Article

Effects of Hydroalcoholic Extract of *Celtis iguanaea* on Markers of Cardiovascular Diseases and Glucose Metabolism in Cholesterol-Fed Rats

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Abstract: *Celtis iguanaea* is popularly used in the treatment of diabetes mellitus. However, thorough chemical and pharmacological investigations regarding its activity are lacking. In this study, we investigated the effects of the hydroalcoholic extract from *C. iguanaea* (CI) on markers of cardiovascular diseases and the glucose metabolism in cholesterol-fed rats. Therefore, hypercholesterolemic rats (1% cholesterol) were orally treated with CI (150, 300, or 600 mg/kg) or simvastatin (4 mg/kg) (n = 6) once a day for 30 days along with a hypercholesterolemic diet. A control group (C) was given saline solution. CI showed significant decreases in serum levels of total cholesterol, LDL-cholesterol, HMG-CoA-reductase, IL-1, IL-6, TNF−α, and IFN−γ when compared to group C ($p < 0.05$). Hypoglycemic effects were observed along with a decrease of the activity of sucrase (CI 600), maltase (CI 150, CI 300), and an increase in muscle glycogen levels (CI 300). Antioxidant effects were observed in plasma, and the histopathological analysis showed a significant decrease in the liver fat area for CI compared to group C ($p < 0.001$). Our results suggest that the biological effects of CI could be related to the presence of flavonoids that possibly exert antioxidant, enzymatic inhibitory, and insulin-mimetic effects.

Keywords: medicinal plants; flavonoids; hypoglycemic; hypolipidemic; antiatherogenic.

1. Introduction

Cardiovascular diseases (CVD), a group of disorders of the heart and blood vessels, are considered the first cause of death globally, being responsible for more than 17 million deaths each year [1]. One of the major risk factors for the development of CVD is diabetes, a chronic disease that occurs when the body is unable to produce or effectively use the hormones responsible for regulating blood glucose levels [2]. Diabetes can contribute to the occurrence of CVD such as hyperlipidemias, characterized by an excess of lipids, mainly cholesterol, triacylglycerides (TG), and low-density lipoprotein (LDL) [3,4,1].

The risk for developing CVD such as atherosclerosis increases significantly with elevated total cholesterol and LDL-cholesterol and decreased HDL-cholesterol values [5]. In addition to hypercholesterolemia, oxidative stress plays an important role in the progress of atherosclerosis. Excessive production of reactive species contributes to converts LDL-cholesterol in oxidized LDL (oxLDL-C) that is recognized by macrophages. Macrophages activated by oxLDL-C induce further oxidative stress, which in turn, contributes to inflammatory response by secreting pro-inflammatory cytokines [6].

For the treatment and prevention of hyperlipidemia, statins are the drugs of choice. However, these molecules can cause several side effects such as musculoskeletal complaints, gastro-intestinal discomfort, fatigue, liver enzyme elevation, peripheral neuropathy, insomnia, and neurocognitive symptoms [7]. Thus, studies to evaluate the hypolipidemic potential of medicinal plants are important for the discovery of drugs with fewer side effects that could contribute to reducing the mortality index caused by CVD [8,9].

Recently, advances have been made in the search for natural products able to reduce hyperlipidemias [10]. Various plants, which contain substances such as saponins, polyphenols, and flavonoids, have shown good results in reducing plasma lipid levels [11]. Due to the high chemical diversity of this compounds, an excellent strategy has been the search for pure substances or even crude extracts that contribute to reduction of both body fat and glycemic levels that may be associated with dyslipidemias [10,12].

Celtis iguanaea (Jacq) Sargent; Cannabaceae family, is popularly known as esporão-de-galo, taleira, sarã and gurrupiá, according to the region where it is found in Brazil, and may develop in temperate or tropical regions]13,14,15]. Previous studies showed the traditional use of leaves of *C. iguanaea* in the treatment of body pain, rheumatism, asthma, cramping, dyspepsia, urinary infections, and for the control of diabetes mellitus [13,15,16,17,18].

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Recently, a gastro-protective effect of the hexane fraction of the ethanolic extract was observed using different models of gastric ulcers [19,20,21]. The aqueous extract administered to mice demonstrated no cytotoxic or genotoxic effects [22], and in a toxicity test in *Artemia salina,* the ethanolic extract of *C. iguanaea* bark and its fractions showed an LC_{50} higher than 1000 µg/mL, indicating the absence of a toxic effect [23].

Among the chemical constituents, the presence of pentacyclic triterpenes of type friedelano, friedelin, and epifriedelinol is reported [23]. Preliminary phytochemical analyses of leaves and stems of *C. iguanaea* also revealed the presence of coumarins, mucilage, and flavonoids [15].

Flavonoids have demonstrated multiple pharmacological effects, including anti-inflammatory, antioxidant, anti-cancer, hypoglycemic, and hypolipidemic activities [24,25,26]. However, studies evaluating protection against the risk factors for CVD have not been performed and the hypoglycemic effects of *C. iguanaea* have not been evaluated. Therefore, the present study aimed to investigate the chemical composition of *C. iguanaea* and to evaluate the effects of *C. iguanaea* on markers of lipids and glucose metabolism in cholesterol-fed rats.

2. Results

2.1. Chemical constituents of C. iguanaea

The chemical profile of *C. iguanaea* obtained via mass spectrometry in TIC mode is shown in Figure 1. The compounds obtained were registered as their respective ions $[M\text{-}H]^-$. For these ions, partitions in multiple stages were obtained, which allowed the structures of some molecules to be suggested based on their fragmentation mechanisms (Table 1). Several free and glycosylated flavonoids were identified among these compounds. It should be noted that orientin is present in both the hydroalcoholic and dichloromethane extract. This molecule is one of the major components of hydroalcoholic extract of the plant.

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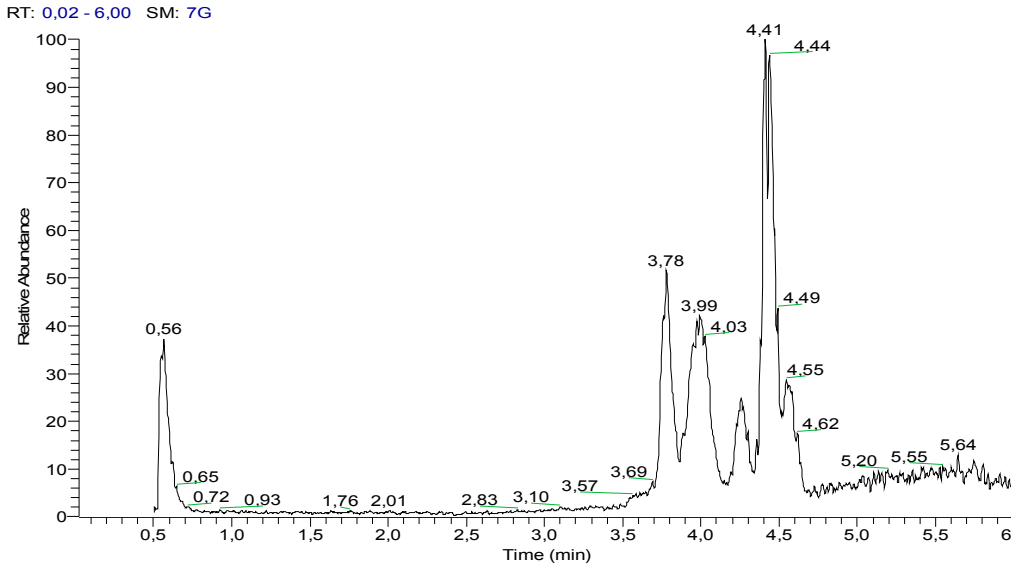
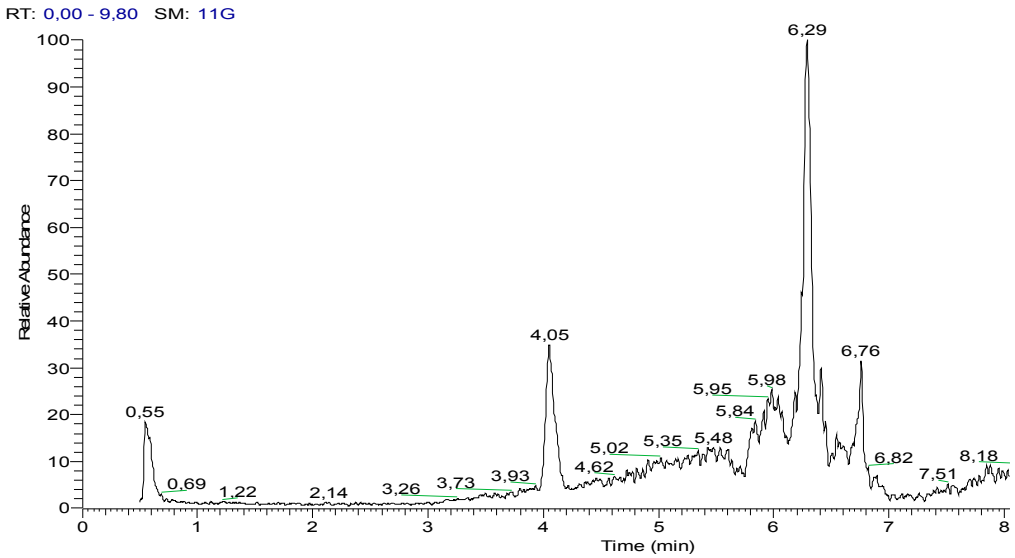


(**a**)



(**b**)

Figure 1. Total ion current chromatogram of the extracts from *Celtis iguanaea* (HPLC-ESI-IT-MS^n negative ion mode). (**a**) Dichlorometane extract; (**b**) Hidroalcoholic extract.

Table 1. ESI-MS and ESI-MS^n product ions of compounds occurring in the extracts of the *Celtis iguanaea*.

Extract	Compound	Retention time	M-H$^-$	MS^n
Dichloromethane	2-O-pentosyl-8-C-hexosyl-apigenin	3.59	563	503, 353, 325
	Luteolin-4'-O-rhamnosyl (1→2) glycoside	3.78	593	473, 353, 269
	Orientin	4.01	547	285
	Genistin	4.26	431	311, 269
	Rutin	4.45	609	447, 301, 285
	Vitexin	6.29	431	311, 269
	Tetrahydroxyisoflavone-O-hexoside	9.5	447	327, 285
Hidroalcoholic	Orientin	4.04	447	285
	(9S,10E,12Z,15Z)-9-Hydroxy-10,12,15-Octadecatrienoic acid	6.30	293	275

2.2. Effects of hydroalcoholic extract of C. iguanaea (CI) on serum lipid profiles

At the end the four-week treatment, hypercholesterolemic rats treated with saline 0.9% (group C), exhibited higher levels of serum TC and LDL- C ($p < 0.001$) than rats fed with a normal diet (group N). The hypercholesterolemic rats treated with CI (300 or 600 mg/kg) or simvastatin (SIMV, 4 mg/kg) exhibited significant decreases in TC (40.7, 37.0 and 41.9 %, respectively) compared with group C ($p < 0.001$) (Figure 2a). All hypercholesterolemic rats treated with CI (150, 300 or 600 mg/kg) or SIMV revealed significant decreases in LDL-C (37.0, 57.5, 52.2 and 46.0%, respectively) compared to group C ($p < 0.001$) (Figure 2b). The analyses also demonstrated an accentuated reduction of the atherogenic index after treatment with CI 300 and 600 mg/kg 51.56% and 46.44%, respectively, compared to group C ($p < 0.05$) (Figure 2c). There were no differences in the serum levels of ALT enzyme activity and urea compared to control group, which suggests the absence of hepatic and renal toxicity (Figure 2d-e). There were also no differences in TG and HDL-C values between the treated groups and group N and C (data not shown).

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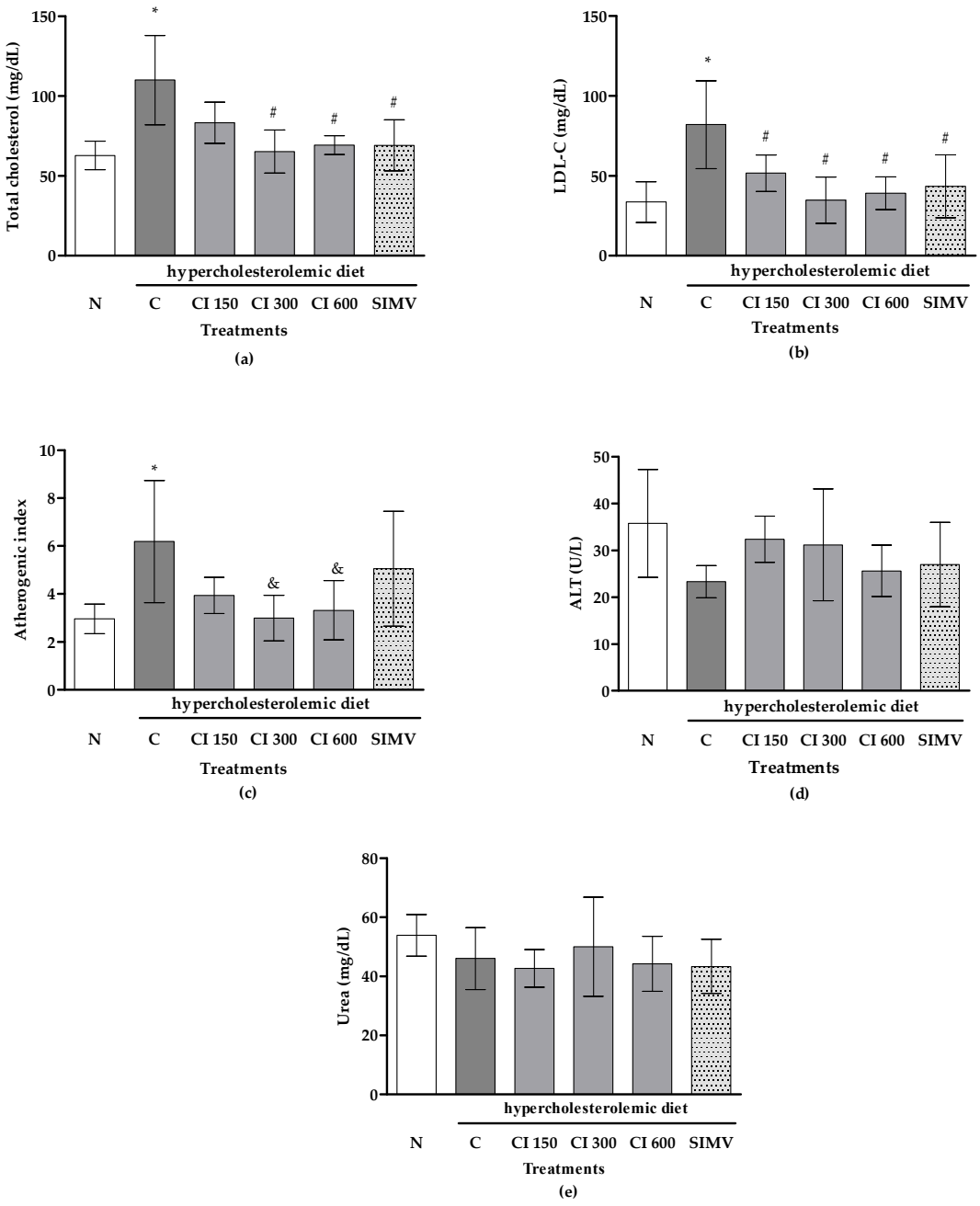

Figure 2. Effect of hidroalcoholic extract from *Celtis iguanaea* (CI; 150, 300, and 600 mg/kg), and simvastatin (SIMV; 4 mg/kg) in the values of total cholesterol (a), LDL-C (b), atherogenic index (c), ALT (d) and urea (e), in cholesterol-fed rats. Rats were either given a normal diet (N) or the following treatment with saline (C). Values are expressed as means ± SD (n = 6). ANOVA one way $^{*}p < 0.001$ compared with group N. $^{\#}p < 0.001$ compared with group C. $^{\&}p < 0.05$ compared with group C.

2.3 Effects of CI on body and liver weights, HMG-CoA/mevalonate ratio, and fecal excretion of cholesterol

All animals gained weight from the beginning to the end of the experiment. However, no differences in weight gain were observed between the groups. The average liver weight was significantly higher in rats that received the high-cholesterol diet as compared to the group fed with the normal diet. To propose a possible mechanism of the hypolipidemic effects of CI, the activity of hepatic 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, which is the regulatory enzyme in cholesterol biosynthesis, was measured indirectly. Analysis of the enzymatic activity revealed an

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accentuated increase in HMG-CoA/mevalonate ratio in the groups treated with CI or SIMV compared to untreated rats fed with the high-fat diet and water (group C) ($p < 0.001$). The fecal cholesterol levels differed between the experimental group C ($p < 0.01$) and the N group. In cholesterol-fed rats, treated with CI (300 and 600 mg/kg), and SIMV exhibited significantly decreased cholesterol excretion values compared to rat belonging to group C ($p < 0.01$) (Table 2).

Table 2. The effects of hydroalcoholic extract of *Celtis iguanaea* on animal body and liver weights, HMGCoA/mevalonate ratio, and in the faecal excretion of cholesterol (n = 6) (mean ± SD).

Treatments	Weights of rats (g)	Weights of livers (g)	HMG-CoA/mevalonate ratio	Faecal excretion of cholesterol (mg/dL)
N	385.3 ± 41.20	10.14 ± 0.84	2.13 ± 0.29	31.32 ± 2.23
C	366.28 ± 7.83	13.39 ± 0.40*	3.19 ± 0.26	54.29 ± 19.12*
CI 150	362.35 ± 30.29	13.47 ± 1.12*	4.22 ± 0.17#	39.68 ± 12.69
CI 300	357.33 ± 29.45	12.41 ± 1.22*	4.85 ± 0.21#	32.87 ± 7.49#
CI 600	385.08 ± 23.14	13.15 ± 1.05*	4.99 ± 0.35#	38.59 ± 23.57#
SIMV	339.98 ± 43.91	12.60 ± 1.31*	5.17 ± 0.53#	38.85 ± 14.26#

*$p < 0.001$, compared to group N; #$p < 0.001$ compared to group C. N - normal; C - control; CI – hydroalcoholic extract of *Celtis iguanaea* (150, 300, and 600 mg/kg); SIMV – simvastatin

2.4. Effects of CI on inflammatory markers

The inflammatory reaction and the associated immune response are the main events that lead to the process of atherogenesis. The analysis of pro-inflammatory interleukins revealed a significant decrease in interleukin-1 (IL-1), IL-6, tumor necrosis factor- α (TNF-α), and interferon gamma (IFN-γ) values ($p < 0.001$) in all hypercholesterolemic rats treated with CI and SIMV compared with group C (Figure 3a-d). Conversely, the groups N, CI 150, CI 300, CI 600, and SIMV exhibited higher levels of anti-inflammatory IL-10 ($p < 0.001$) compared to group C ($p < 0.001$) (Figure 3e).

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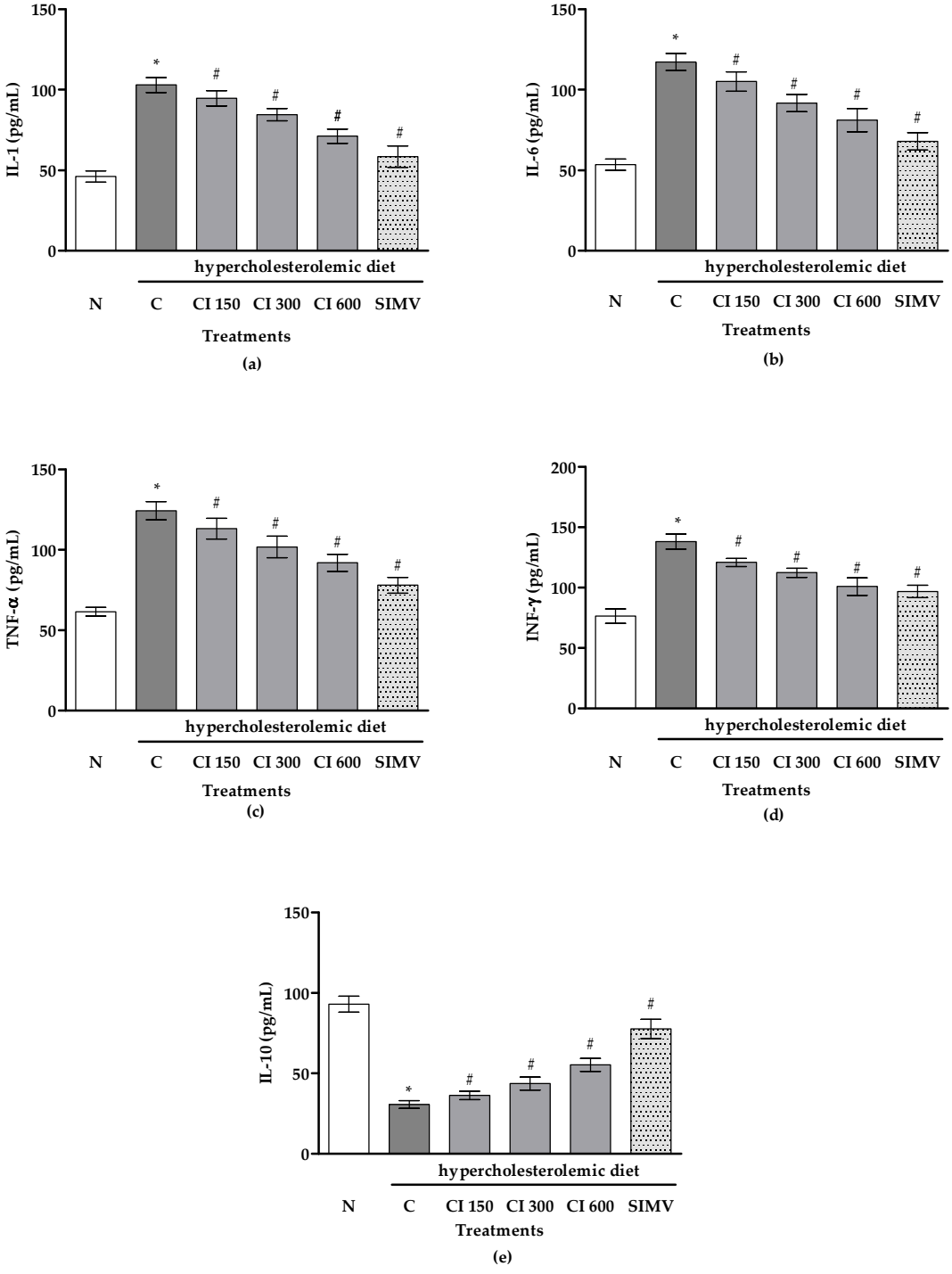

Figure 3. Effect of hidroalcoholic extract from *Celtis iguanaea* (CI; 150, 300, and 600 mg/kg), and simvastatin (SIMV; 4 mg/kg) on the values of IL-1 (a), IL-6 (b), TNF-α (c), IFN-γ (d), IL-10 (e) (mean ± SD; n = 6) in rats fed a high-fat diet. Rats were either given a normal diet (N) or the following treatment with saline (C). ANOVA one way $^{*}p < 0.001$ compared with group N. $^{\#}p < 0.001$ compared with the group C.

2.5 Effects of CI on glycemia, disaccharidase activity, and concentration of hepatic and muscular glycogen

The serum glucose levels of CI 300, CI 600 and SIMV rats display a significant decrease (20.11, 19.85 and 19.84%, respectively), when compared to the C group ($p < 0.001$) (Figure 4a). The

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disaccharidase activity was significantly decrease by CI; maltase activity was also inhibited at concentrations of 150 and 300 mg/kg when compared to group C ($p < 0.01$) (Figure 4b). The sucrase activity was reduced by 600 mg/kg and SIMV ($p < 0.05$) compared to group C (Figure 4c). Lactase activity was not detected in any of the evaluated groups (data not shown). Moreover, analysis showed that CI 300 mg/kg increased significantly muscular glycogen ($p < 0.001$) (Figure 4d), and that none of the doses of CI affected hepatic glycogen content (Figure 4e).

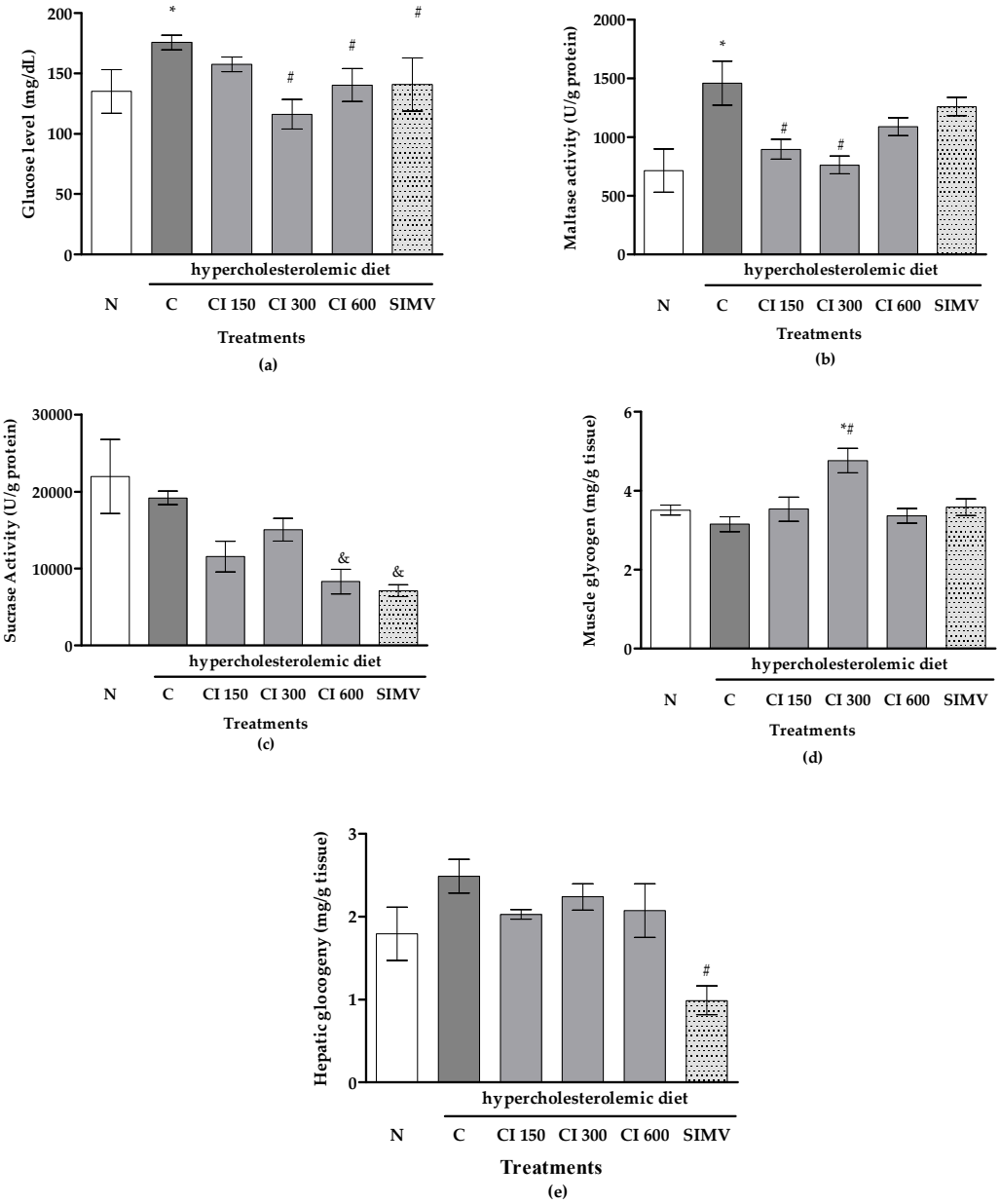

Figure 4. Effect of hidroalcoholic extract from *Celtis iguanaea* (CI; 150, 300, and 600 mg/kg), and simvastatin (SIMV; 4 mg/kg) on serum glucose levels (a), disaccharidases (maltase and sucrase activity) (b - c), muscular and hepatic glycogen (d - e) in rats fed a high-fat diet (mean ± SD; n = 6). Rats were either given a normal diet (N) or the following treatment with saline (C). ANOVA one way $^{*}p < 0.001$ compared with group N. $^{\#}p < 0.001$ compared with the group C. $^{\&}p < 0.05$ compared with the group C.

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2.6 Effects of CI on the serum and liver markers of oxidative damage and antioxidant enzyme activity

The results for plasma thiobarbituric acid reactive substances (TBARS) revealed a significant increase in lipid peroxidation in group C compared to group N ($p < 0.001$), while CI 600 mg/kg induced a lipid peroxidation decrease compared to group C levels ($p < 0.01$) (Figure 5a). The analyses of hepatic TBARS demonstrated a significant increase in lipid peroxidation after high cholesterol diet (C group) ($p < 0.01$) (Figure 5b), and this effect was not protected by CI extract or simvastatin treatments. A significant increase in the values of plasma non-protein thiols (NPSH) was noted after administration of CI 600 mg/kg CI as compared to that of group C ($p < 0.001$) (Figure 5c). Regarding antioxidant enzymes activity, all treatments with CI and SIMV have caused a decrease of hepatic SOD activity in comparison to both groups N and C ($p < 0.001$) (Figure 5d). However, plasma SOD was increased in the CI 300 group compared to group N ($p < 0.01$) (Figure 5e). The results for hepatic CAT activity have also shown the effect in the dose of 150 mg/kg compared with group C ($p < 0.05$) (Figure 5f). The hepatic SOD/CAT ratio decreased after CI (150 and 600) and SIMV treatment when compared to the ratio obtained for group N ($p < 0.05$) (Figure 5g).

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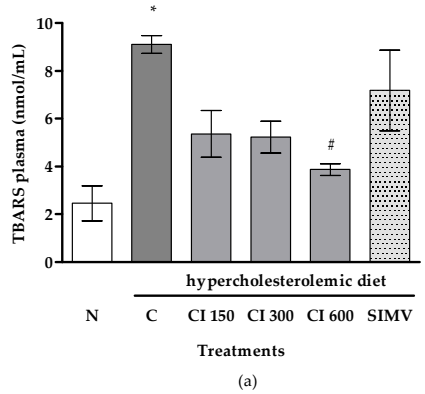

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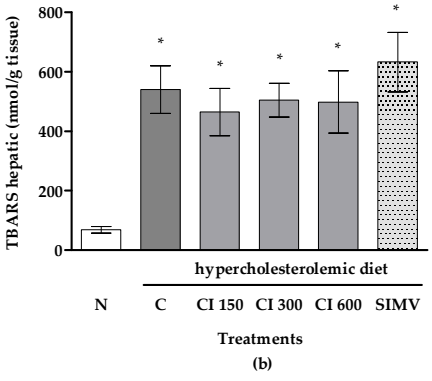

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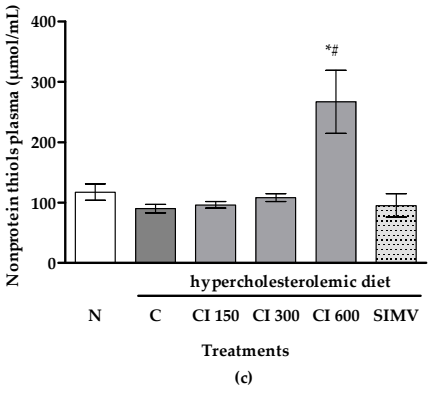

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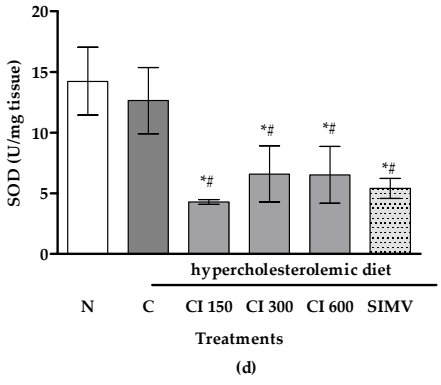

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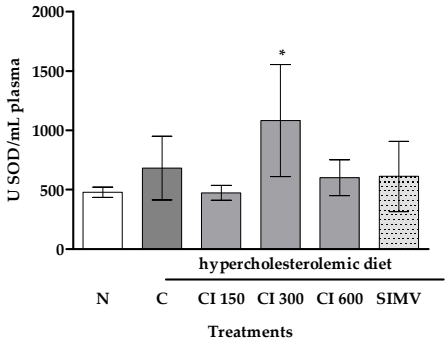

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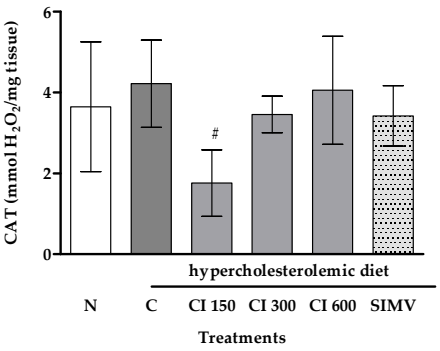

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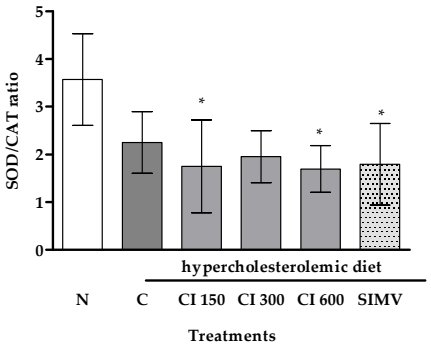

(g)

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Figure 5. Effect of hidroalcoholic extract from *Celtis iguanaea* (CI; 150, 300, and 600 mg/kg), and simvastatin (SIMV; 4 mg/kg) on TBARS plasma (a), TBARS hepatic (b), NPSH (c), plasma and hepatic SOD activity (d - e), CAT activity (f) and hepatic SOD/CAT ratio (g) in rats fed a high-fat diet (mean ± SD; n = 6). Rats were either given a normal diet (N) or the following treatment with saline (C). ANOVA one way $^{*}p < 0.001$ compared with group N. $^{\#}p < 0.05$ compared with the group C. $^{\&}p < 0.001$ compared with the group C.

2.7 Histological results

Histopathological analyses of liver tissue did not show any increase in liver fat area in group N compared to group C (data not shown). However, all animals treated with CI (150, 300 and 600 mg/kg) and SIMV revealed a significant decrease on the fat liver area (24.26, 22.91, 20.63 and 24.60, respectively) compared to group C ($p < 0.001$). CI 150 and 300 mg/kg as well as SIMV decreased the liver fat area where compared with the N group as well group C ($p < 0.05$) (Figure 6a). The evaluation of liver glycogen content in histological sections did not show differences between group N and any of the treated groups (Figure 6b).

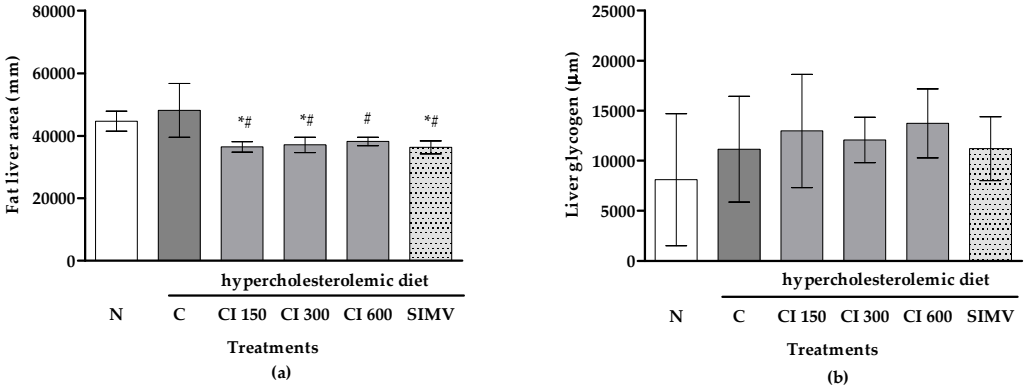

Figure 6. Effect of hidroalcoholic extract from *Celtis iguanaea* (CI; 150, 300, and 600 mg/kg), and simvastatin (SIMV; 4 mg/kg) on fat liver area (a) and liver glycogen (b) in rats fed a high-fat diet (mean ± SD; n = 6). Rats were either given a normal diet (N) or the following treatment with saline (C). ANOVA one way $^{*}p < 0.05$ compared with group N. $^{\#}p < 0.001$ compared with the group C.

3. Discussion

Hyperlipidemia is a serious global public health problem, as it is the main cause for the occurrence of atherosclerosis and CVD [27]. The relevance of the search for new drugs for its treatment and plants compounds with hypolipidemic potential for the treatment and prevention of atherosclerosis and other injuries related to the cardiovascular system is well-recognized by the scientific community [8]. Polyphenols are a large group of molecules categorized according to their chemical structure as flavonoids, stilbenes, and lignans, and have been investigated for their benefits in reducing the atherosclerotic processes and the risks for CVD [28]. These benefits are because of their antioxidant and anti-inflammatory potential that can prevent thrombus formation, modulate blood lipid levels, and contribute to the modulation of glucose metabolism and increased endothelial function [28].

This is the first report on the hypolipidemic and hypoglycemic effects of a hydroalcoholic extract of the *Celtis iguanaea* (CI) in cholesterol-fed rats. The results of the present study show that a cholesterol-enriched diet causes an increase in serum TC and LDL-C levels, which can be mitigated by CI at doses of 150, 300 and 600 mg/kg (for LDL-C) and 300 and 600 mg/kg (for TC). The atherogenic index (AI), an important predictor of cardiovascular diseases and atherosclerosis, decreased in all

groups treated with CI when compared to group C. This index has been associated with the risk for developing atherosclerosis, since it may indicate the formation of atheromatous plaques from the infiltration of cells and lipids in the blood vessels, heart, liver, and kidney. Increased AI values are associated with an increased risk of organ damage, due to the oxidative stress, which is in turn related to the increase in blood lipid levels [29]. Several flavonoids and polyphenolic compounds are present in *C. iguanaea* extracts of different polarities and orientin is one of the major components of the hydroalcoholic extract (CI). The hypolipidemic potential of CI must be related to the presence of flavonoids in their composition, since these substances, as found in similar studies, are able to reduce TG, CT and LDL-C levels and further increase HDL-C levels [26,30]. The reduction of lipid levels in the blood stream in cholesterol fed-rats, is related to decreased enzyme activities of HMG-CoA reductase and acyl-CoA acetyltransferase, which cause decreased levels of cholesterol esters available to form very low density lipoproteins (VLDL), resulting in reduced VLDL secretion by the liver [30]. The results of this study, showed a significant reduction of the activity of the HMG-CoA reductase enzyme, consistent with the observed reduction of the fecal cholesterol levels of the rats. These effects may be related to flavonoids that exhibited potent anti-adipogenesis activity and inhibition of intracellular triglyceride accumulation, by inhibiting the expression of C/EBPα and PPARγ proteins [31]. A previous study showed that dietary polyphenols can bind the HMG-CoA reductase enzyme that catalyzes the reaction converting HMG-CoA to mevalonate, which leads to the production of cholesterol and blocks the binding of nicotinamide adenine dinucleotide phosphate (NADP+) [32].

It is well established that high levels of LDL-C precede inflammatory processes that cause damage to the blood vessel wall and atherosclerosis [33]. Many inflammatory markers associated with atherosclerosis have been described over the years [34], including the pro-inflammatory cytokines involved in the atherosclerotic process such as IL-1, IL-6, TNF-α, CRP, and INF-γ [35,36,37,38,39]. In this study, the group of animals that was fed a hypercholesterolemic diet showed higher concentrations of pro-inflammatory interleukins; however, in the treatment groups (all concentrations of CI), these levels decreased significantly. The decrease in pro-inflammatory interleukins can be related to inhibition of transcription factors, STAT1 and nuclear factor-κB (NF-kB), major polyphenol targets in the vascular system [40]. Orientin, one of the main components of CI, has already demonstrated to inhibit the high mobility group box-1 protein HMGB1 levels in lipopolysaccharide (LPS)-induced human umbilical vein endothelial cells (HUVECs) and HMGB1-mediated cytoskeletal rearrangements [41]. In addition, in human endothelial cell lines, orientin suppressed LPS-induced membrane disruption, migration of monocytes, expression of cell adhesion molecules (CAMs), and LPS-induced endothelial cell protein C receptor (EPCR) detachment [42]. Furthermore, *in vivo* assessments showed that orientin inhibited HMGB1-mediated and LPS-induced hyperpermeability, cecal ligation and puncture (CLP)-induced release of HMGB1 level, leukocyte migration, LPS-induced TNF-α level, IL-6 level, NF-kB level, extracellular regulated kinases (ERK) 1/2 level, and lethality of mice [42]. In addition, the pre-treatment with orientin or isoorientin may contribute to decreased vascular inflammatory effects of high glucose levels in HUVECs and in mice, through the inhibition of NF-κB [43].

However, the inflammatory process related to atherosclerosis appears to be regulated by anti-inflammatory cytokines such as IL-10. This cytokine has a powerful impact on macrophage cholesterol metabolism, stimulating both the absorption of cholesterol from modified lipoproteins, as well as cholesterol efflux from the cell, typical for atherosclerotic processes [44]. The major mechanisms for these anti-atherogenic effects is the activation of the PPARγ-LXR-ABCA1/ABCG1 [45] pathway in macrophages and the ability of IL-10 to inhibit the expression of inflammatory mediators, probably by regulating the expression of extracellular matrix (ECM)-degrading enzymes [46] and by inhibiting NF-kB activity [47,48]. Thus, the increase of IL-10 at doses of 300 and 600 mg/kg of CI might be involved in both cholesterol-lowering and anti-inflammatory effects observed in cholesterol-fed rats. Further studies are needed to elucidate the mechanisms responsible for these effects induced by CI.

In addition to a hypolipidemic potential, CI groups showed to reduce blood glucose levels and promote changes in carbohydrate digestion and metabolism. Carbohydrates are stored in the form of

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glycogen in humans in the skeletal muscles and liver. This mechanism is regulated by insulin, leading to difficulties in reducing blood glucose levels for individuals with diabetes and insulin resistance [49]. This study demonstrated that CI at a concentration of 300 mg/kg increases significantly the levels of glycogen in the soleus muscle without changing the hepatic glycogen, inhibits the activity of maltase, and decreases the activity of sucrase (at 600 mg/kg), what probably caused the reduction of blood glucose. Flavonoids such as orientin, can decrease the disaccharidase activity [50,51], promote alterations in the carbohydrate metabolism and glucose absorption in the intestine, stimulate the insulin secretion from the pancreatic β–cells, modulate glucose release from the liver, activate the insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulate the intracellular signaling pathways and gene expression [52].

Another important condition related to the atherogenic process is the presence of oxidative stress. Our results demonstrated an increase in the lipid peroxidation in the hypercaloric diet group, which could be due to an elevated production of reactive oxygen species (ROS), an important condition for the development of metabolic syndrome and some disorders such obesity, systemic arterial hypertension (SAH), atherosclerosis, and diabetes [53,54]. However, an increase in the plasma TBARS was prevented by the CI extract or simvastatin treatments. In addition, the analyses demonstrated a significant increase in the plasma NPSH and SOD in the groups treated with 600 and 300 mg/kg of CI, respectively. SOD enzyme catalyzes the dismutation of superoxide anions into hydrogen peroxide (H_2O_2) that easily diffuses across cell membranes and cytosol. To attenuate this action, CAT and other enzymes are able to neutralize this reactive species, producing water (H_2O) and molecular oxygen [55,56], while NPSH groups protect the cells against the cytotoxic effects of endogenous or exogenous electrophiles metabolites [57]. Thus, the increase of these antioxidants may have contributed to protect against lipid peroxidation in plasma. In addition, flavonoids such as orientin, isoorientin, vitexin and also statins exhibit radical scavenging activity, which may explain the protective effect against lipid peroxidation at all doses of CI and simvastatin [58,59]. On the other hand, the lack of protection against the increase of liver TBARS levels induced by high-fat diet may indicate that ROS production overcome the antioxidant capacity of liver.

In addition to the protective oxidative damage by CI, the histological evaluation showed a reduction of the fat liver in all groups treated with the extract, which might be related to the decrease of hepatic HMGCoA activity with reduced cholesterol synthesis. No differences in the glycogen liver and in the tunica intima and media thickness of the aorta were noted. Over the years, research has shown that drugs with hypolipidemic potential, such as statins (pleiotropic effects), may increase endothelial function, modulate the inflammatory response related to atherogenesis and reduce platelet aggregation [60].

This study revealed that the administration of CI in cholesterol-fed rats can significantly reduce the LDL and TC levels related to the decreased HMG-CoA activity, inhibition of pro-inflammatory cytokines, and reduction of the glucose levels and lipid peroxidation. In addition, the ALT levels do not differ between treatments groups, indicating the absence of CI toxicity at the doses tested. Thus, these results indicate that CI may be beneficial for may be beneficial for lipids and glucose metabolism, and most likely, for the prevention of atherosclerosis.

4. Materials and Methods

4.1. Solvents and chemicals

The solvents MeOH and acetic acid used for HPLC analysis were purchased from Merck® (São Paulo; SP, Brazil). Ethanol for the production of extracts was purchased from Vetec® (Rio de Janeiro; RJ, Brazil). Nylon membrane filters (0.45 mm) were purchased from of Flow Supply®, and the water was purified using a Milli-Q plus system from Millipore®.

4.2. Plant material

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The leaves of *C. iguanaea* were collected in Chapecó (SC), Brazil (27° 01′ 55.14″ S e 52° 47′ 29.42″ O) in September 2015, and authenticated by Professor Adriano Dias de Oliveira of the Community University of the Region of Chapecó (Unochapecó), where a voucher specimen is deposited (#3463).

4.3 Production of hydroalcoholic extract of Celtis iguanaea (CI)

The leaves of *C. iguanaea* were dried at room temperature (25 ± 5°C), pounded in a knife mill (Ciemlab®, CE430), selected in a sieve (425 µm; 35 Tyler/Mesch), identified, and stored with protection from light. The extracts were produced via maceration (5 days) at room temperature using dry-milled leaves of the plant (100 g) and ethanol 70% (1:20, w/v). After filtration through Büchner funnel, the hydroalcoholic extract (CI) was concentrated via evaporation under reduced pressure, lyophilized, weighed, and stored at -20°C.

4.4. Chemical analysis of Celtis iguanaea

To increase the phytochemical study of *C. iguanaea* in addition to the CI extract, a dichloromethane extract of the leaves (1:20 w/v) was prepared via maceration (5 days). Both the extracts were analyzed via liquid chromatography tandem mass spectrometry. The samples (5.0 mg) were dissolved in methanol (3.0 mL) and filtered in a Sepak RP-18® cartridge, and then through a nylon membrane (Flow Supply®) with a 22.25 mm diameter and a 0.22 µm pore size.

4.4.1. HPLC-ESI-IT-MS^n Analyses

An aliquot of CI and dichloromethane extract were analyzed separately via in-line HPLC-ESI-IT-MS^n, using a SURVEYOR MS micro system coupled in-line to an LCQ Fleet ion-trap mass spectrometer (Thermo Scientific). HPLC separation was conducted on a chromatographic column (250 × 4.6 mm i.d. 5 micron) using a gradient mobile phase with a flow rate of 0.8 mL/min of water) and MeOH plus 0.1% acetic acid. Initial conditions were 5% MeOH increasing to reach 100% MeOH and hold at 100% MeOH at 80 min and held at 100% MeOH for 10 min. Both extracts were analyzed by ESI-MS^n in negative ion mode with a LCQ Fleet ion-trap instrument from Thermo Scientific. The capillary voltage was set at -20 kV, the spray voltage at -5 kV and the tube lens offset at 100 V, sheath gas (nitrogen) flow rate at 80 (arbitrary units) and auxiliary gas flow rate at 5 (arbitrary units). Data were acquired in MS1 and MS^n scanning modes. The capillary temperature was 275°C. Xcalibur 2.1 software (Thermo Scientific) was used for data analysis.

4.4.2. ESI-MS^n Analysis

For analysis via mass spectrometry, CI and dichloromethane extract (5.0 mg) were dissolved in methanol (3.0 mL) and filtered in a Sepak RP-18 cartridge, and then through a nylon membrane (Flow Supply®) with a 22.25 mm diameter and a 0.22 µm pore size. The samples were analyzed online via the LCQ Fleet, Thermo Scientific® mass spectrometer, equipped with a direct sample insertion device for streaming injection analysis (FIA). The samples were ionized via electrospray ionization (ESI) and fragmentations into multiple stages (MS^n) were held in an Ion-Trap (IT) interface. The negative mode was chosen for the generation and analysis of all the spectra, and the experimental conditions were as follows: capillary voltage -35 V, spray voltage -5000 V, capillary temperature 350°C, drag gas (N2) and flow rate 60 (arbitrary units). The acquisition track was m/z 100-2000, with two or more scan events held simultaneously in the spectrum. The experiment was performed by Laboratory of Bioprospecting of Natural Products (LBPN), UNESP, Coastal Campus IB-CLP.

4.5. Animals

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The International Guidelines for Care and Use of Laboratory Animals were followed for all experiments, and the experimental protocol was approved by the Ethics Committee on Animal Use (CEUA: 013/2015) of Unochapecó, Brazil. Male adult Wistar rats (n = 36), weighing 200 ± 15 g, were purchased from the animal facility of Unochapecó. The animals were housed in wire-bottomed 17 × 33.5 × 40.5 cm cages in a controlled environment at 22 ± 2°C with a 12 h light-dark cycle (lights on at 07:00 am and off at 07:00 pm) and minimal noise.

4.6 Hyperlipidemic diet

The high cholesterol diet was prepared by mixing a normal basal diet [composition (w/w): 48.3% carbohydrate, 23.5% crude protein, 5.9% crude fat, 5.9% crude ash, and 3.9% crude fiber] with cholic acid and cholesterol (989.9:10:1 w/w). The mixture was pelleted prior to use [61].

4.7 Experimental design

After 7 days of acclimatization the animals were divided randomly in two groups: a normal group (N) (n = 6) fed on pelleted food (Biobase®) and an induced group (I) (n =30) fed with hyperlipidemic diet (pelleted food Biobase® + 1% cholesterol + 0.1% cholic acid Sigma-Aldrich®, St. Louis, MO, USA). The rats of both groups had access to water *ad libitum*. After 15 days, group I was divided randomly into 5 groups (n = 6). The groups were treated intragastrically once a day for 30 days and were divided as follows: Control (saline 0.9%) (C); hydroalcoholic extracts of CI (150, 300 or 600 mg/kg, respectively) (CI: 150, 300 or 600) and simvastatin (4 mg/kg) (SIMV) [62]. All treatments (at defined doses) were administered in a volume of 0.5 mL/200 g body weight diluted in saline (0.9% saline) and subjected to an ultrasonic bath (20°C) to facilitate solubility. Dietary intake was measured daily and body weights of animals were recorded before and every three days following the initiation of treatment. All animals were fasted for a 12 h period before euthanasia, but with free access to water. The rats were anesthetized with a mixture of lidocaine and thiopental sodium (10 and 40 mg/kg, respectively), administered intraperitoneally. Blood aliquots were collected via cardiac puncture and the animals were euthanized using an overdose of sodium thiopental (120 mg/kg). The right soleus muscle, liver samples, intestine, and the aorta arteries were collected for biochemical and histopathological analyses.

4.7.1. *Biochemical analysis of blood samples*

Upon collection, the blood samples were immediately centrifuged (3000 × *g*) for 15 min. Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triacylglycerides (TG), urea, IL-1 (sensitivity: 2 pg/mL; range: 1–50 pg/mL), IL-6 (sensitivity: 2 pg/mL; range: 2–200 pg/mL),TNF-α (sensitivity: 4 pg/mL; range: 4–500 pg/mL), IFN-γ (sensitivity: 4 pg/mL; range: 4–200 pg/mL), IL-10 (sensitivity: 2 pg/mL; range: 1–80 pg/mL), glucose and ALT levels were determined by enzymatic colorimetric methods (UV/Vis) using commercial kits and according to the manufacturer's instructions [63]. Serum LDL-C and VLDL levels were calculated using the Friedewald equation: LDL-C = TC− [HDL-C − (TG/5) [64], and the atherogenic index calculated using the equation: TC/HDL-C) [65]. For the evaluation of lipid peroxidation, a blood sample with heparin was preserved and EDTA tubes containing blood samples were centrifuged (3000 × *g*) for 10 min, and the plasma was separated and used for analysis of the antioxidant activity

4.7.2. Estimation of HMG-CoA reductase activity (HMG-CoA/mevalonate ratio)

HMG-CoA reductase activity was measured in liver homogenates using the ratio of HMG-CoA to mevalonate, an index of enzyme activity which catalyzes the conversion of HMG-CoA to mevalonate. Therefore, the liver tissue was removed as quickly as possible and a 10% w/v homogenate was prepared in saline arsenate solution. The homogenate was deproteinized using an

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equal volume of dilute perchloric acid and allow to stand for 5 min, followed by centrifugation. To 1 mL of the filtrate, 0.5 mL of freshly prepared (alkaline hydroxylamine reagent in the case of HMG-CoA) was added. It was mixed and 1.5 mL of ferric chloride reagent was added after 5 min. The absorbance was read after 10 min at 540 nm versus a similarly treated saline arsenate blank. The ratio of HMG-CoA/mevalonate was calculated [66].

4.7.3 Fecal cholesterol

Fecal material was collected in the last 3 days of the experiment and stored at -20°C. The stools were thawed e dried at 60°C (24 h). To the powdered samples (0.5 g), isopropanol (5 mL) was added and the solutions were agitated and stored at -20°C for 24 h with subsequent centrifugation (12.000 × *g*) for 10 min. The supernatant was analyzed for levels of fecal cholesterol at 500 nm via enzymatic colorimetric methods (UV/Vis) using commercial Labtest® kits according to the manufacturer's instructions [67].

4.7.4 Glycogen measurements

The harvested liver and soleus muscle were assessed for glycogen content. Glycogen was isolated from these tissues. The tissue was weighed, homogenized in 33% KOH, and boiled at 100°C for 30 min, with occasional stirring. After cooling, 96% ethanol was added to the samples, which were then heated to boiling 100°C temperature and cooled in an ice bath to aid glycogen precipitation. The homogenate was centrifuged (1300 × *g*) for 15 min, the supernatant was discarded, and the resulting pellet was washed and resolubilized in water. Glycogen content was determined via treatment with an iodine reagent, and the absorbance was measured at 460 nm. The results were expressed as milligrams of glycogen per gram of tissue [68].

4.7.5 Disaccharidase extraction and assays

The extracted small intestine segment was washed in 0.9% NaCl solution, dried on filter paper, weighed, trimmed, and homogenized (300 × *g*) with 0.9% NaCl (400 mg of duodenum per 1.0 mL of 0.9% NaCl) for 1 min at 4°C. The resulting extract was centrifuged at (1300 × *g*) for 8 min. The supernatant was assessed to measure *in vivo* maltase, sucrase, and lactase activity as well as protein determination. The activity of maltase (EC 3.2.1.20), lactase (EC 3.2.1.23), and sucrase (EC 3.2.1.48) was determined using a glucose diagnosis kit based on the reagent glucose oxidase. To determine dissaccharidase activity, duodenum homogenates (10 µL) were incubated at 37°C for 60 min with 10 µL of the substrate (equivalent to 0.056 µM of maltose, sucrose, or lactose dissolved in sodium maleate buffer (pH 6.0). Then, the reagent solution containing glucose oxidase and peroxidase was added and incubated at 37°C for 10 min. The absorbance was read at 500 nm, and the activity calculation was based on a glucose standard [69,70]. One enzyme unit (U) was defined as the amount of enzyme that catalyzed the release of 1 µmol of glucose per minute under the assay conditions. The specific activity was defined as enzyme activity (U) per milligram of protein. The protein concentration was determined using bovine serum albumin as a standard. The assays were performed in duplicate along with the appropriate controls [71].

4.7.6 Lipid peroxidation

To evaluate lipid peroxidation and antioxidant enzyme activity, the liver samples were homogenized in three volumes of Tris HCl (150 mM; pH 7.4) and centrifuged (3000 ×*g* at 4°C) for 10 min yielding a low-speed supernatant, which was used for further analysis. Lipid peroxidation was evaluated by measuring the TBARS level [72] after the addition of 7.2 mmol/L of butylated hydroxytoluene to the homogenates or plasma to prevent further oxidation. Liver homogenates and plasma samples were deproteinized with 10% trichloroacetic acid (TCA) and preincubated with

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0.67% thiobarbituric acid (TBA) at 100°C for 1 h. The colored product of the reaction was then extracted with *n*-butanol and measured at 535 nm using a standard curve of 1,1,3,3-tetraethoxypropane.

4.7.7 Antioxidant activity

Epinephrine (5 µL, 60 mM) was added to a medium containing glycine buffer (50 mM, pH 10.2) and an aliquot of sample was added in a final volume of 200 µL. The inhibition of epinephrine auto-oxidation to adrenochrome at alkaline pH was spectrophotometrically determined at 480 nm. Liver SOD activity was expressed as U/g of tissue (1U is the amount of enzyme that inhibits the oxidation of epinephrine by 50%) whereas plasma SOD was expressed as U/mL of plasma [73]. Liver catalase (CAT) activity was determined by adding hydrogen peroxide (0.5 M H_2O_2) in a medium containing 30 µL of sample and 50 mM potassium phosphate buffer, pH 7.0 (final volume of 2 mL). The rate of decrease in H_2O_2 absorbance was monitored at 240 nm. The pseudo-first order reaction constant (*k*) of the decrease in H_2O_2 absorption at 25°C was determined and specific activity was expressed as *k*/g wet tissue [74].

4.7.8 Histopathological analysis

Aorta and hepatic tissues were fixed in 10% formalin and trimmed to obtain 2-mm-thick cross sections. All tissues were dehydrated in an ascending graded series of ethanol, cleared in xylene, and embedded in paraffin. Sections of 3 µm were obtained with a standard microtome and were mounted on glass slides. The sections were later stained with hematoxylin and eosin for histological analyses and morphometry of fat area in the liver and thickness of tunica intima and media of aorta. The fat area was measured from 10 pictures (400 x magnification) per sample using the Image J 1.45s program. All white area was considered to contain fat. To evaluate the thickness of tunica intima, 10 pictures (1000 x magnification) per sample were captured and three measurements were made per picture. Tunica intima was considered from endothelium to internal elastic lamina, including both. For tunica media, 10 pictures (400 x magnification) per sample were captured and three measurements were made per picture as well. Tunica media was considered from the end of intima to internal border of adventitia. To identify and visualize the glycogen, each liver sample was sectioned and stained to the Periodic Acid Schiff (PAS) [75]. Each PAS glass slide was used to capture 10 pictures (400 x magnification). The Image J 1.45s program was used to measure the glycogen area considering all PAS positive areas as glycogen area. All pictures were obtained using a binocular microscope Leica DM 500 coupled to an ICC 50 HD camera. The software Leica Application Suite EZ (LAS-EZ) version 3.0 was used to capture the images and to measure thickness of tunica.

4.8 Statistical analysis

The data were expressed as means ± standard derivation (SD). Statistical analysis between the treated and the control groups were performed using one-way ANOVA followed by Tukey post hoc test. A difference in the mean values of $p < 0.05$ was considered statistically significant.

5. Conclusions

The hydroalcoholic extract of CI may be effective in the prevention of hypercholesterolemia and the protection against atherosclerosis and hyperglycemia. These biological activities are possibly related to its antioxidant effect, enzymatic inhibitory activity, and insulin mimetic potential.

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Author Contributions

All authors contributed substantially to the work reported. BZ and WARJ conceived and designed the experiments, analyzed the data and wrote the paper; DBG, VSC, KAPD, APS, CF, PN, FB, RC, AS, CP, TB, NSA, JCOM, FC, GS, ATR and FCZ performed the experiments; LGM, APZ, SMW, LZ, MMMFD, GMMC and CQR performed the experiments and contributed with materials, analysis tools and wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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