



## RESEARCH ARTICLE

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# Beneficial effects of hydro-alcoholic extract of *Zuccagnia punctata* against atherosclerosis in normal weight obesity accompanied by hypercholesterolemia rabbit model

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## ABSTRACT

Insulin resistance (IR) increases the synthesis of cholesterol so hypercholesterolemia is one regular feature of obesity. Rich in flavonoids *Zuccagnia punctata* extract (Zp-E) is a traditional herbal medicine found to be beneficial against hypercholesterolemia-induced oxidative damage. This study aimed to evaluate the effects of Zp-E in a diet-induced rabbit model of IR accompanied by hypercholesterolemia. The major components of the Zp-E were analyzed by using a reversed-phase HPLC method. Male hybrid rabbits (cross between New Zealand and Californian certificated breeds) were separated into six groups: 1: fed on regular chow (SD), 2: fed on SD supplemented with 18% fat and 0.3% cholesterol (HC-HFD), 3, 4, 5: fed on HC-HFD and orally administered 2.5 mg, 5 mg or 10 mg GAE/day of Zp-E, respectively, 6: fed on HC-HFD and orally administered 2.5 mg ezetimibe/kg/day. All diets were administered for 6 weeks. The major compounds of Zp-E identified were chalcones: 2',4'-dihydroxy-3'-methoxychalcone and 2',4'-dihydroxychalcone. Zp-E only at 2.5 mg GAE/day reduced total cholesterol and did not modify fasting glucose, visceral abdominal fat, or IR at any of the doses tested. Zp-E normalized TBARS levels, sudanophilic area, and intima/media ratio at all the doses tested while significantly improving acetylcholine relaxation only at 5 mg GAE/day. Despite Zp-E's failure to prevent IR under hypercholesterolemic conditions, the extract showed protective effects on blood vessels by preventing the formation of atherosclerotic plaque through its strong antioxidant properties.

## 1. Introduction

Hypercholesterolemia in obesity contributes to atherosclerosis plaque development, and vascular dysfunction and could be accompanied by insulin resistance. Atherosclerosis plays a crucial role in the development of cardiovascular diseases, affecting both the heart and blood vessels. The primary risk factor for atherosclerosis and endothelial dysfunction is high blood levels of cholesterol, especially oxidized LDL cholesterol (LDL-C) (Boren et al., 2020).

Obesity is characterized by abnormal or excessive fat accumulation. When fat accumulates in the abdomen, obesity is referred to as visceral or central obesity. This enlarged adipose tissue mass becomes dysfunctional and causes systemic insulin resistance. In addition, increased cholesterol synthesis has been found in obesity (Mc Auley, 2020). Since comparable results were demonstrated in individuals with type 2 diabetes mellitus regardless of their body weight (Simonen et al., 2002), insulin resistance could explain the increase in cholesterol synthesis in

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patients with obesity and type 2 diabetes mellitus (Pihlajamäki et al., 2004). An increase in cholesterol synthesis is always accompanied by low rates of cholesterol absorption; therefore, it has been difficult to determine which of these two is primarily affected in subjects with obesity or type 2 diabetes mellitus (Gylling et al., 2010).

Hypercholesterolemia could trigger insulin resistance or vice versa, where both processes are regularly found in obesity. Hypercholesterolemia induces the production of free radicals and the subsequent elevation of lipid peroxides, which can initiate atherogenic processes (Yang et al., 2017). Studies have indicated impaired endothelium-dependent vasodilation in isolated arteries from rabbits with diet-induced hypercholesterolemia and atherosclerosis (Jerez et al., 2008; Jerez et al., 2010). Regarding obesity, some authors claim that oxidative stress is a common feature of obesity (Manna & Jain, 2015). However, Alarcon et al. (2018) demonstrated that in an early stage, normal-weight obesity is accompanied by a pro-inflammatory status without oxidative stress.

High costs and potential adverse effects associated with synthetic chemical drugs have fostered and intensified the search for alternative and complementary treatments for obesity and hypercholesterolemia. As such, medicinal plants and their products are gaining attention as potential therapeutic agents for these conditions, even among patients and healthcare providers (Payab et al., 2020). According to several studies, the incorporation of natural flavonoids into the diet has been associated with a reduction in the risk of cardiovascular diseases in obese individuals (Sandoval et al., 2020).

*Zuccagnia punctata* Cav. (Zp), a monotypic species belonging to the Fabaceae family is commonly found throughout western Argentina. It is popularly named "jarilla" in Argentina. The infusions and extracts of the plant are used in Argentinian folk medicine for the treatment of bacterial and fungal infections, asthma, arthritis, rheumatism, inflammation, and tumors (Isla et al., 2016). It is also claimed that extracts from Zp inhibit enzymes involved in obesity pathologies such as glucosidase and pancreatic lipase (Salas et al., 2020). Phytochemical analysis of Zp has revealed that it is a rich source of flavonoids such as flavanones, flavones, and chalcones, as well as caffeoyl esters. The leaf resin of Zp contains 2',4'-dihydroxychalcone (DHC) and 2',4'-dihydroxy-3'-methoxychalcone (DHMC) as its major constituents (Solorzano et al., 2017). Oral administration of a hydroalcoholic extract from Zp (Zp-E) is non-toxic, lower total cholesterol (TC) and triglyceride (TG) levels, improves oxidative status, restores endothelial function, and reduces the contractile response to vasoconstrictors in rabbits fed a high cholesterol diet (Roco et al., 2018). In addition, Zp-E has been found to improve insulin resistance in a rabbit model of normal-weight obesity without oxidative stress (Valoy et al., 2023). At present, however, no studies were performed on models having both insulin resistance and hypercholesterolemia conditions. Taking into account that insulin resistance regulates cholesterol metabolism (Gylling et al., 2010), the effects of Zp-E may not necessarily be the same as the effect of each model separately. Thus, the present study was carried out to investigate the effects of oral administration of Zp-E extract in a high fat-high cholesterol diet-induced insulin resistance accompanied by a hypercholesterolemia rabbit model.

## 2. Materials and methods

### 2.1. Plant materials

*Z. punctata* is a glutinous and aromatic shrub 1 to 2.5 m high, pseudo-paripinnate resinous leaves of 3-5 cm with sub-opposite leaflets (5 to 13 pairs), nanophyll with acuminate apex, rounded base, and entire margin (Mercado et al., 2013). Epidermal surfaces of the leaflets present epidermal cells with straight anticlinal walls, thick cuticles, cyclocytic stoma, sunken capitate glandular trichomes located in crypts, and non-glandular one-celled trichomes arranged in the margins (Mercado et al., 2013). In the section, leaflets are isolateral and amphistomatic. The middle vein presents a collateral vascular bundle with sclerenchymatous layers at the phloem pole. Idioblasts containing druses in the mesophyll are abundant. In cross-section, the stem of the primary structure presents an unstratified epidermis, raised stomata, unicellular and glandular trichomes. The parenchymatic cortex of 7-8 cell layers (Mercado et al., 2013).

Moreno et al. (2015) reported the content of total polyphenolic compounds on *Z. punctata* foliar surface ( $177 \pm 13 \mu\text{g GAE}/\text{cm}^2$ ). Analysis of the foliar washings revealed the presence of two major constituents, DHC and DHMC. The content of both major compounds ( $\text{cm}^2$  of leaf) was  $99.2 \mu\text{g DHC}$  and  $73.4 \mu\text{g DHMC}/\text{cm}^2$ . Histochemical analysis (fluorescence microscopy and emission scanning electron microscopy coupled with energy dispersive X-ray spectrometry) revealed on the foliar surface a high accumulation of chalcones.

The aerial parts of Zp, including the leaves and stems, were collected from Amaicha del Valle in Tucuman, Argentina, at an altitude of 2000 meters above sea level, between January and February 2016. The samples were dried in the dark at room temperature for one week, and voucher specimens (IML 605935) were deposited at the Miguel Lillo Foundation-Herbarium in Tucumán, Argentina. The authenticity of the samples was verified by Dr. Soledad Cuello.

### 2.2. *Z. punctata* extract

#### 2.2.1. Preparation of the Zp extract

Zp-E was prepared according to Roco et al. (2017). Briefly, 20 g of ground air-dried plant material were macerated in 100 ml of 80:20 ethanol:water solution for seven days with shaking (40 cycles/min) at room temperature. The resulting extracts were filtered using Whatman No. 4 filter paper and analyzed for total phenolic (TP) content using the Folin-Ciocalteu method. The non-flavonoid (NFP) and flavonoid (FP) phenolic compounds were measured in accordance with Isla et al. (2014) and Popova et al. (2005), respectively. Afterward, the solvent was evaporated to dryness in a rotary evaporator at 40 °C. The dry extract obtained (3.6 g) was stored at -20 °C until required. The quantities of TP and NFP were expressed as mg gallic acid equivalent per gram of dry Zp-E (mg GAE/g Zp-E), while FP was expressed as mg quercetin equivalent per gram of dry Zp-E.

#### 2.2.2. Assessment of total phenolic and flavonoids

The Zp-E was analyzed by HPLC attached to a diode array detector (DAD) according to Valoy et al. (2023). UV spectra and co-injection with standards were utilized to identify flavonoid markers in the Zp-E. The major compounds identified were DHMC and DHC, which were also quantified. A calibration curve was prepared using commercial standards to determine the relationship between the

concentration and peak area. The concentration of the compounds was expressed as mg/g of dry Zp-E.

### 2.3. Animals

The animal experimental procedures were authorized by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL), from the Universidad Nacional de Tucumán, Argentina-Approval number: 021/2019; Date: November 15<sup>th</sup>, 2019; Date endorsed: March 23<sup>rd</sup>, 2021. All animal care and use programs were performed following the guidelines from the Guide for the Care and Use of Laboratory Animals (NIH Publication 8<sup>th</sup> edition, updated 2011). Male hybrid rabbits from the “Cunicola Modulo” (Agronomy and Zootechnic Faculty of UNT) slaughterhouse initially weighing 650–800 g were housed in single cages in a humidity and temperature-controlled room with a 12-h light cycle. They were fed 100 g/day of standard rabbit chow. Water was given ad libitum. The animals were weighed before experimental manipulation and every day throughout the experiment. Environmental enrichment practices were carried out to reduce stress before, during, and after the experimental interventions.

### 2.4. High fat-high cholesterol diet

A high-fat high cholesterol diet consisted of 18% fat (10% corn oil, Mazola®, Argentina, and 8 % lard, Paladini®, Argentina) and 0.3% cholesterol (Cayman Chemicals, United States). Nutritional composition is described in the supplementary file.

### 2.5. Experimental protocol

A longitudinal, prospective, randomized, vehicle-controlled bioassay was conducted in rabbit models. After a 1-week acclimatization period, once animals were raised to a weight of 1000 g, they were randomly allocated into six groups ( $n = 6$  rabbits for each group), with food and water freely available. The individual rabbit was considered the experimental unit within the studies. The 3Rs (Replacement, Reduction, and Refinement) were taken into account to define the experimental number of animals in each group. The first group received a standard diet (SD, control group), while the other groups (groups 2-6) were fed with the high-fat high cholesterol diet. In addition, groups 1 and 2 received DMSO in water (1/1000) per day as the vehicle (HC-HFD), group 3 received 2.5 mg/day of ezetimibe (inhibitor of Niemann-Pick C1-Like 1, intestinal cholesterol transporter; Eze) as the positive control, and groups 4, 5 and 6 received 2.5, 5, and 10 mg GAE/day Zp-E, respectively. All diets were administered for 6 weeks. The doses and the feeding period were chosen using previous works as a reference (Roco et al., 2018; Valoy et al., 2023). Ezetimibe and extracts were suspended in water and were given orally at 8 a.m. for the 6 weeks of the experimental period.

### 2.6. Clinical and biochemical parameters

An intraperitoneal glucose tolerance test (IGT) and intravenous insulin tolerance test (IVITT) were performed two days before the end of the 6 weeks of feeding according to Alarcon et al. (2018) and Georgiev et al. (2011). Plasma glucose concentrations were measured by using colorimetric reactions with commercial kits (Wiener 864122524/02, Rosario, Argentina). The area under the curve (AUC) was calculated from the IGT and IVITT by the trapezoidal rule. Furthermore, the following kinetic parameters from IVITT were also calculated: minimal glucose concentration ( $C_{\min}$ ) and its corresponding time ( $T_{\min}$ ).

After treatment, rabbits were fasted for 12 h, weighed, and anesthetized using ketamine (20 mg/kg) and diazepam (0.5 mg/kg). Direct measurements of mean arterial blood pressure (MAP) and heart rate (HR) were carried out using a catheter inserted into the carotid artery and connected to a pressure transducer (Gould-Statham P23, California, USA). The data was recorded using a data acquisition system (Biopac MP100, Aero Camino Goleta, USA). After recording the MAP, blood samples were drawn from the carotid artery and collected into centrifuge tubes. The tubes were centrifuged to obtain serum for biochemical and hematological analyses.

Fasting glucose and lipid profile: total cholesterol (TC), high-density cholesterol lipoprotein (HDL-C), LDL-C, and triglycerides (TG) were measured using commercial kits (Wiener, Rosario, Argentina) through colorimetric reactions. Renal and liver functions were checked to determine if there is synergistic toxicity when high-fat and cholesterol-rich diets are administered simultaneously. Creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin (total, direct, and indirect) were measured using commercial kits (Wiener, Rosario, Argentina). The hematological study included an estimation of white blood cell count and hematocrit.

Following blood collection, the animals were immediately euthanized for tissue studies. The thoracic aorta was quickly removed and divided into sections. The aortic arch and the adjacent segment (about 6 mm long) were immersed in a 10% neutral-buffered formaldehyde solution for histological studies. The following segment was immersed in Krebs solution for isolated blood vessel preparation. The liver was dissected immediately, rinsed with ice-cold saline solution, blotted dry using filter paper, and stored at  $-80^{\circ}\text{C}$  for further analysis. Adipose tissues from the abdominal region (visceral and retroperitoneal, VAT) were collected and weighed. The VAT, liver, kidney, and heart were expressed as a percentage of the total body weight: (fat weight/animal weight)  $\times 100$  (Shuster et al., 2012).

The TyG index is a simple marker that correlates well with the degree of insulin resistance determined by hyperinsulinemic-euglycemic clamp studies (Guerrero-Romero et al., 2010). The TyG index was calculated as  $\ln(\text{fasting triglycerides (mg dl}^{-1}) \times \text{fasting glucose (mg dl}^{-1})/2)$ .

### 2.7. Lipid peroxidation in serum and liver

The levels of malondialdehyde (MDA), a thiobarbituric acid reactive substance (TBARS), from serum and liver homogenates were analyzed as a marker of oxidative stress in accordance with the method described by Karbinger et al. (2013). Lipid peroxides were expressed as mmoles MDA production/g liver and mmoles/mg protein.

### 2.8. Histological examination

For the histological study, previously fixed biopsies adjacent to aortic arch segments were cut into 2 segments. Specimens from all diet groups were embedded in paraffin and sectioned at  $5\ \mu\text{m}$ . The sections were stained with hematoxylin and eosin, and examined by light microscopy (Schmitz et al., 2010). Media and intima thickness were measured by image analysis with the software Media Cybernetics® Image-Pro Plus TM. The ratio of tunica intima/ tunica media was calculated.

Aortic lipid staining with Sudan IV was performed to check atherosclerotic lesions (Shahid et al., 2011). Fixed aortic arches were rinsed with 70% (v/v) ethanol, placed in Herxheimer's solution, and decolorized in 80% (v/v) ethanol. Neutral resins were used to block specimens. The percentage of plaque coverage (sudanophilic area) was calculated using the software Media Cybernetics® Image-Pro Plus™. Pixels from the total intimal area and sudanophilic area were quantitatively measured using the MacScope system and expressed as % of microscopic lesion areas: (sudanophilic area pixels/total intimal area pixels) x 100.

### 2.9. Isolated thoracic aorta preparation

The segment of the thoracic aorta previously immersed in Krebs solution was cut into 5 mm ring segments (4 from each aorta); care was taken to avoid any damage to the endothelium. Each aortic ring was placed in a 10 ml organ bath. The isometric force was measured using a force transducer connected to a data-acquisition system (BIOPAC) and analyzed with appropriate software (Acknowledge 3.4). The rings were allowed to equilibrate for 120 min at a resting tension of 2 g, with the bath solution changed every 15 min (Jerez et al., 2008). After the equilibration period, aortic rings were contracted with phenylephrine ( $5 \times 10^{-6}$  M) and cumulatively exposed to acetylcholine ( $10^{-8}$ - $10^{-6}$  M) to construct a concentration-response curve (CRC) to assess the endothelium-dependent relaxation. The endothelium-independent relaxation was checked by adding sodium nitroprusside  $10^{-5}$  M on the phenylephrine plateau. To evaluate vascular reactivity, contractile response to KCl (96 mM) and vasoconstrictor agonists were 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.

### 2.10. Statistical analysis

Normal distribution was tested with Kolmogorov-Smirnov goodness-of-fit test. The results were presented as mean  $\pm$  standard error of the mean (SEM). For each experimental condition, a concentration-response curve (CRC) was plotted, and the maximal response ( $R_{max}$ ) and the negative log molar concentration of the agonist producing 50% of the maximum contraction or relaxation ( $pEC_{50}$ ) were determined using Prism Version 3.0 (GraphPad Software, USA). One-way ANOVA followed by Duncan's test was used to compare the mean values among the six diet groups. A  $p$ -value less than 0.05 was considered statistically significant.

## 3. Results and discussion

### 3.1. Phytochemical screening of Zp-E

Zp-E contained  $1238 \pm 52$  mg GAE/g dry extract of TP compounds: 53% were FP ( $656 \pm 12$  mg QE/g dry Zp-E) and 47% were NFP ( $582 \pm 15$  mg GAE/g dry Zp-E). The phenolic compound profile was analyzed, and the major bioactive compounds DHMC and DHC were identified by UV spectra and co-chromatography with the commercially obtained pure compounds (Indofine Chemical Company, New Jersey, USA). Results showed the following flavonoid quantities: DHMC:  $63 \pm 2.5$  mg/g dry Zp-E, DHC:  $41 \pm 2.1$  mg/g dry Zp-E. These findings were consistent with those of earlier studies (Roco et al., 2017; Valoy et al., 2023).

### 3.2 Effect of Zp-E on clinical and biochemical parameters

The body weight and food consumption of all diet groups were monitored during the 6 weeks of the feeding period. No differences were found between the groups both in the final body weight (Table 1) and the weight gain throughout the experiment. Compared to the SD group, the addition of fat and cholesterol to the diet significantly increased total cholesterol, AUC-IGT, AUC-IVITT, and VAF and decreased HDL-C (Tables 1 and 2). These results mean that the present model mimics pathological alterations from normal weight obesity (NWO) individuals. NWO is a relatively new category of obesity that is characterized by the presence of high body fat despite having a normal body mass index (BMI). Individuals with this phenotype of obesity have a higher risk for cardiometabolic diseases than individuals with normal weight and without high body fat (Wijayatunga & Dhurandhar, 2021). Metabolic dysregulations include insulin resistance, atherogenic dyslipidemia, central obesity, and hypertension. Obesity is always accompanied by an expansion of white adipose tissue, which is a source of cytokines and contributes to a proinflammatory status (de Lorenzo et al., 2007). Furthermore, oxidative stress (di Renzo et al., 2010) and increased cholesterol synthesis have been observed in NWO (Mc Auley, 2020; Miettinen & Gylling, 2000). The oral administration of Zp-E (2.5 mg GAE/day) for 6 weeks to the HC-HFD group significantly reduced the serum level of total cholesterol and LDL-C, while ezetimibe 10 mg/day reached that of SD. Higher doses of Zp-E did not affect total cholesterol levels. These results partially agree with Roco et al. (2018) and may imply that the Zp-E effect was not dose-dependent. Valoy et al. (2023) find that rich in flavonoids Zp-E inhibits pancreatic lipase. This mechanism may account for the hypocholesterolemic effect of Zp-E, in part due to its polyphenolic compounds (Padilla-Camberos et al., 2015). Furthermore, no treatment modified glucose intolerance, TyG index, or VAF (Table 2). Even more, fasting glucose, AUC-IGT, AUC-IVITT, and Cmin were higher in HC-HFD orally treated with ezetimibe than those of SD or HC-HFD (Table 2). These results agree with those of Takeshita et al. (2014) and disagree with several data from the bibliography claiming the beneficial effect of ezetimibe on fasting glucose and insulin resistance (Nakamura et al., 2019). More studies beyond the scope of the present work are necessary to elucidate this phenomenon. The failure of the Zp-E to improve glucose intolerance was unexpected considering that Valoy et al. (2023) find oral administration of a similar Zp-E improves insulin sensitivity in a rabbit model of high-fat diet induced-NWO. However, such a model has a normal oxidative status and hypertriglyceridemia (Alarcon et al., 2018), while in the present work, rabbits fed on HC-HFD have both serum and liver MDA levels significantly higher than those of rabbits fed on SD and normal levels of serum triglycerides (Figure 1a and 1b). These results may imply that pathophysiological mechanisms of insulin resistance are different in both animal models. In the high fat diet induced-NWO model elevated triglyceride levels can contribute to insulin resistance. Excess triglycerides can accumulate in tissues such as skeletal muscle and liver, leading to the development of intracellular lipid droplets. These lipid deposits can interfere with insulin signaling pathways, impair glucose uptake, and disrupt insulin sensitivity, ultimately contributing to insulin resistance. Zp-E inhibits pancreatic lipase and reaches to control triglyceride levels improving insulin resistance. However, in the HC-HFD model, unexpectedly, triglyceride levels are similar to SD. This result would mean another mechanism is involved in glucose intolerance. Insulin resistance is associated with increased cholesterol synthesis and decreased cholesterol absorption (Pihlajamäki et al., 2004). In turn, insulin resistance and high cholesterol levels form a vicious circle, in which each condition exacerbates the other and leads to the acceleration of the

atherosclerosis process in conditions of oxidative stress (de Mutsert et al., 2018). Oral administration of Zp-E 2.5, 5, and 10 mg GAE/day reduced liver and serum MDA levels to those of SD (Figure 1a and 1b) suggesting that ZP extract acts mainly as an antioxidant due to the high content of flavonoids. Taking these data into account, the failure of the Zp-E to improve glucose intolerance despite its antioxidant effect may be related to its weak hypocholesterolemic

effect. Supporting this view, the oral treatment of HC-HFD rabbits with ezetimibe 2.5 mg/day as the positive control, while reduced total cholesterol levels did not modify MDA levels and significantly increased fasting glucose and worsened insulin resistance. In addition, Zp-E significantly reduced MAP (Table 1). No treatment modified hematological, hepatic, or renal parameters (Table 1).

**Table 1.** Clinical, biochemical, and hemodynamic parameters

	SD	HC-HFD	Zp-E 2.5 mg	Zp-E 5 mg	Zp-E 10 mg	Eze
BW (g)	2088 ± 99	1883 ± 113	1947 ± 83	2123 ± 130	2163 ± 183	2003 ± 62
MAP (mmHg)	60.5 ± 3.5	47 ± 2	36 ± 2.8 <sup>a</sup>	41 ± 3.8 <sup>a</sup>	30 ± 3.8 <sup>a</sup>	50 ± 5
HR (beats/min)	277.6 ± 29	245 ± 4.8	263 ± 15.2	262 ± 9.2	262 ± 8.4	255 ± 17
FG (mg/dl)	113.2 ± 2.7	115.5 ± 5.8	115.3 ± 3.8	117.6 ± 21.1	117.6 ± 19.8	154.9 ± 22.6 <sup>a</sup>
AUC-IGT	1038 ± 19	1223 ± 90 <sup>a</sup>	1235 ± 72 <sup>a</sup>	1240 ± 138 <sup>a</sup>	1193 ± 86 <sup>a</sup>	1390 ± 184 <sup>a</sup>
TC (mg/dl)	78.4 ± 6.4	499.7 ± 55.1 <sup>a</sup>	222 ± 79 <sup>a,b</sup>	429.8 ± 99.5 <sup>a</sup>	391.4 ± 45.9 <sup>a</sup>	97.4 ± 28.3 <sup>b</sup>
HDL-C	54.7 ± 2.8	16.8 ± 2.7 <sup>a</sup>	14.6 ± 2.2 <sup>a</sup>	14 ± 1 <sup>a</sup>	11.8 ± 2.2 <sup>a</sup>	16.4 ± 2.8 <sup>a</sup>
LDL-C	35.1 ± 2.3	391.8 ± 68.8 <sup>a</sup>	180.3 ± 77.5 <sup>a</sup>	330.9 ± 93.6 <sup>a</sup>	271.8 ± 20.6 <sup>a</sup>	65.1 ± 29.1 <sup>b</sup>
TG (mg/dl)	113.7 ± 14.3	89.7 ± 25.8	139.4 ± 36.9	123.7 ± 23.8	112.4 ± 25.7	79.1 ± 10.3
TyG index	8.2 ± 0.2	8.7 ± 0.4 <sup>a</sup>	8.8 ± 0.3 <sup>a</sup>	8.7 ± 0.3 <sup>a</sup>	8.8 ± 0.2 <sup>a</sup>	8.7 ± 0.2 <sup>a</sup>
Creatinine (mg/dl)	0.81 ± 0.04	1 ± 0.1	1 ± 0.05	1 ± 0.11	0.9 ± 0.12	1 ± 0.2
AST (U/l)	21.1 ± 1.5	13.3 ± 2.9	15.7 ± 1.4	7.2 ± 1.3	5.4 ± 1.7	7.3 ± 1
ALT (U/l)	19.6 ± 3.9	12.8 ± 2.8	11 ± 2.1	11 ± 2.8	14.1 ± 7.6	4.1 ± 1.1 <sup>b</sup>
T-bilirubin (mg/dl)	0.32 ± 0.03	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.3	0.34 ± 0.04
D-bilirubin (mg/dl)	0.086 ± 0.02	0.1 ± 0.04	-	0.1 ± 0.01	0.1 ± 0.04	0.03 ± 0.02
I-bilirubin (mg/dl)	0.2 ± 0.03	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.04	0.2 ± 0.04	0.27 ± 0.02
VAf (%)	1.3 ± 0.05	2.9 ± 0.1 <sup>a</sup>	2.6 ± 0.3 <sup>a</sup>	3.2 ± 0.2 <sup>a</sup>	3.6 ± 0.3 <sup>a</sup>	3.9 ± 0.3 <sup>b</sup>
Liver (%)	3.19 ± 0.14	3.8 ± 0.3	3.7 ± 0.3	3.3 ± 0.2	3.3 ± 0.2	2.9 ± 0.3
Kidney (%)	0.34 ± 0.05	0.27 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.25 ± 0.01
Heart (%)	0.27 ± 0.02	0.24 ± 0.01	0.23 ± 0.01	0.21 ± 0.003	0.21 ± 0.01	0.23 ± 0.01
Hematocrit (%)	41.0 ± 1.2	43.6 ± 0.7	43.8 ± 1.6	40.7 ± 1.3	40.3 ± 1.3	44.7 ± 1
White cells (x10 <sup>-3</sup> )	3.9 ± 0.9	2.4 ± 0.3	3.2 ± 0.9	1.5 ± 0.2 <sup>a</sup>	2 ± 0.4 <sup>a</sup>	2.5 ± 0.3

Control diet (SD), high cholesterol-high fat diet (HC-HFD), and HC-HFD orally treated with *Z. punctata* extract (Zp-E) or ezetimibe (Eze). Data are expressed as mean ± SEM of 6 animals. <sup>a</sup> indicates statistically significant differences as compared with SD. <sup>b</sup> indicates statistically significant differences as compared with HC-HFD (one-way ANOVA and Duncan's post test).

**Table 2.** Glycaemia kinetic parameters measured during an intravenous insulin tolerance test

	SD	HC-HFD	Zp-E 2.5 mg	Zp-E 5 mg	Zp-E 10 mg	Eze
AUC-IVITT (mmol/l.min)	612 ± 43	760 ± 35 <sup>a</sup>	796 ± 28 <sup>a</sup>	699 ± 46 <sup>a</sup>	704 ± 97 <sup>a</sup>	951 ± 73 <sup>a,b</sup>
C <sub>min</sub> (mmol/l)	3.6 ± 0.4	4.8 ± 0.4	4.5 ± 0.4	3.9 ± 0.3	4.8 ± 2.1	6.4 ± 0.9 <sup>a,b</sup>
T <sub>min</sub> (min)	24 ± 3.7	25 ± 7.2	26.4 ± 6.4	20 ± 4.1	20 ± 0	30 ± 10

Control diet (SD), high cholesterol-high fat diet (HC-HFD), and HC-HFD orally treated with *Z. punctata* extract (Zp-E) or ezetimibe (Eze). Data are expressed as mean ± SEM of 6 animals. <sup>a</sup> indicates statistically significant differences as compared with SD. <sup>b</sup> indicates statistically significant differences as compared with HC-HFD (one-way ANOVA and Duncan's post test).

### 3.3. Effect of ZP extract on lipid accumulation in the aorta and liver

Neutral lipids (cholesterol esters and triglycerides, stained with Sudan IV) can show different degrees of aortic lesions (red areas stained). Sudanophilic area was higher in the aorta from HC-HFD (27.1 ± 4.5%) than SD (1.3 ± 0.07%). Treatments with Zp-E and ezetimibe reduced sudanophilic area in the aorta from rabbits fed on HC-HFD (Figure 2; 2.5-Zp-E: 5.7 ± 0.6%; 5-Zp-E: 13.6 ± 1.9%; 10-Zp-E: 7.9 ± 0.9%; Eze: 2.1 ± 0.3 %; *p* < 0.05, one-way ANOVA and Duncan's post-test). The microscopic study of the tissue slices from the HC-HFD aortae revealed an increase in intima/media ratio (Figure 3; SD: 0.009 ± 0.002 vs HC-HFD: 0.16 ± 0.009; *p* < 0.05; Student test). Thickening of the intima/media is generally considered to be an early marker of generalized atherosclerosis because such thickening (intimal hyperplasia) has been associated with an unfavorable cardiovascular risk profile (Bonithon-Kopp et al., 1996). Zp-E and ezetimibe significantly reduced the intimal hyperplasia (Figure 3; 2.5-Zp-E: 0.02 ± 0.004; 5-Zp-E: 0.04 ± 0.002; 10-Zp-E: 0.07 ± 0.008; Eze: 0.016 ± 0.03; *p* = 0.0023; one-way ANOVA).

These beneficial effects were present despite the high levels of serum cholesterol. The phytochemical screening of the Zp-E showed that quantitatively the quantities of chalcones were predominant. Flavonoids are antioxidants and reduce the oxidation of LDL-C,

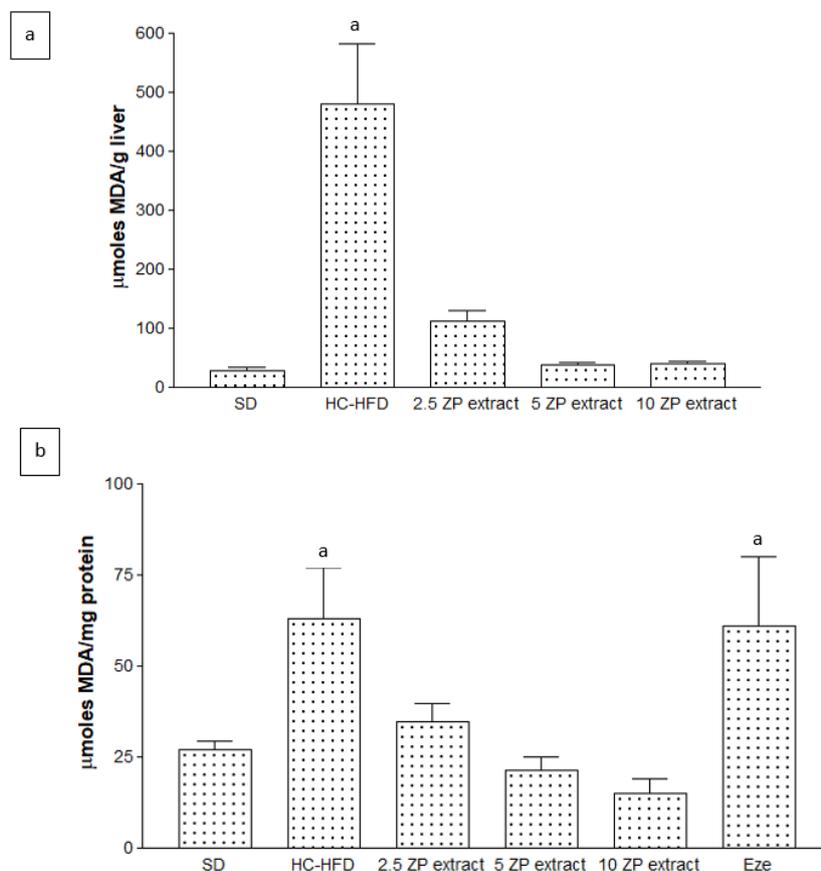
therefore suggesting their role in preventing hyperlipidemic damage in cardiovascular diseases (Simonen et al., 2002). This result supports the view that ZP extract acts mainly as an antioxidant.

### 3.4. Effect of Zp extract on the vascular function

The cardiovascular continuum is a sequence of cardiovascular events, which begins from a cluster of cardiovascular risk factors consisting of diabetes mellitus, dyslipidemia, hypertension, smoking, and visceral obesity (Chrysant, 2011). Vascular dysfunction is at the beginning of the cardiovascular continuum. For that reason, the prevention of vascular dysfunction is essential to avoid the progression of cardiovascular disease. A high-cholesterol diet induces vascular dysfunction (Jerez et al., 2008; Jerez et al., 2010). This dysfunction is characterized by reduced response to the endothelium-dependent vasorelaxant agonist acetylcholine and by increased response to Angiotensin II. Roco et al. (2018) demonstrate that Zp-E improves vascular dysfunction. Thus, the present study investigated, on one hand, whether HC-HFD induces vascular dysfunction and, on the other hand, whether the Zp-E improves it. The acetylcholine-induced endothelium-dependent relaxation was lower in the aortae from the HC-HFD group than that of the SD group. The mechanisms underlying the impairment of endothelium-dependent relaxation may involve NO inactivation by oxygen-derived free radicals between other mechanisms (Sena et al., 2018).

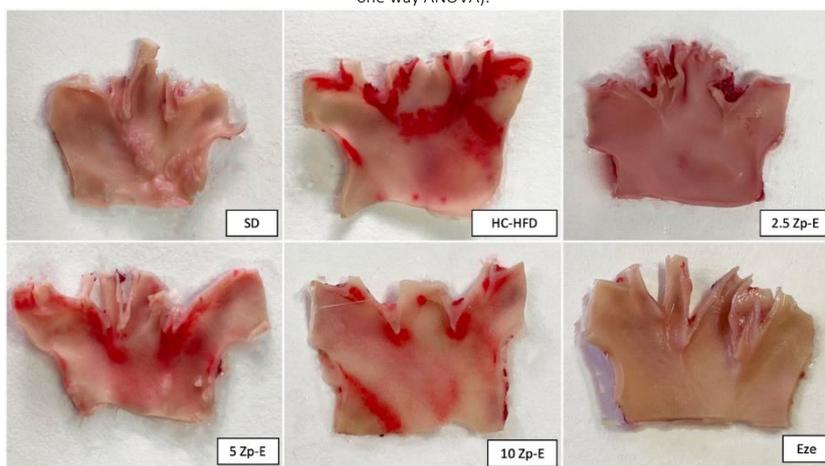
Administration of Zp-E 5 mg GAE/day and ezetimibe significantly reached SD the acetylcholine relaxation (Table 3). Considering that Zp-E is a strong scavenger of free radicals, one of the main inactivating agents of NO (Moran Vieyra et al., 2009), its antioxidant effect may account for the morphological and functional

improvements. Endothelium-independent vascular relaxations induced by sodium nitroprusside were similar in all diet groups (Table 3).



**Figure 1.** Serum and liver levels of the thiobarbituric acid substances (TBARS)

in rabbits fed a control diet (SD), a: high cholesterol-high fat diet (HC-HFD), b: HC-HFD orally administered with either 2.5, 5 or 10 mg GAE/day of Zp-E; Eze: HC-HFD with oral administration of 2.5 mg ezetimibe/day. The values shown represent the mean  $\pm$  SEM ( $n = 12$ ). The bars marked with "a" are significantly different as compared with SD ( $p < 0.05$ , one way ANOVA).



**Figure 2.** Representative photographs of aortic arches stained with Sudan IV

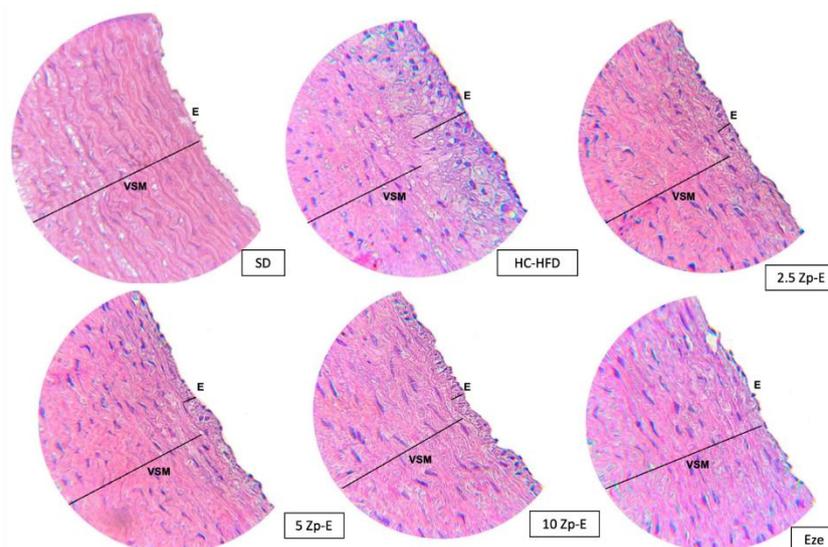
SD: control diet, HC-HFD: high cholesterol-high fat diet, Zp-E: HC-HFD orally administered with either 2.5, 5 or 10 mg GAE/day, Eze: HC-HFD with oral administration of 2.5 mg ezetimibe/day

Arteries from the HC-HFD group showed a decreased  $R_{max}$  to angiotensin II and norepinephrine as compared to the SD group. This result was unexpected because hypercholesterolemia has been found to increase contractile response to angiotensin II (Jerez et al., 2008). However, the relationship between insulin resistance and

blunted contractile responses to vasoconstrictor agonists is also reported (Jerez et al., 2012; Velloso et al., 2006). This HC-HFD-induced blunted response was not modified by oral treatment with ZP extract. However, ezetimibe-induced  $R_{max}$  to angiotensin II reaches that of SD. Furthermore, the ZP extract did not modify

angiotensin II-affinity (Table 3). Zp extract blunted angiotensin II-contractile response both in vitro and in vivo studies by a mechanism that involves a dose-dependent competitive or noncompetitive antagonism (Roco et al., 2018; Roco et al., 2017). Thus, as the effect of HC-HFD does not add up to that of Zp extract, insulin-induced angiotensin II receptors' downregulation may be responsible for reducing the angiotensin II contractile response in

the present model even under hypercholesterolemic conditions. Supporting this view is the finding that ezetimibe was reached to control total cholesterol levels and angiotensin II response in rabbits fed on HC-HFD. Further experimental designs are necessary to prove this hypothesis.



**Figure 3.** Representative microphotographs of aorta sections stained with hematoxylin/eosin

SD: control diet, HC-HFD: high cholesterol-high fat diet, Zp-E: HC-HFD orally administrated with either 2.5, 5 or 10 mg GAE/day, Eze: HC-HFD with oral administration of 2.5 mg ezetimibe/day, E: endothelium, VSM: vascular smooth muscle. The microphotographs were taken using an AxioCam HRC digital camera attached to a Zeiss Imager A2 microscope. Images were captured using Axio Vision Release 4.8.2 software at 40x magnification.

**Table 3.** Maximal response ( $R_{max}$ ) and  $pEC_{50}$  to acetylcholine (Ach), angiotensin II (Ang II), norepinephrine (NE), KCl and sodium nitroprusside (SNP)

	SD	HC-HFD	Zp-E 2.5 mg	Zp-E 5 mg	Zp-E 10 mg	Eze
Ach ( $R_{max}$ )	66.2±5.2	38.9±3.7	24.9±5	57.7±3.7 <sup>b</sup>	41±7.9	69±6.4 <sup>b</sup>
Ach ( $pEC_{50}$ )	7.01±0.07	7.8±0.2 <sup>a</sup>	8.2±0.3 <sup>a</sup>	7.9±0.2 <sup>a</sup>	7.5±0.1	7.3±0.4
AngII ( $R_{max}$ )	4055±320	2292±308 <sup>a</sup>	2031±615 <sup>a</sup>	2178.4±675 <sup>a</sup>	1410±328 <sup>a</sup>	3545±255
AngII ( $pEC_{50}$ )	7.8±0.2	8.2±0.1 <sup>a</sup>	8.4±0.1 <sup>a</sup>	8.5±0.2 <sup>a</sup>	8.2±0.2 <sup>a</sup>	8.4±0.2 <sup>a</sup>
NE ( $R_{max}$ )	11237±1234	7858±522 <sup>a</sup>	7489±615 <sup>a</sup>	10164±944	10567±1064	9756±544
NE ( $pEC_{50}$ )	6.4±0.1	7.3±0.3 <sup>a</sup>	8.4±0.1 <sup>a</sup>	6.6±0.1 <sup>b</sup>	6.3±0.2 <sup>b</sup>	6.2±0.2 <sup>b</sup>
KCl (mg tensión)	3907±270	3400±381	3591±966	4832±204	4040±477	3897±451
SNP (% relaxation)	93.3±4	93.3±0.9	93.1±1.0	99.7±0	98±2	97±2

Control diet (SD), high cholesterol-high fat diet (HC-HFD), and HC-HFD orally treated with *Z. punctata* extract (Zp-E) or ezetimibe (Eze). Data are expressed as mean ± SEM of 8 experiments. <sup>a</sup> indicates statistically significant differences as compared with SD. <sup>b</sup> indicates statistically significant differences as compared with HC-HFD (one-way ANOVA and Duncan's post test).

Concerning norepinephrine contractile response, Zp-E 5 and 10 mg GAE/day reached the norepinephrine  $R_{max}$  to that of SD values (Table 3). These results would mean that the norepinephrine contractile response is independent of insulin resistance under hypercholesterolemic conditions. No treatment modified the response to KCl (Table 3).

#### 4. Conclusions

The results showed that oral administration of a Zp-E reduced cholesterol levels and prevented the aortic morphological and atherogenic changes observed in conditions of insulin resistance accompanied by hypercholesterolemia. The Zp-E prevented HC-HFD-induced oxidative stress, which might be explained by its strong antioxidant properties. One of the main limitations of the use of the Zp-E is that no dose-dependent relationship has been observed regarding its effects. However, the use of the Zp-E as a supplement to prevent damage caused by hypercholesterolemia accompanied

by oxidative stress can be promising, considering that the extract does not have toxic effects at any of the doses used.

The main projections for future research are to analyze the effect of Zp-E and dietary fat on cholesterol transporters using Caco-2 cells and to study mechanisms involved in changes in vascular function using inhibitors or activators of ion channels.

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#### Conflict of interest

The authors confirm that there are no known conflicts of interest.

## Statement of ethics

All experimental procedures were authorized by the Comité Institucional de Uso y Cuidado de Animales de Laboratorio (CICUAL) from the Universidad Nacional de Tucumán, Argentina, with approval number 021/2019 and endorsed on March 23<sup>rd</sup>, 2021, in accordance with the guidelines of the NIH Publication 'Guide for the Care and Use of Laboratory Animals' (updated in 2011).

## Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Supplementary File

The supplementary file accompanying this article is available at <https://ijpbp.com/index.php/ijpbp/libraryFiles/downloadPublic/15>.

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