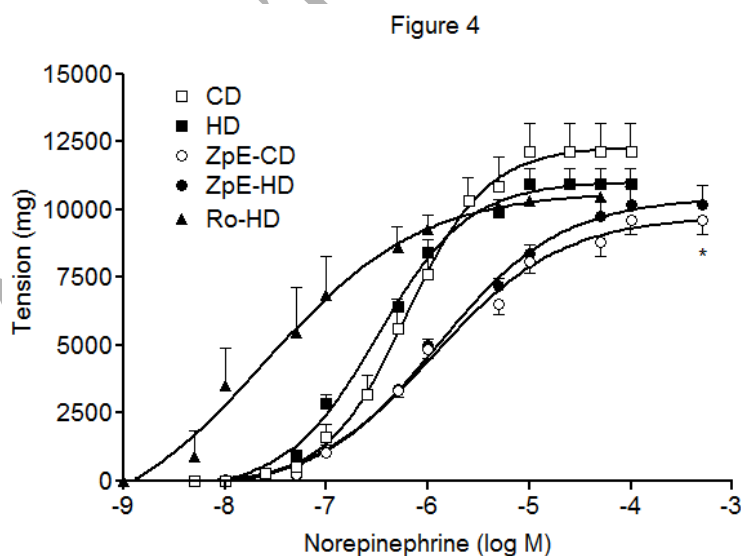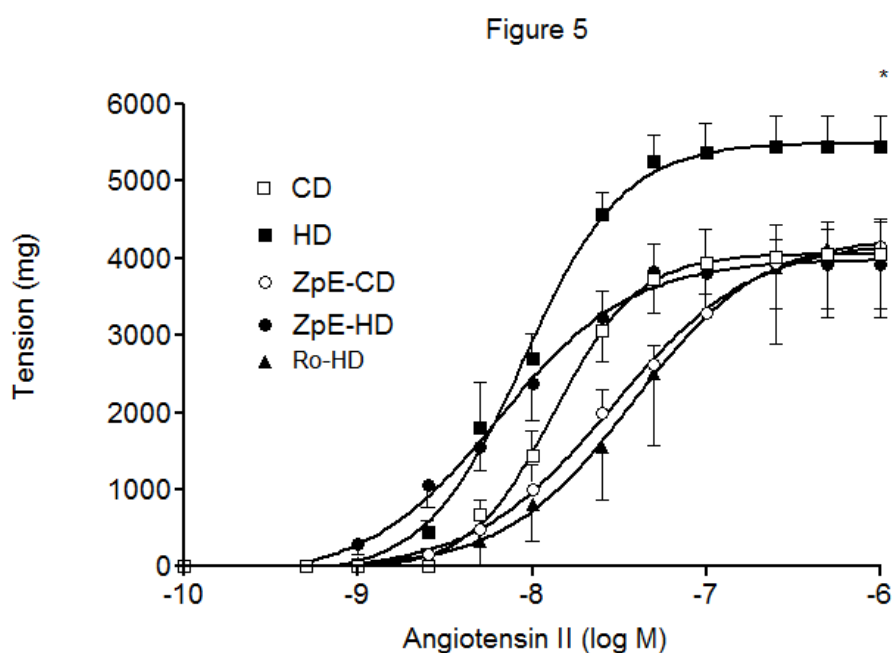

Fig. 4. Concentration response curves to norepinephrine in aortic rings from: CD: rabbits fed a control diet; HD rabbits fed a high cholesterol diet (HD); ZpE-CD: rabbits fed a control diet and treated with 2.5 mg/day of hydroalcoholic *Z. punctata* extract; ZpE-HD: rabbits fed a high cholesterol diet and treated with 2.5 mg/day of hydroalcoholic *Z. punctata* extract. Ro-HD: rabbits fed a high cholesterol diet and treated with 2.5 mg/day of rosuvastatin. * *$p < 0.05$* indicates statistically significant differences between maximal response of HD with respect to CD, ZpE-CD and ZpE-HD (one way ANOVA). *ƒ $p < 0.05$* indicates statistically significant differences in pEC_{50} between 2.5 mg/day ZpE and rosuvastatin treated with respect tountreated
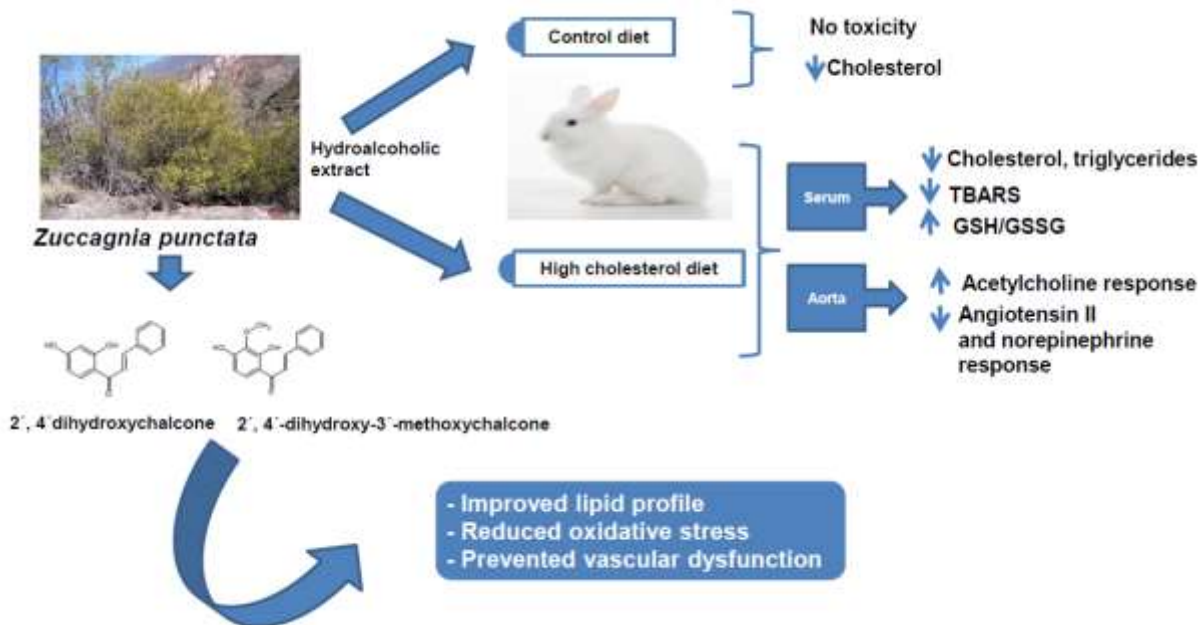

rabbits.

Fig. 5. Concentration response curves to angiotensin II in aortic rings from: CD: rabbits fed a control diet; HD rabbits fed a high cholesterol diet (HD); ZpE-CD: rabbits fed a control diet and treated with 2.5 mg/day of hydroalcoholic *Z. punctata* extract; ZpE-HD: rabbits fed a high cholesterol diet and treated with 2.5 mg/day of hydroalcoholic *Z. punctata* extract; Ro-HD: rabbits fed a high cholesterol diet and treated with 2.5 mg/day of rosuvastatin. *p < 0.05* indicates statistically significant differences in maximal response between HD, ZpE-HD and Ro-HD. *f p < 0.05* indicates statistically significant differences in pEC_{50} between CD and ZpE-

Figure 5

C

GraficalAbstract