RENOPROTECTIVE AND HEPATOPROTECTIVE EFFECTS OF *HIPPOCRATEA EXCELSA* ON METABOLIC SYNDROME IN FRUCTOSE-FED RATS

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Abstract

The metabolic syndrome is associated with the development of chronic kidney disease and liver damage. The aim of this research was to determine the effect of the ethanol bark extract of *Hippocratea excelsa* (HE) on high fructose consumption-induced adverse effects in the kidney and liver of rats. Rats with 20% fructose feeding for 12 weeks showed arterial hypertension, obesity, dyslipidaemia and developed oxidative stress, proteinuria, the activities of antioxidant enzymes in the renal cortex and liver were decreased, TGF-β1 increased, and kidney and liver damage were observed. After the treatment for 6 weeks with HE (30 and 100 mg/kg bw) renoprotective and hepatoprotective effects in high fructose induced metabolic syndrome in rats, were demonstrated.

Rezumat

Sindromul metabolic este asociat cu dezvoltarea bolilor renale cronice și a afectării hepatice. Scopul acestei cercetări a fost de a evalua acțiunea extractului etanolic din scoarța de *Hippocratea excelsa* (HE) asupra efectelor adverse induse de consumul ridicat de fructoză, la nivel renal și hepatic, la șobolani. Șobolanii cu hrana îmbogățită cu 20% fructoză, timp de 12 săptămâni, au prezentat hipertensiune arterială, obezitate, dislipidemie și au dezvoltat stres oxidativ, proteinurie. Activitățile enzimelor antioxidante din cortexul renal și ficat au scăzut, TGF-β1 a crescut și au fost observate leziuni la nivelul rinichilor și ficatului. După tratarea timp de 6 săptămâni cu HE (30 și 100 mg/kgc) s-au observat efecte renoprotectoare și hepatoprotectoare la șobolanii cărora li s-a indus sindromul metabolic prin consumul ridicat de fructoză.

Keywords: *Hippocratea excelsa*, kidney disease, metabolic syndrome, liver damage

Introduction

Metabolic syndrome is a serious threat to public health because it is closely related to the modern lifestyle, diet plays an important role in growth and development as a source of nutrition, but the composition of the diet decides its nutritional status. The modern diet, especially in Western countries, is rich in carbohydrates such as fructose and sucrose as well as saturated fat. This increased caloric intake affects multiple metabolic functions and has been associated with a higher incidence of the metabolic syndrome [1]. Excess weight and obesity are associated with hemodynamic, structural and histological renal and liver changes, in addition to metabolic and biochemical alterations that lead to kidney disease and liver injury [2].

Combinations of carbohydrate and fat-rich dietary components have been used in rodents to mimic these

signs and symptoms of human metabolic syndrome and his association with renal and liver damage [3]. *Hippocratea excelsa* HBK. (*Hippocrateaceae*) (syn.: *Hemiangium excelsum* HBK) is a liana native to Mexico and Central America. The root bark of this plant, known as "Cancerina", is used in the Mexican traditional medicine for the treatment of peptic ulcers, gastrointestinal infections, skin ailments, kidney disease, menstruation disorders and as antihypertensive [4]. Root bark of *H. excelsa* has been widely studied in México for its anti-inflammatory, antiparasite and *in vitro* anti-tumour effects [5]. *H. excelsa*, as anti-inflammatory agent produced a significant inhibition of carrageenan-induced paw oedema and reduced the weight of cotton pellet-induced granuloma at doses of 25 - 100 mg/kg bw [5]. *H. excelsa*, for its anti-tumour effect, was used in bioscreening studies to detect the cytotoxic activity against human tumour cells in three different extracts (petroleum ether, ethylacetate and methanol) [5]. In the present study, we examined the effect of *H. excelsa* administration on liver damage and kidney disease, in a high fructose induced metabolic syndrome rat model.

Materials and Methods

Preparation and identification of the ethanol extract of H. excelsa
The root bark of *H. excelsa* was collected on April 2016 at Costa Grande, Guerrero, Mexico and authenticated by Edith López Villafranco, biologist. A voucher specimen (2483) has been deposited at the Herbarium of the Botany Department of the Faculty of Superior Studies Iztacala, National Autonomous University of Mexico (UNAM).
For the *in vivo* evaluation, powered *H. excelsa* root bark (3 kg) was extracted twice by maceration with ethanol (30:l v/w) at room temperature for 72 h, filtered and evaporated *in vacuo* (50°C). The dry ethanol extract was stored at 4°C. The yield of obtaining the ethanol extract of *H. excelsa* (HE) was 6.5%.
Phytochemical profiling
For the chromatographic analysis of HE it was used a high-resolution liquid chromatograph Hewlett Packard Mod. 1100, equipped with an automatic injector (Agilent Technologies Mod. 1200), a diode array detector (Hewlett Packard Mod. 1100) and a quaternary pump HP Mod. 1100.
Chromatography for the analysis of phenolic acids in HE was performed on a nucleosil 100A 125 x 4 mm column, adjusted to 30°, using a linear gradient of 1 mL/min of water (pH 2.5 with trifluoroacetic acid) (Solution A) and acetonitrile (solution B). Initially, (0 to 0.1 min) 85% solution A and 15% solution B, (0.1 to 20 min) 65% solution A and 35% solution B and (20 to 23 min) 65% solution A and 35% solution B; injection volume: 20 µL; the phenolic acids were detected at 280 nm.

For the flavonoids in HE, the chromatography was performed on a Hypersil ODS 100A column of 123 × 4.0 mm, adjusted to 30°. The system was operated with gradient elution with solution A: water (pH 2.5) with trifluoroacetic acid and solution B: acetonitrile, with a linear gradient of 1 mL/min. Initially, (0 to 0.1 min) 85% solution A and 15% solution B, (0.1 to 20 min) 65% solution A and 35% solution B and (20 to 25 min) 65% solution A and 35% solution B; injection volume: 20 µL; flavonoids were detected at 254, 316 and 365 nm.
The terpenoid analysis was performed with a ZORBAX Eclipse XDB-C8 column (4 mm × 125 mm, 5 µm). The major constituents were separated with gradient mobile phase; and the flow was adjusted to 1 mL/min for 21 min; that consists of water 20% and acetonitrile 80%; the detection wavelength of 215 and 220 nm; 20 µL injection volume.
In vitro antioxidant capacity
Determination of total phenolic content (TPC). The determination of TPC of the ethanol extract of *H. excelsa* was performed by Folin-Ciocalteu method with little modifications, using gallic acid as a standard phenolic compound [6]. The extract was diluted with distilled water to a known concentration in order to obtain the readings within the standard curve range of 0.0 to 600.0 µg of gallic acid/mL. A volume of 250 µL of diluted extract or gallic acid solution was mixed with 1 mL of distilled water in a test tube followed by the addition of 250 µL of Folin-Ciocalteu reagent. The samples were mixed and then allowed to stand for 5 min at room temperature in order to allow complete reaction with Folin-Ciocalteu reagent. Then 2.5 mL of 7% sodium carbonate aqueous solution was added and the final volume was made up to 6 mL with distilled water. After incubating the samples for 90 min at room temperature, the absorbance of the resulting blue colour solution was measured at 760 nm using a spectrophotometer. The result was expressed as mg of gallic acid equivalents (GAE)/g extract by using an equation that was obtained from standard gallic acid curve. All the experiment was conducted in triplicate.
DPPH radical scavenging assay. The DPPH assay was carried out as described by Hsu *et al.* with some modifications [7]. A volume of 1.5 mL of 0.1 mmol/L DPPH solution was mixed with 1.5 mL of various concentrations (10 to 500 µg/mL) of bark extract. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm by a spectrophotometer. The solution with DPPH and methanol was used as negative control. The experiment was replicated in three independent assays. Quercetin was used as positive control. Inhibition of DPPH free radical in percentage was calculated by the formula:

DPPH radical scavenging activity (%) = (A$_{control}$ - A$_{test}$)/A$_{control}$ × 100,

where, A$_{control}$ is the absorbance of the negative control and A$_{test}$ is the absorbance of samples. The antioxidant activity of each sample was expressed in terms of IC$_{50}$ (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the graph after plotting inhibition percentage against the extract concentration.

ABTS radical scavenging assay. In order to assess the ABTS radical scavenging assay, the method of Re *et al.* was adapted [8]. The stock solutions included 7 mmol/L ABTS solution and 2.4 mmol/L potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The resulting solution was then diluted by mixing 1 mL of freshly prepared ABTS solution to obtain an absorbance of (0.706 ± 0.001) units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. The plant extract (1 mL) was allowed to react with 2.5 mL of the ABTS solution and the absorbance was registered at 734 nm after 7 min using a spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of Trolox and the percentage inhibition was calculated as:

ABTS radical scavenging activity (%) = (A$_{control}$ - A$_{test}$)/A$_{control}$ × 100,

where A$_{control}$ is the absorbance of ABTS radical + methanol; A$_{test}$ is the absorbance of ABTS radical + sample extract/standard.

Reducing Power Assay (FRAP). For FRAP (ferric reducing antioxidant power) assay, extract/fraction solution (0.1 mL) was added to reagent (2 mL) in acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (10 mM) in 40 mM HCl and ferric chloride (20 mM) in a final ratio of 10:1:1 (v/v/v). Then, the absorbance at 593 nm was read after 30 min of incubation at room temperature. Similarly, a blank sample (prepared in the same manner, but without the extract) was prepared. Millimoles of Trolox equivalents per gram of ethanolic extract *H. excelsa* (TEs/g extract) were the measurement unit [9].

Ethical consideration and animals used
The study was submitted to the Animal Use Ethics Committee of Faculty of Superior Studies Iztacala, UNAM. It was approved under Protocol No. CE/FESI/102016/1110. The handling of the laboratory animals followed the rules for the Care and use of laboratory animals of the Official Mexican Rule (NOM-062-ZOO-1999, revised in 2001); the International Guide for Caring and Use of Laboratory Animals NRC 2002; all procedures and experimental protocols are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Thirty male Wistar rats were used, each with a weight of around 200 - 250 g. During the study, the animals were housed in individual stainless steel metabolic cages, measuring 60 cm × 50 cm × 22 cm. They were kept in an air-conditioned environment, with a temperature of 25 ± 3°C, and a humidity of 50 ± 10%, a photoperiod of 12 h of light and dark, and they were fed with standard balanced food ratios for rodents and water *ad libitum.*

Induction of metabolic syndrome
The control diet (2018s Teklad Global 18% protein rodent diet from Harlan Laboratories) contained proteins (18.6%), carbohydrates (44.2%) and fat (6.2%). Chow and drinking water with 20% fructose were elaborated [10]. Rats were initially divided into two groups: control group (n = 6) and fructose feed (F) group (n = 24), and treated for 12 weeks under the next conditions: the control group with regular chow and drinking water, and the fructose fed group with 20% fructose in chow and drinking water.

Experimental design
After 12 weeks of fructose treatment, the rats were randomly divided into four groups (n = 6), were maintained under initial diet conditions and treatments, and were orally administrated for 6 weeks, as follows: Control group; Metabolic syndrome (F); Metabolic syndrome treated with losartan 10 mg/kg bw (F + Los); Metabolic syndrome group treated with vitamin E 500 mg/kg bw (F + Vit E); Metabolic syndrome treated with ethanol extract of *H. excelsa* (HE): 30 mg/kg bw and 100 mg/kg bw (F + HE 30 and F + HE 100). The HE doses used were based on the toxicity study, the lowest dose that did not present toxic effect (30 mg/kg bw and 100 mg/kg bw) were used.

After 6 weeks of treatment, rats were kept in metabolic cages, for evaluating water intake, food intake, and urinary volume at 24 hours; urine samples were used for protein concentration measurement by Bradford method (Bio-Rad) [11].

Blood pressure
Systolic arterial blood pressure (SBP) was measured noninvasively using a tail-cuff computer-aided monitoring device (Automatic Blood Pressure Computer, Model LE 5007; Letica Scientific Instruments, Barcelona, Spain) using the procedures described [12], at the beginning (0 week), middle (12 weeks) and end (6 weeks) of the experiment.

Biochemical analyses
Blood concentrations of glucose, total cholesterol and triglycerides were measured using an Accutrend Sensor glucometer (Roche), at the beginning (0 week), middle (12 weeks) and end (6 weeks) of the experiment.
On the 6th week of treatment, the blood was collected (3 mL) for the biochemical assessment. High-density lipoprotein (HDL) (Spinreact, Cat. 1001097) and LDLc (Spinreact, Cat. 41023) cholesterol levels, aspartate aminotransferase (AST) (Spinreact, Cat. 12531) and alanine aminotransferase (ALT) (Spinreact, Cat. 12533)

were measured using commercially available kits following the manufacturer´s protocol.

Very-low-density lipoprotein cholesterol (VLDLc) was calculated using the formula: VLDLc = 0.2 x TAG. Cardiac index was calculated as TC/HDLc. Atherogenic index was calculated TC-HDLc/HDLc and coronary artery index was calculated as LDLc/HDLc [13].

The plasma concentration of angiotensin II, angiotensin (1-7), nitric oxide and endothelin were measured by capillary zone electrophoresis. Plasma was deproteinized with methanol 10:1 (v:v) and centrifuged at 16,000 x g for 10 min at 4°C (Sorvall RC-28S, rotor SS34; DuPont, Newtown, CT, USA). The pellet was discarded, and supernatant was deproteinized by the addition of 20% trichloroacetic acid, homogenized and centrifuged at 16, 000 x g for 10 min at 4°C. The supernatant was filtered through a 0.22 μm nitrocellulose membrane filter (Millipore, Billerica, MA, USA) and diluted 1:10 with 0.1 M NaOH. The sample (2 mL) was then passed through a Sep-Pak Classic C-18 cartridge (Waters Corporation, Milford, MA, USA) as described by [10, 11]. These experiments were performed using a Beckman Coulter (Fullerton, CA, USA) P/ACE^{TM} MDQ Capillary Electrophoresis System equipped with PDA and controlled by means of the P/ACE MDQ Capillary Electrophoresis System software (version 7.0; Beckman Coulter Inc., Fullerton, CA, USA) [14, 15].

Histopathological analysis

At the end of the treatments, animals were weighted and anesthetized with sodium pentobarbital (45 mg/kg bw, intraperitoneally). The mass of each organ and tissue was measured: kidneys, retroperitoneal adipose tissue and omental adipose tissue. The histo-pathological analysis of the organs was realized following the technique previously described [16], kidneys and livers were placed in paraformaldehyde 4%, were dehydrated through ethanol graded series, embedded in paraffin, sectioned in 5 μm thick slices, mounted on glass slides and stained with haematoxylin-eosin. Sections of renal cortex further subjected to morphometric analysis 10 adjacent non-overlapping fields from each group were randomly chosen and examined by the light microscope (Leica DMD 108) using a magnification of 40X.

Western blotting assessment

The kidneys were perfused and rapidly removed. The cortex was isolated before western blotting and enzyme activity measurements. The renal tissue was homogenized in 100 mM Tris (hydroxymethyl-aminomethane-tris-hydrochloride, Sigma, St Louis, MO, USA), pH 7.4, incubated with a protease-inhibitor cocktail (Complete Mini, EDTA-free protease inhibitor cocktail, Roche, Germany) and centrifuged at 10,000 x g for 10 min to remove insoluble debris. Aliquots containing 80 μg of protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted

to polyvinylidene difluoride membranes. Coloured molecular weight standards (GE Healthcare, Piscataway, NJ, USA) were run simultaneously. Membranes were blocked for 2 h in 5% (w/v) non-fat milk and incubated overnight in the presence of the corresponding anti-bodies (rabbit polyclonal antibody to AT_1R, mouse monoclonal antibodies to transforming growth factor beta 1 (TGF-β_1) and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA)) (1:1000 dilution) in 5% (w/v) BSA in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20, at 4°C. After incubation for 2 h at room temperature in the presence of the corresponding horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) (1:1000 dilution). Complexes were visualized by chemiluminescence detection. Films were scanned, and densitometric analysis was performed using the software Multi Gauge, Fuji Film Science, Lab2003 (Fuji Photo Film Co., LTD).

Evaluation of oxidative stress

Renal and liver tissue catalase (CAT) activity was assayed at 25ºC, method which is based on the disappearance of H_2O_2 from a solution containing 30 mmol/L H_2O_2 in 10 mmol/L potassium phosphate buffer (pH 7) at 240 nm [17]. The glutathione peroxidase (GPx) activity was assayed by a previously described method [18]. Results were expressed as UI/mg protein. Superoxide dismutase (SOD) activity in renal cortical homogenates was measured by a competitive inhibition assay using xanthine–xanthine oxidase system to reduce NBT [19]. Results were expressed as UI/mg protein.

Statistical Analysis

The data represent the mean ± SEM from 6 rats per treatment. All statistical analyses were performed using GraphPad Prism 5.00 (GraphPad Software, La Jolla, California, USA). C, F, F + Los, F + Vit E, F + HE 30 and F + HE 100 groups were tested for effects of diet, treatment, and their interactions by two-factor analysis of variance (ANOVA). When the interaction and/or the main effects were significant, means were compared using Tukey´s multiple comparison *post hoc* test.

Results and Discussion

MS is a progressive health disorder associated with different risk factors, including hyperglycaemia, dyslipidaemia, hypertension and obesity, and that predisposes to cardio-renal dysfunction [20-22]. The chemical composition of the extract of *Hippocratea excelsa* is presented in Table I. Fructose is a highly lipogenic sugar [22]; the administration of 20% fructose to rats for 12 weeks induced the classic symptoms of MS; blood tri-glycerides (TGs), body weight gain, body mass index and abdominal circumference increased, correlated with the increase of weight of total abdominal adipose

tissue (mesentery, retroperitoneal and epididymal fat) compared to animals with normal diet (Table II and III). Hence, we have used this fructose induced metabolic syndrome rat model [10] to investigate whether the oral administration of ethanol extract of *H. excelsa* (HE) for 6 weeks can reverse the alterations in liver and renal parameters.

Table I

Phytochemical constituents of the ethanol extract of *Hippocratea excelsa*

Retention time (min)	Area (mAU * s)	Identification	Quantification (%)
2.502	85.00604	gallic acid	0.045
4.420	24.63361	chlorogenic acid	0.16
5.491	683.4551	vanillinic acid	0.11
6.703	500.3157	caffeic acid	0.22
9.416	28.79682	ferulic acid	0.05
10.020	43.70384	p-cumaric	0.03
4.260	6500.446	oleanolic acid	1.6
2.539	127.2414	ursolic acid	0.26
6.386	110.4542	α-amyrin	9.3
6.718	23.818	phloridzin	0.017
12.275	9.0686	naringenin	0.015
21.428	26.4578	galangin	0.013

Table II

Effect of fructose feeding over body, plasma, and urinary parameters

Variables	Control	Fructose
Initial body weight (g)	231 ± 4.37	225 ± 2.99
Body weight at 12 weeks (g)	398 ± 19	436 ± 10 *
Body weight gained (1 - 12 weeks) (%)	7.2 ± 1.74	9.4 ± 1.2
Body mass index (g/cm^3)	0.89 ± 0.04	0.88 ± 0.05
Abdominal circumference (cm)	17 ± 0.58	22 ± 0.085 *
Lee index	0.28 ± 0.01	0.34 ± 0.01 *
Plasma glucose (mmol/L)	4.64 ± 0.48	4.89 ± 0.24
Plasma triglycerides (mmol/L)	1.044 ± 0.25	2.72 ± 0.46 *
Plasma cholesterol (mmol/L)	2.6 ± 4	2.7 ± 5
Urinary volume (mL)	7 ± 2	27 ± 6 *
Food intake (g/day)	20 ± 2	48 ± 3 *
Water intake (mL/day)	35 ± 9	79 ± 13 *
Urine protein excretion (mg/24 h)	27 ± 4	115 ± 24 *
Systolic blood pressure (mmHg)	116 ± 15	140 ± 5 *

Mean $\pm$ SEM; n = 6 for control group and n = 30 for fructose fed group. Statistically significant compared with the control group; * = p < 0.05
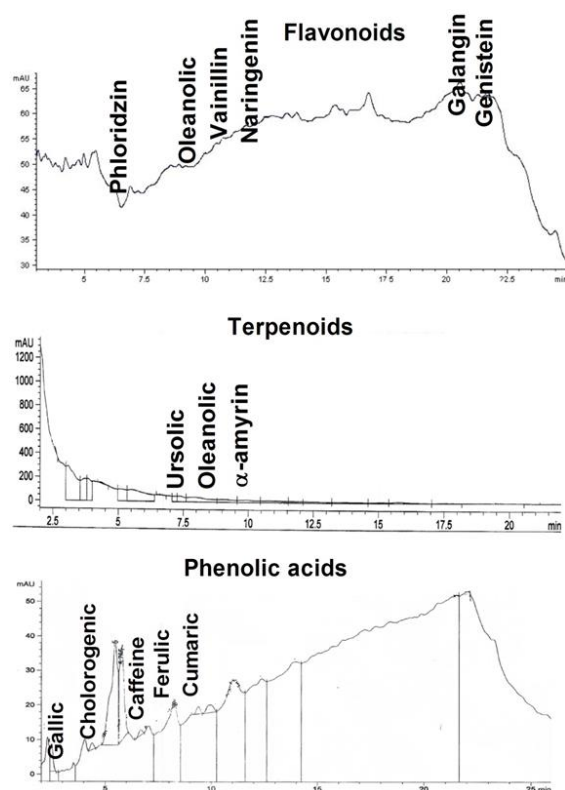
Table III

Effect of HE treatment on metabolic variables in fructose fed rats

Variables	Control	F	Los	Vit E	HE 30	HE 100
Food intake (g/day)	42 ± 4	14 ± 5	15 ± 2	15 ± 4	16 ± 4	18 ± 4
Water intake (mL/day)	42 ± 4	70 ± 5	66 ± 8	87 ± 8	69 ± 9	76 ± 10
Body weight at 18 weeks (g)	434 ± 20	572 ± 19 *	505 ± 16	491 ± 31	522 ± 16	551 ± 16 *
Body weight gained (12 - 18 weeks) (%)	14 ± 3	30 ± 2 *	15 ± 2 $^{\&}$	16 ± 2 &	16 ± 2 &	19 ± 2 &
Visceral adiposity index (%)	2.53 ± 0.46	4.93 ± 0.57 *	3.95 ± 0.44 *	4.37 ± 0.68	4.72 ± 1.13	3.86 ± 0.30 *
Body mass index (g/cm^3)	0.73 ± 0.02	1.05 ± 0.11 *	0.76 ± 0.03 &	0.74 ± 0.05 &	0.75 ± 0.02 &	0.77 ± 0.02 &
Abdominal circumference (cm)	19 ± 0.37	22 ± 0.6 *	20 ± 0.32	19 ± 0.5	20 ± 0.48	21 ± 0.48
Lee index	0.31 ± 0.01	0.36 ± 0.02 *	0.31 ± 0.01 &	0.30 ± 0.008 &	0.30 ± 0.003 &	0.30 ± 0.004 &
Tissue wet weight (mg/mm)						
Retroperitoneal adipose tissue	152 ± 32	326 ± 47 *	135 ± 20 &	196 ± 39 &	148 ± 12 &	205 ± 45 &
Omental adipose tissue	144 ± 34	255 ± 40 *	184 ± 47	138 ± 23 &	150 ± 27 &	188 ± 67
Plasma glucose (mmol/L)	4.42 ± 0.30	5.25 ± 0.23	5.55 ± 0.24	5.06 ± 0.12	5.07 ± 0.04	5.6 ± 0.22
Plasma triglycerides (mmol/L)	1.18 ± 0.19	2.62 ± 0.58 *	3.4 ± 0.28 *	2.53 ± 0.34 *	2.57 ± 0.12 *	3.17 ± 0.55 *
Plasma cholesterol (mmol/L)	2.0 ± 3.7	2.0 ± 4	2.34 ± 6	2.5 ± 5	2.2 ± 4	2.3 ± 8
AST (UI/L)	6.4 ± 3	26 ± 2 *	18 ± 3 &	14 ± 2 &	16 ± 3 &	8.6 ± 2 &
ALT (UI/L)	9.3 ± 2	37 ± 3 *	25 ± 4 &	19 ± 4 &	21 ± 3 &	17 ± 3 &

F = fructose fed rats; Los = F + losartan 10 mg/kg bw; Vit E = F + vitamin E 500 mg/kg bw; HE 30 = F + HE 30 mg/kg bw; HE 100 = F + HE 100 m/kg bw; n = 6; * = p < 0.05 control *vs*. treatment, & = p < 0.05 fructose fed rats *vs*. treatment

The fructose fed groups treated with losartan, vitamin E and HE showed lower Lee index, blood triglycerides, weight of retroperitoneal adipose tissue and omental adipose tissue compared with fructose fed animals (Table III). Losartan treatment significantly elevated the serum concentrations of total adiponectin in patients with essential hypertension [23]. In rats, losartan reduced leptin concentration in both losartan and high fat diet and losartan groups [24]. Leptin is released from adipose tissues into the blood stream and regulates appetite, feeding and energy expenditure [24]. Liver functions were evaluated in the rat by determining the serum concentrations of ALT and AST. Activities of AST and ALT are most commonly used as biochemical markers for liver damage. Since these enzymes are cytoplasmic in nature, upon liver injury these enzymes enter into the circulatory system due to altered permeability of membrane [25]. As shown in Table III, serum levels of AST and ALT were significantly increased after 18-weeks of high fructose feeding (p < 0.05) [26]; HE significantly prevented high fructose induced elevation of AST and ALT, indicating the hepatoprotective activity of HE. Oral treatment with α-amyrin (20 mg/kg bw), a pentacyclic triterpenoid that is a component of HE, attenuated the increase of AST and ALT enzymes activities in a rat model of CCl4-induced hepatic oxidative stress and a subsequent recovery towards normalization of these enzymes [27].

Figure 1.
Chromatographic profile of root bark ethanol extract of *H. excelsa*

The chromatographic profile of HE revealed the presence of several phytoconstituents (Figure 1 and Table I): α-amyrin, oleanolic acid, ursolic acid, caffeic acid, and chlorogenic acid. The components of HE: α-amyrin and oleanolic acid, cause the effects attributed to *H. excelsa*. Alpha-amyrin treatment prevented the increase in blood triglycerides [28], weight of retroperitoneal adipose tissue and omental adipose tissue [29], observed effects in HE treated groups in this work (Table III).
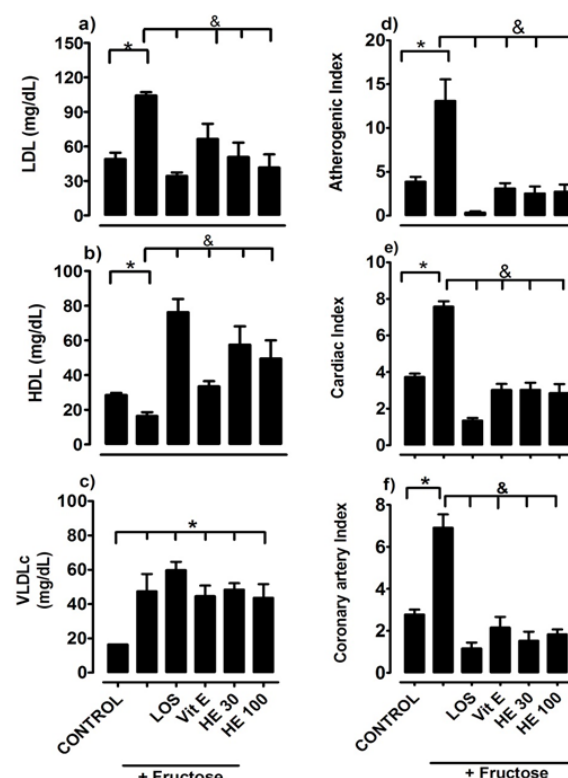
Figure 2.
Lipid profile: (a) LDL, (b) HDL, (c) VLDL and (d) atherogenic, (e) cardiac and (f) coronary artery indexes in rats with fructose induced metabolic syndrome after six weeks of administration of ethanol extract of *H. excelsa* (HE)

* = p < 0.05 control *vs.* treatment; & = p < 0.05 MetS *vs.* treatment: Control, fructose fed rats (F), F + losartan (Los), F + vitamin E (Vit E), F + HE 30 mg/kg bw (HE 30) and F + HE 100 mg/kg bw (HE 100)

Impact of treatment with HE on lipid profile
Dietary fructose in the liver is rapidly taken up by the liver, where it can be converted to glycerol-3-phosphate, favouring the esterification of unbound fatty acids to form TGs [30]. Hypertriglyceridemia occurs following 12 weeks of consumption of fructose, marked by elevated levels of plasma triglycerides; VLDLc and LDLc in rats with MS increased significantly (p < 0.05) compared with the control (Figures 2a and 2c). Conversely, the HDLc significantly lowered (Figure 2b); these alterations are features of atherosclerosis and cardiovascular disease [31]. These changes

in rats with MS were attenuated by HE compared with losartan and vitamin E treated groups (Figures 2a and 2c). Furthermore, calculated atherogenic, cardiac and coronary artery indexes were increased in rats with MS compared with the control (Figure 2d); administration of losartan, vitamin E and HE in fructose fed rats, reversed the increases in these indexes (Figures 2d, 2e and 2f). The attenuation of decrease of HDLc by HE (Figure 2b) shows its ability to prevent the development of atherosclerosis [32]. Oleanolic acid reduced serum triglycerides, total cholesterol, and LDL cholesterol [33]. Chlorogenic acid reduced total cholesterol and LDL-cholesterol, increased HDL cholesterol, and improved both the atherogenic index and the cardiac risk factor, to inhibit fatty acid synthase and hydroxyl methyl glutaryl coenzyme A reductase [34]. Alpha-amyrin reduced serum triglycerides, total cholesterol, LDL-cholesterol, atherogenic index, and increased HDL cholesterol [28].

Antihypertensive effect of HE

In the fructose-fed rats, the systolic blood pressure (SBP) was increased compared with the control (148 ± 3 mmHg for MS group compared with 106 ± 2 mmHg in control group); this increase was prevented by losartan (115 ± 3 mmHg) and HE 30 mg/kg bw (119 ± 3 mmHg). Increase in SBP after chronic fructose feeding

was partially abolished with HE 100 mg/kg bw treatment (130 ± 6 mmHg) (Figure 3).
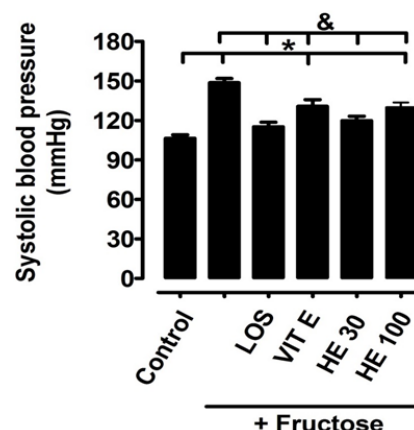
Figure 3.

Effect of the ethanol extract of *H. excelsa* (HE) on systolic blood pressure of fructose fed rats (F) 6 weeks after the establishment of metabolic syndrome

Values are the mean ± SEM (n = 6); * = p < 0.05 control *vs*. treatment; & = p < 0.05 F *vs*. treatment: F + losartan (Los), F + vitamin E (Vit E), F + ethanolic extract of *H. excelsa* (HE) 30 mg/kg bw (HE 30) and F + ethanolic extract of *H. excelsa* 100
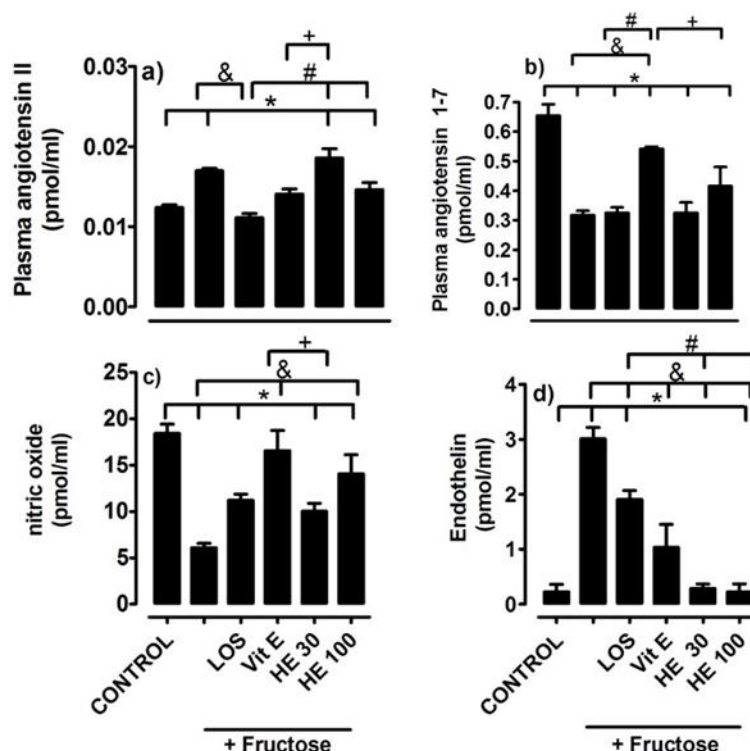
Figure 4.

Effect of ethanol extract of *H. excelsa* (HE) on the plasma level of (a) angiotensin II, (b) angiotensin 1-7, (c) nitric oxide and (d) endothelin in fructose fed rats (F)

All values are represented as mean ± SEM; n = 6; * = p < 0.05 control *vs*. treatment; & = p < 0.05 F *vs*. treatment: F + losartan (Los); # = p < 0.05 Los *vs*. treatment; + = p < 0.05 Vitamin E (Vit E) *vs*. treatment, F + HE 30 and HE 100

The administration of fructose decreased the systemic synthesis of vasodilator NO and Ang 1-7 with an increase of vasoconstrictor endothelin 1 (Figures 4a - 4c); this effect was associated with increase in the

SBP at 12 weeks of fructose fed, as has been shown by other authors [10, 35]. The compression of kidney by the adipose tissue around it causes activation of the RAS [36]. The activation of RAS causes retention of sodium and water by angiotensin II and leads to the development of hypertension. The pharmacological inhibition of RAS reduced blood pressure to about 50% to 60% (Figure 3) [32-35, 37]. The administration of HE prevented the increase of SBP induced by administration of fructose, so the release of angiotensin 1-7 and release of NO seems to be one mechanism of action in the anti-hypertensive effect of HE (Figure 4d).

Histopathological study

In this study, we found that fructose feeding conducted to kidney hypercellularity (gh), which is an indicator of proliferative glomerulonephritis associated with degenerative changes, atrophy characterized by decrease in kidney size, number of renal corpuscles *per* field and thickness of the cortex, necrosis, thyroidization and protein deposits located in the proximal convoluted tubule (TCP) and in the space of the Bowman's capsule (Figures 5a and 5d).

Pathologically, kidney damage is characterized by a number of structural changes of kidney cells including a decreased GFR that can lead to the development of glomerulosclerosis and tubulointerstitial fibrosis [38, 39]. Distortion in the architecture of the cortex and medulla and the significant reduction of the glomerulus diameter suggest sclerosis in the MS of the current study. All these events observed in fructose fed rats were partially ameliorated by treatment with HE in doses of 30 and 100 mg/kg bw and renal corpuscles showed a diffuse mild hypercellularity (Figures 6e and 6f).
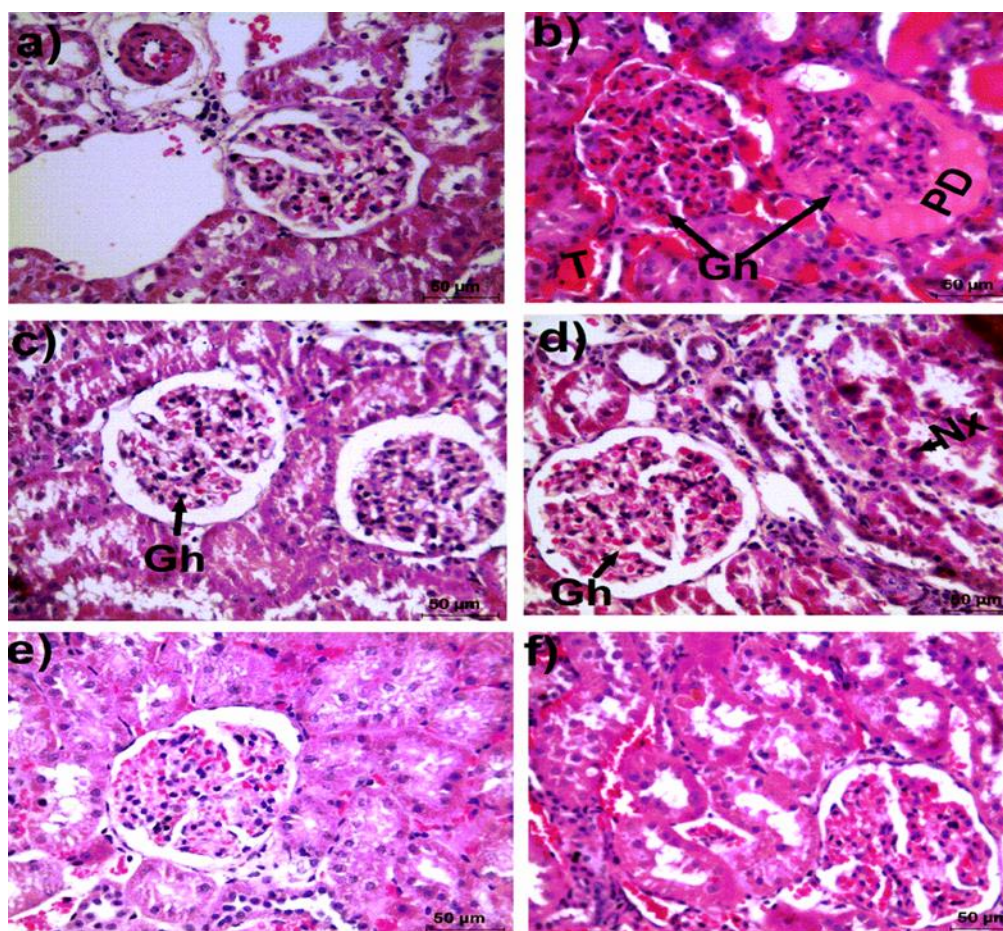
Figure 5.
Photomicrographs (40X magnification) showing histopathological changes in different groups, (a) control. Note that the group treated with fructose (b) is the one that presents the most notorious changes, in this group, the changes were diagnosed as moderate diffuse extension proliferative glomerulonephritis, hypercellularity (Gh), protein deposits (pd) in the Bowman capsule space, obliterated capillary lumen. In the rest of the groups, lighter and multifocal changes were identified. (c) In F + losartan treated group glomerulus only presents hypercellularity (Gh) and the capillary lumen is not obliterated. (d) In F + vitamin E treated group, glomeruli present hypercellularity (GH), multifocal extension. (e) and (f) In F + ethanolic extract of *Hippocratea excelsa* (HE) treated group, the kidney has an almost normal appearance
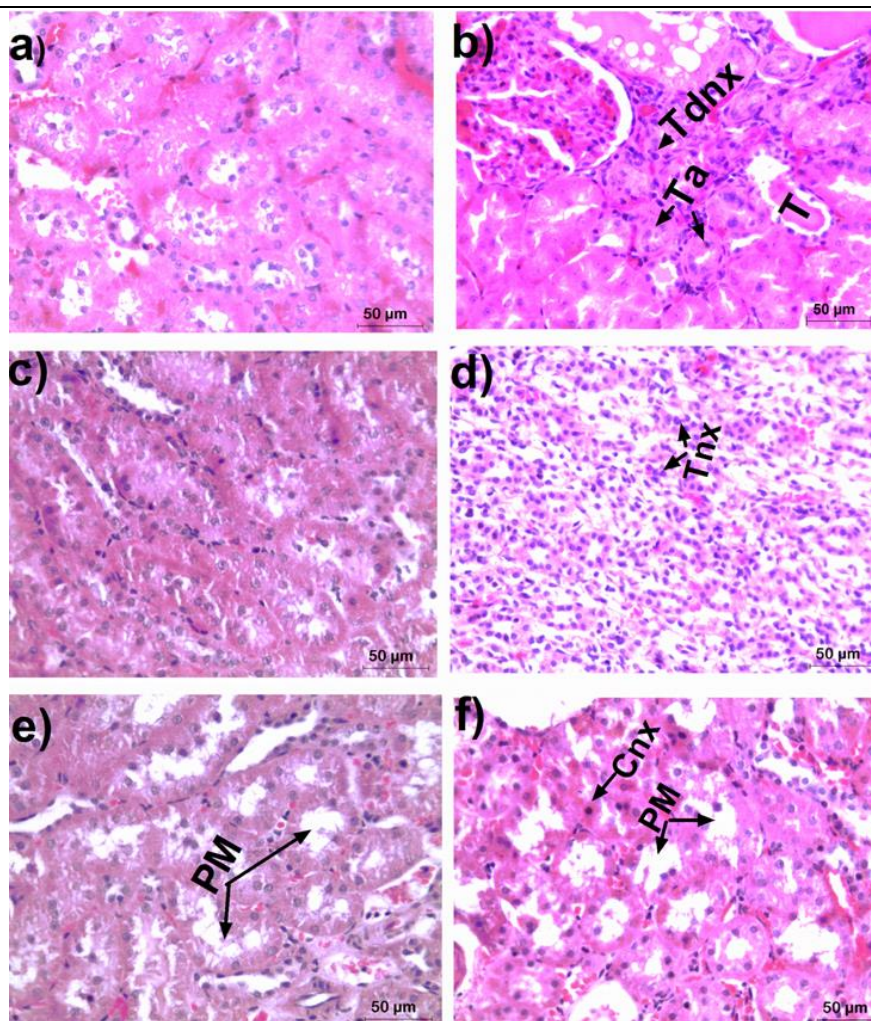
Figure 6.
Photomicrographs (40X magnification) of contoured tubules in the different groups. Control group (a). Note that in the group treated with fructose (b), the most affected tubules correspond to the distal tubules (TCDnx), in them we find multifocal changes that consist of dilatation that is due to thyroidization, tubular atrophy (Ta) and necrosis. In the group treated with F + vitamin E (d), tubules with degenerative and necrotic changes of multifocal distribution are identified. In the fructose groups treated with losartan (c), HE 30 and HE 100 (e and f), the tubules only show the loss of microvilli, but the tubular arrangement and cellular vitality is maintained (Cn); in them, only some cells suggest necrosis (Cn)

At the hepatic level, the MS group exhibited cellular degeneration, massive fatty changes, cytoplasmic vacuolation and the loss of cellular boundaries (Figure 7b). The liver displayed near normal appearance with well-preserved cytoplasm and prominent nuclei (Figures 7e and 7f); renoprotective and hepatoprotective effect of HE was demonstrated in an experimental model of metabolic syndrome on rats.

We found that the fructose-treated rats showed renal dysfunctions such as reduced kidney weight, diminished number of renal corpuscles *per* field and proteinuria (Table IV).

Table IV

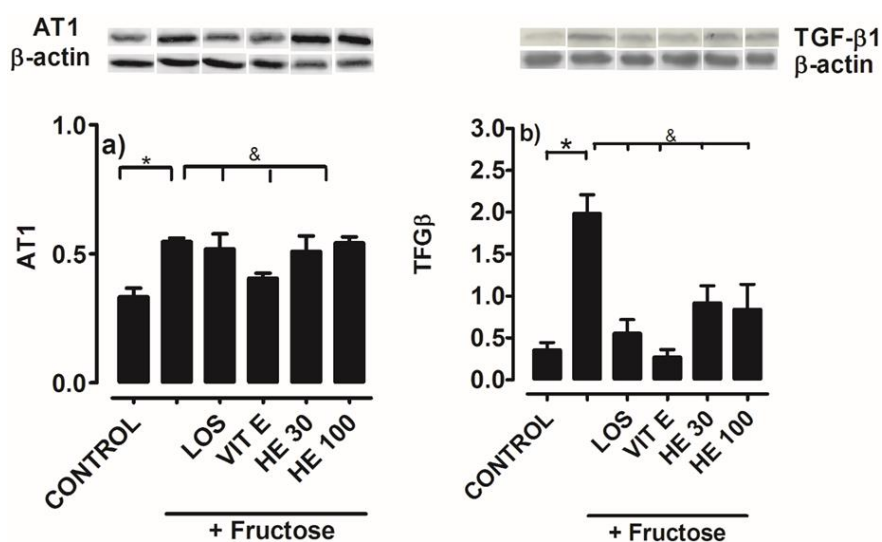Effect of ethanol extract of *H. excelsa* (HE) over renal parameters in fructose fed rats

Variables	Control	F	Los	Vit E	HE 30	HE 100
Kidney weight (g)	1.32 ± 0.035	1.21 ± 0.03 *	1.45 ± 0.04	1.3 ± 0.07	1.32 ± 0.024	1.28 ± 0.03
Rat body weight ratio (mg/g)	2.79 ± 0.10	2 ± 0.086 *	2.74 ± 0.15 &	2.59 ± 0.17 &	2.54 ± 0.09 &	2.38 ± 0.04 &
Thickness of the cortex (μm)	2721 ± 50	2185 ± 90 *	2355 ± 22 &	1946 ± 103 *	2415 ± 39 &	2453 ± 33 &
Number of renal corpuscles *per* field (10X)	9 ± 0.4	5.7 ± 1.0 *	7.5 ± 0.85 &	4.5 ± 0.5 *	8 ± 1.4 &	7.5 ± 0.95 &
Proteinuria (mg/24 h)	17 ± 6	89 ± 14 *	32 ± 2 &	31 ± 4 &	37 ± 8 &	31 ± 5 &

F = rats with fructose induced metabolic syndrome; Los = F + losartan; Vit E = F + vitamin E; HE 30 = F + HE 30; HE 100 = F + HE 100; n = 6; * = $p < 0.05$ control *vs.* treatment; & = $p < 0.05$ F *vs.* treatment
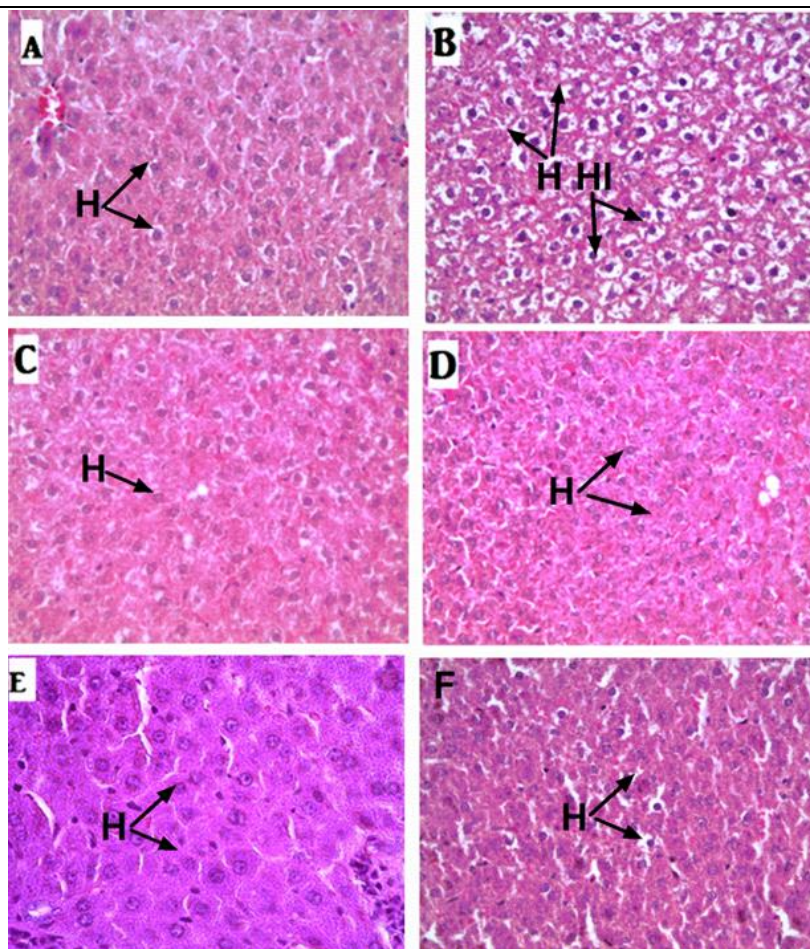
Figure 7.
Representative haematoxylin and eosin (H&E) staining photos of liver tissue (40X magnification) showing histopathological changes in different groups: (A) control; (B) metabolic syndrome (F); (C) F + losartan treated group, (D) In F + vitamin E treated group, (E and F) In F + ethanolic extract of *Hippocratea excelsa* (HE) treated group

Figure 8.
Effect of ethanol extract of *H. excelsa* (HE) on the expression of (a) AT1R and (b) TGF-β1 proteins in renal cortex of rats with fructose induced metabolic syndrome (F)
F = fructose; Los = F + losartan; Vit E = F + vitamin E; HE 30 = F + HE 30; HE 100 = F + HE 100
Values are expressed as mean ± SEM; n = 5; * = $p < 0.05$ control *vs.* treatment; & = $p < 0.05$ F *vs.* treatment

These findings are in line with previous studies demonstrating that high fructose resulted in proteinuria [13, 34]. The overexpression of TGF-β1 in the rat glomeruli induces proteinuria [40]. However, the parameters of kidney function in the fructose fed rats treated with *H. excelsa* were comparable to those of the control rats; these results demonstrate that *H. excelsa* slowed the progression of functional and structural damage to the kidney in fructose-fed rats. Inappropriate activation of renin-angiotensin aldosterone system (RAS) is a pathophysiologic factor in the link between hypertension and metabolic syndrome [41]; the profibrogenic cytokine TGF-β1 participates in kidney damage in high fructose induced MS [42-44]. In this study, it was found an elevated expression of AT1R and TGF-β1 in MS compared to the lean controls (Figures 8a and 8b). Conversely, HE treatment did not change the expression of AT1R, (Figure 8a) and decreased TGF-β1 protein expression (Figure 8b).

It has been shown that local RAS is significantly up-regulated during liver fibrosis where angiotensin II stimulates contraction and proliferation of the activated hepatic stellate cells and increases the expression of TGF-β1 through angiotensin II type 1 receptors [45]. The present results showed that losartan and extract of bark of *H. excelsa* treatments significantly alleviated the histological injury of liver, displaying near normal appearance with well-preserved cytoplasm and prominent nuclei (Figure 7); hepatoprotective effect of HE was demonstrated in an experimental model of metabolic syndrome on rats [46].

Antioxidant capacity in vitro

Other mechanisms involved in renal and liver damage are concerning oxidative stress. Fructose consumption increases levels of lipid peroxides and decreases activities of antioxidant enzymes in the kidney and liver [47, 48]. High fructose produces reactive oxygen species (ROS) *in vitro* and *in vivo*. In this study was determined the antioxidant activity *in vivo* and *in vitro* of the ethanol extract *H. excelsa*.

In the present study, *H. excelsa* bark extract possessed high phenolic contents (286.4 mg GAE/g of extract), was calculated using the standard curve of gallic acid (standard curve equation: $Y = 11.747x + 0.0262$, $R^2 = 0.998$). Other plant that showed relevant anti-oxidant and medicinal properties is *Buddleja cordata*; the methanol extract of *B. cordata* showed 177.13 ± 1.97 mgEq gallic acid/g, which presented 17.71% phenolic compounds in the extract; *B. cordata* showed antioxidant and neuroprotective effects in the 1-methyl-4-phenylpyridinium Parkinson disease rat model [49, 50].

DPPH radical scavenging assay

It is well known that the antioxidant activity of plant extracts containing polyphenol components is due to the capacity to be donors of hydrogen atoms or electrons and to capture the free radicals [51]. In the present study, *H. excelsa* ethanolic bark extract showed a significant effect in inhibiting DPPH, reaching up to 88% at concentration of 50 µg/mL, showing a dose response curve of DPPH radical scavenging activity of *H. excelsa* compared with standard quercetin. The IC_{50} value of *H. excelsa* extract was 18.05 µg/mL while the IC_{50} value of standard antioxidant quercetin was 5.3 µg/mL. The DPPH assay is one of the most widely used methods for screening the antioxidant activity of plant extracts. The antioxidant plant, *B. cordata* showed an IC_{50} value of 64.19 ± 2.09 µg/mL [51].

ABTS radical scavenging activity

The ethanolic bark extract of *H. excelsa* were fast and effective scavengers of the ABTS radical and this activity was comparable to that of BHT. It exhibited potent scavenging effects against ABTS with an IC_{50} value of 21.73 µg/mL almost equivalent to that of standard Trolox (IC_{50} value 5.3 µg/mL) [48]. The percentage of inhibition was 99% for the bark extract at 70 µg/mL concentration. Another root extract with antioxidant and hepatoprotective properties, the extract of *Pueraria thunbergiana* Benth. showed an IC_{50} value of 138.0 ± 2.7 µg/mL [53].

The reducing power of Fe^{2+} by the tested plant was evaluated. The radical scavenging activity of the plant extract showed a concentration-dependent reducing power of 379.23 µg/ET/g/extract, compared to standard Trolox [49].

Antioxidant enzymes

Oxidative stress is a well-recognized phenomenon playing an important role in the pathogenesis of endothelial dysfunction, hypertension, inflammation and atherosclerotic cardiovascular disease. It is defined as an impaired balance between free radical production and endogenous antioxidant capacity, resulting in the accumulation of oxidative products [13].

The SOD, catalase and GPx activities in renal cortex and liver were reduced in fructose fed rats compared with control group (Figures 9a and 10a); administration of the ethanol bark extract of *H. excelsa* effectively prevented the decrease of SOD, CAT, and GPx (p < 0.05, respectively).

These results indicated that ethanol extract of bark of *H. excelsa* exerted protective effects against kidney and hepatic injury induced by high fructose diet, at least in part, through decreasing oxidative stress (Figures 9b and 9c); (Figures 10b and 10c), through enhancing ROS-detoxifying enzymes, possibly by activating redox transcription factors as nuclear factor erythroid 2 (Nrf2), perhaps by effect of oleanolic acid [54]. It has been shown that oleanolic acid inhibited oxidative stress and activated heme oxygenase 1 (HO-1)/Nrf2 [54].
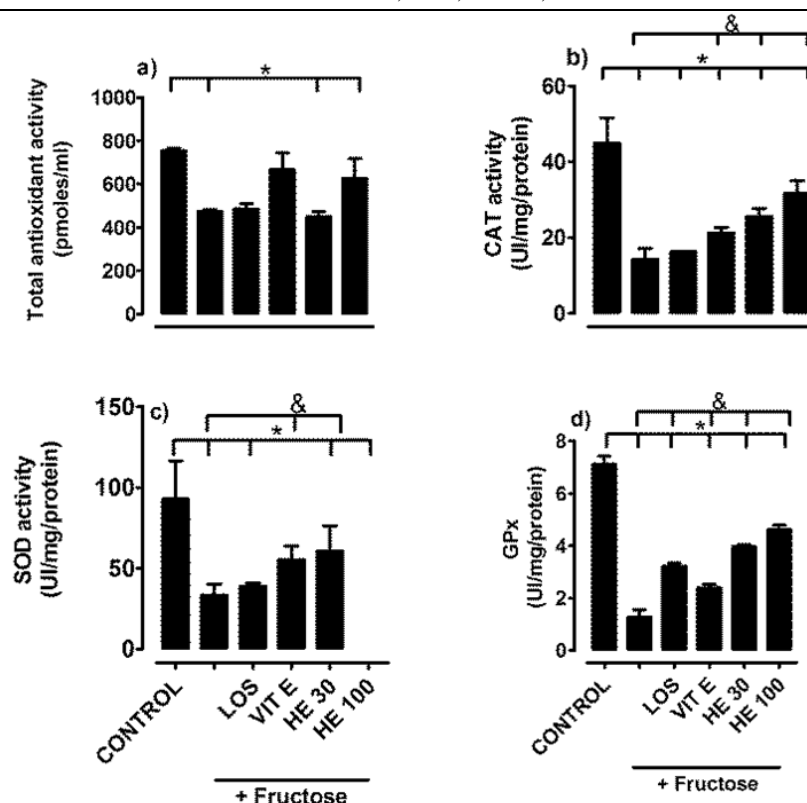
Figure 9.

Effects of ethanolic extract of *H. excelsa* (HE) on antioxidant enzymes in fructose induced metabolic syndrome rats (F). Plasma total antioxidant activity (a), renal cortical catalase (CAT) (b), superoxide dismutase (SOD) (c) and glutathione peroxidase activities (GPx) (d)

Los = F + losartan; Vit E = F + vitamin E; HE 30 = F + HE 30; HE 100 = F + HE 100

All values are represented as mean ± SEM; n = 5; * = p < 0.05 control *vs.* treatment; & = p < 0.05 F *vs.* treatment
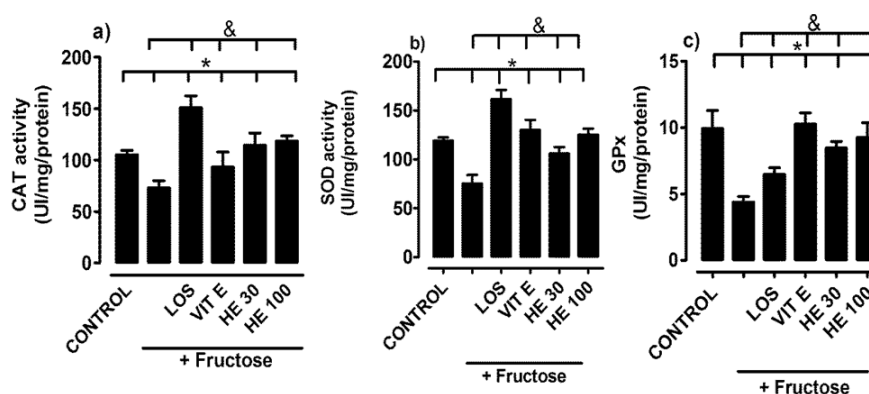

Figure 10.

Effects of ethanolic extract of *H. excelsa* (HE) on antioxidant enzymes in fructose induced metabolic syndrome rats (F). Hepatic catalase (CAT) (b), superoxide dismutase (SOD) (c) and glutathione peroxidase activities (GPx) (d)

Los = F + losartan; Vit E = F + vitamin E; HE 30 = F + HE 30; HE 100 = F + HE 100

All values are represented as mean ± SEM; n = 5; * = p < 0.05 control *vs.* treatment; & = p < 0.05 F *vs.* treatment

Conclusions

The ethanol extract of *H. excelsa* showed nephroprotective and hepatoprotective effects by decreasing of arterial hypertension, dyslipidaemia, proteinuria and the expression of TGF-β1.

Acknowledgement

Conflict of interest

The authors declare no conflict of interest.

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