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Evaluation of phenolic compounds and lipid-lowering effect of *Morus nigra* leaves extract

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ABSTRACT

Morus nigra L. (Moraceae) is a tree known as black mulberry and the leaves are used in folk medicine in the treatment of diabetes, high cholesterol and menopause symptoms. The aim of this study was to evaluate the *M. nigra* leaves phytochemical profile in different extractions and the hypolipidemic effect of the infusion comparing to the fenofibrate. *Morus nigra* infusion (MN) showed higher amounts of phenolics and flavonoids (83.85 mg/g and 79.96 µg/g, respectively), as well as antioxidant activity (83.85%) than decoction or hydromethanolic extracts. Although, decoction showed the best result for ascorbic acid (4.35 mg/100 g) than hydromethanolic or infusion (2.51 or 2.13 mg/100 g, respectively). The phenolic acids gallic, chlorogenic and caffeic and the flavonoids quercetin, rutin and catechin were found in the *M. nigra* extracts. Hyperlipidemic rats treated with 100, 200 or 400 mg/kg of MN decreased serum cholesterol, triglycerides and normalized lipoproteins. Furthermore, MN inhibited lipid peroxidation in liver, kidney and brain of hyperlipidemic rats. This study provides evidence that *M. nigra* leaves extracts are rich in polyphenols, mainly chlorogenic acid, which normalized hyperlipidemic disturbance. The results suggest a potential therapeutic effect of the *M. nigra* leaves infusion on dislipidemic condition and related oxidative stress.

Key words: *Morus nigra*, leaves, phenolics, Triton WR-1339.

INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide with an increasing incidence rate (Mendis 2011). Cholesterol is a constituent of membranes and plays a role in synthesis of bile acids, hormones and vitamins. Although the hypercholesterolemic together with the hypertrigliceridemic state of the serum are both risk factors to the development of coronary heart disease and atherosclerosis progression (Lusis 2000). On the other hand, the decrease in low density lipoprotein cholesterol (LDL) and increase in high density lipoprotein cholesterol (HDL) serum levels contribute to an anti-atherogenic condition (Lusis 2000, West et al. 1983, Assman and Nofer 2003).

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Lipid-lowering substances such as statins and fibrates reduce the number of events related to cardiovascular complications, but having potential side effects and great drug dependence; many patients have been choosing alternative ways for the treatment. In addition, oxidative stress is an early event in hyperlipidemic conditions and it has been suggested that antioxidants can break a vicious cycle in the progress of the disease (Rony et al. 2014). Therefore, it has been growing steadily the interest in seeking drugs to decrease side effects and treat the disease using multiple targets; in this line, plant material is an available option.

Morus nigra L. belonging to Moraceae family usually known as black mulberry is a tree worldwide distributed and its fruits are consumed regarding its nutritional value (Gundogdu et al. 2011). The leaves are used traditionally for therapeutical purposes as for the treatment of diabetes, hypercholesterolemia, menopause and obesity (Oliveira et al. 2013, Silva and Naves 2001, Volpato et al. 2011, Miranda et al. 2010). Moreover, Volpato et al. (2011) studied antihyperglicemic effect from *Morus nigra* leaf decoction in pregnancy diabetic rats although, obtained positive effects only in lipids levels. Additionally, Araújo et al. (2015) demonstrated an increased insulinemia and improved oxidative stress state in diabetic rats treated with hydroethanolic leaf extract. *Morus nigra* leaves have shown evidence of anti-inflammatory, antinociceptive and hepatoprotective effects (Padilha et al. 2009, 2010, Malhi et al. 2014). Although, Queiroz et al. (2012), did not confirmed the estrogenic activity, the most popular use of *M. nigra*. Moreover, *M. nigra* did not exert a toxic effect on the female reproductive system or on the embryonic development of rats contributing to reduce incidence of abnormalities in offspring from diabetic dams (Volpato et al. 2011, Queiroz et al. 2012). Furthermore, Oliveira et al. (2013) have considered *M. nigra* leaf aqueous extract as being of low toxicity after a treatment by oral route during 30 days in rats.

Recent studies developed by Memon et al. (2010), Malhi et al. (2014), Araújo et al. (2015), Sánchez-Salcedo et al. (2015) with black mulberry leaves extracts using organic solvents demonstrated the richness of phenolic compounds which provides a potential antioxidant activity. Since oxidative stress has been implicating on the improvement of cardiovascular and neurodegenerative diseases this source of phenolics could be usefull therapy (Heo and Lee 2006, Tarozzi et al. 2013, Wiczkowski et al. 2013).

Therefore, to the best of our knowledge, this is the first study with the goals to investigate phenolics in different extracts, mainly aqueous, from black mulberry leaves (infusion, decoction and hydromethanolic). And also, to inspect the hypolipidemic and antioxidant activities from the *M. nigra* leaves infusion in hyperlipidemic rats, compared to the fenofibrate.

MATERIALS AND METHODS

COLLECTION, PREPARATION OF PLANT MATERIAL AND EXTRACTS

The leaves of *Morus nigra* L. from the city of Blumenau (Santa Catarina State, Southern Brazil - latitude 26°54'10" S, longitude 49°04'44" W) harvested in February, 2014. The species were identified, taxonomically authenticated and a voucher specimen (N° 42265) deposited at the Regional University of Blumenau's herbarium, Santa Catarina, Brazil. The plant material was submitted to drying at 45°C with forced ventilation, grinded and then stored at -10°C.

Briefly, 2 g of milled leaves were extracted with 100 mL of boiled at 100°C distilled water resting 15 min (infusion), 100 mL of distilled water boiling for 10 min (decoction) or 100 mL of methanol: distilled water, 70%:30% (v/v) stirred for 15 min (hydromethanolic).

DETERMINATION OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT CAPACITY

The concentration of total phenolics (TP) was measured using the Folin–Ciocalteu assay previously described by Singleton and Rossi (1965), reading the absorbance at 725 nm. The TP content calculations were standardized through gallic acid ($y = 0.1893x - 0.1429$, $r^2 = 0.99$) and expressed as gallic acid equivalent (GAE) mg/g dry weight. The total flavonoids (TF) quantification of extracts was performed by mixing the samples (0.5 mL) with $AlCl_3$ (2%, w/v) and 2.5 mL ethanol. The absorbance was determined at 420 nm and the TF content was standardized through quercetin ($y = 0.1755x - 0.3139$, $r^2 = 0.99$). The results expressed as quercetin equivalent (QE) μg/g dry weight (Woisky and Salatino 1998). For the total carotenoids analysis, a hexane: acetone solution (1:1, v/v) containing 100 mg of butyl hydroxytoluene (BHT) was added to 300 mg of biomass sample. After this, the absorbance was determined at 450 nm and the quantification based on the absorption coefficient ($A^{1\%}_{1cm}$ 2300, hexane - 450 nm). The results defined as β-carotene equivalent mg/g dry weight (Britton 1995). The quantification of ascorbic acid carried out with 20 mL of *Morus nigra* extracts titrated by potassium iodide solution (KIO_3 0.01N). The titrations of ascorbic acid on the samples using the starch solution indicator (1%, w/v) and the results expressed as mg/100 g dry weight (Rebollo et al. 2005).

The antioxidant activity determined spectrophotometrically using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH - Sigma Chemical Co., St Louis, MO, USA). After storage at room temperature during 30 min in the dark, the absorbance of the samples was determined at 517 nm (Brand-Williams et al. 1995). DPPH radical-scavenging activity was calculated according to the following equation:

DPPH discoloration % = $1- (A_{sample}/A_{blank}) \times 100$;

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. All tests were performed in triplicate.

HPLC ANALYSIS

RP-HPLC was performed using a Varian ProStar230 chromatograph equipped with a C_{18} reverse-phase column (Agilent Technologies, California, USA, 250 mm x 4.6 mm, 5 μm), protected by a 5 μm C_{18} reverse-phase guard column, and a UV-visible detector (330 nm). The samples were eluted in isocratic mode at a flow rate of 0.9 mL/min, using acidified water (0.5% of formic acid):methanol (70:30, v/v) and the solvents were purchased from Tedia Brazil (Rio de Janeiro, Brazil). The chromatographic analyses lasted for 30 minutes. The phenolic compound identification was performed by comparing retention times of standard compounds, chlorogenic, gallic and caffeic acids and rutin, quercetin, catechin, purchased from Sigma Chemical Co. (St Louis, MO, USA). The injection volume was 20 μL from each extract.

ANIMALS AND EXPERIMENTAL PROTOCOL

The biological tests have been approved by the local Ethics Committee on Animal Use at Regional University of Blumenau (FURB), Protocol nr. 015/2013. Male Wistar rats were used between 9-10 weeks of age. All animals were housed in groups of four per cage, on 12 h light/dark cycle, and air temperature at 22±2°C with food and water *ad libitum.*

The rats were allowed to acclimatize for 1 week before the experiments and then divided into six groups (n=6-8 animals/group). Hyperlipidemic control group (HCG) was performed according to Cruz et al. (2016) through a single intraperitoneal injection (i.p.) with Triton WR-1339 (400 mg/kg - Sigma Chemical Company, St Louis, MO, USA) before administration of other substances. The

normal control group (NCG-distilled water), *Morus nigra* infusion extract (MN - 100, 200 or 400 mg/kg), or fenofibrate (FF- 65 mg/kg–EMS S/A, SP, Brazil) was given by blunt gavage twice a day for three consecutive days. In this study, the infusion extract was chosen for biological tests based on its higher phenolic content and antioxidant activity showed in the phytochemical results.

At the end of the experiment, rats fasted overnight were anesthetized by intraperitoneal (i.p.) injection of sodium thiopental (Cristália – Produtos Farmacêuticos, SP, Brazil). Blood samples were collected into tubes and centrifuged (5000 rpm, 5 min) to obtain serum for lipid profile analysis. Briefly, liver, kidneys and brain were removed, rinsed out with 0.9% cold saline, blotted with filter paper, and frozen for further estimation of lipid peroxidation. In addition, the brain was dissected obtaining cerebral cortex and hippocampus.

BIOCHEMICAL ANALYSIS

The contents of serum total cholesterol (TC), triglycerides (TG) and high-density lipoprotein (HDL-c) determined in a semiautomatic analyzer BIO-2000 (Bioplus, SP, Brazil) using commercial kits (Labtest Diagnóstica SA, Lagoa Santa, MG, Brazil) according to the manufactures' instructions. The results of low-density lipoprotein-cholesterol (LDL) and very low-density lipoprotein cholesterol (VLDL) were estimated by the Friedewald et al. (1972) equations as the following: VLDL = TG/5 and LDL= TC – (HDL + VLDL). The atherogenic index (AT) and cardiovascular risk factor (CR) were calculated by the following equations: AT = (TC – HDL)/HDL and CR = TC/HDL according to Castelli's indexes (Castelli et al. 1986).

EVALUATION OF LIPID PEROXIDATION

Thiobarbituric acid-reactive substances (TBARS) assay levels were determined according to the method described by Ohkawa et al. (1979) that measures malondialdehyde (MDA), a product of lipoperoxidation caused mainly through hydroxyl free radicals by the absorbance at 535 nm. The calibration curve developed using 1,1,3,3-tetramethoxypropane and TBARS levels calculated as nanomol of malondialdehyde formed per milligram of protein.

PROTEIN DETERMINATION

Protein was measured by Lowry et al. (1951) method, using serum bovine albumin as standard.

STATISTICAL ANALYSIS

The chemical data expressed as mean ± S.D., biological data as mean ± S.E.M. and, both compared by one-way ANOVA followed by the Tukey test at $p \leq 0.05$. The statistical package GrapPad Prism 6.0 Version for Windows (GraphPad Software, San Diego, CA, USA) was used for statistics.

RESULTS AND DISCUSSION

ANALYSIS OF TOTAL PHENOLICS, FLAVONOIDS, CAROTENOIDS, ASCORBIC ACID, AND ANTIOXIDANT CAPACITY OF *Morus nigra* EXTRACTS

Several studies have shown that the intake of phenolics and flavonoids can be beneficial to reducing the risk of atherosclerosis development (Costa and Martinez 1997). In this context, flavonoids have been related to inhibition of LDL oxidation, platelet aggregation promoting vasodilatation, and also modification of eicosanoid synthesis (Sesso et al. 2003).

This study, to our knowledge, is the first comparing two aqueous forms and methanolic *Morus nigra* extracts (Table I) detecting a significant (p<0.01) higher concentration of total flavonoids from the infusion and hydromethanolic (79.96 ± 0.71 and 76.76 ± 0.78 μg/g, respectively) than the decoction (67.83 ± 1.24 μg/g). Correspondingly, the antioxidant capacity from the infusion (83.85

TABLE I
DPPH-scavenging activity, total content of phenolics (TP), flavonoids (TF) and ascorbic acid (AA) from *Morus nigra* according to the extraction method[a].

Extracts (Yield %)	DPPH-scavenging activity[b]	TP[c]	TF[d]	AA[c]
Infusion (25.5 ± 0.02)	83.85 ± 0.99a	75.86 ± 0.87a	79.96 ± 0.71a	2.13 ± 0.13a
Decoction (30.0 ± 0.03)	74.37 ± 0.2b	64.59 ± 0.14b	67.83 ± 1.24b	4.35 ± 0.14b
Hydromethanolic (16.0 ± 0.01)	81.71 ± 0.05a	51.01 ± 0.86c	76.76 ± 0.78a	2.51 ± 0.13a

[a]Mean ± standard deviation (n= 3).
[b]Values expressed as % discoloration.
[c]Values expressed as mg/g GAE dry weight.
[d]Values expressed as µg/g QE dry weight.
[e]Values expressed as mg/100 g dry weight.
Different letters in the same column represent significant differences ($p< 0.05$) among extracts.

± 0.99%) and hydromethanolic (81.71 ± 0.05%) extracts shared superiority to the decoction (74.37 ± 0.20%) one. The amount of total phenolics was higher in the infusion (75.86 ± 0.87 mg/g) than the hydromethanolic and decoction (51.01 ± 0.86 and 64.59 ± 0.14 mg/g, respectively). On the contrary, decoction showed better results for ascorbic acid (4.35 ± 0.13 mg/100 g) than the infusion (2.13 ± 0.14 mg/100 g) or hydromethanolic (2.51 ±0.13 mg/100 g) extracts, the same results were obtained by Guimarães et al. (2011). In regard, Fata et al. (2016) pointed that there are several variables to considerer besides temperature since, ascorbic acid quantity is influenced also by oxygen, light intensity, pH, water activity, presence of metallic ions and the presence of sugars (Hsu et al. 2012) could be at least in part influenced the result. Regarding antioxidant effect and amounts of ascorbic acid obtained in this study, it seems that the amounts of phenolics and flavonoids were determinants while ascorbic acid did not. Finally, total carotenoids quantification revealed 12.12 ± 0.56 mg/g.

The evaluation of phenolics and antioxidant capacity found in this study was greater than demonstrated by Araújo et al. (2015) also studying leaves of *Morus nigra*. Moreover, ascorbic acid and carotenoids could have contributed to the observed antioxidant activity showed here. According to Singh et al. (2006) and Silva and Naves (2001), ascorbic acid and phenolic compounds are capable to act against superoxide and hydroxyl radicals and to reduce C-reactive protein (CRP) levels, a marker of inflammation and a predictor of heart disease (Singh et al. 2006).

RP-HPLC ANALYSIS OF PHENOLIC COMPOUNDS

The analysis identified 06 phenolic compounds in *Morus nigra* extracts (Figure 1), although a different phenolic profile was demonstrated for each extract. The phenolic acids present in all extracts were gallic and chlorogenic acids which the last was the major compound detected. Therefore, the constituens as quercetin, gallic and chlorogenic acids maybe involved in the higher antioxidant capacity found in the infusion and hydromethanolic extracts. The acids caffeic, chlorogenic and gallic, and the flavonoids quercetin and rutin, were already found in leaves extracts of *Morus nigra* (Araújo et al. 2015, Sanchez-Salcedo et al. 2015, Freitas et al. 2016), although, catechin was not identified previously. In fact, the cinnamic acid derivatives has been thoroughly studied as anti-atherogenic agents causing alterations in cholesterol storage and transport, LDL-oxidation, and HDL particle size rearrangement (Cai et al. 2004, Ahmad et al. 2012, Balzan et al. 2013). Of note, the fixed wavelength

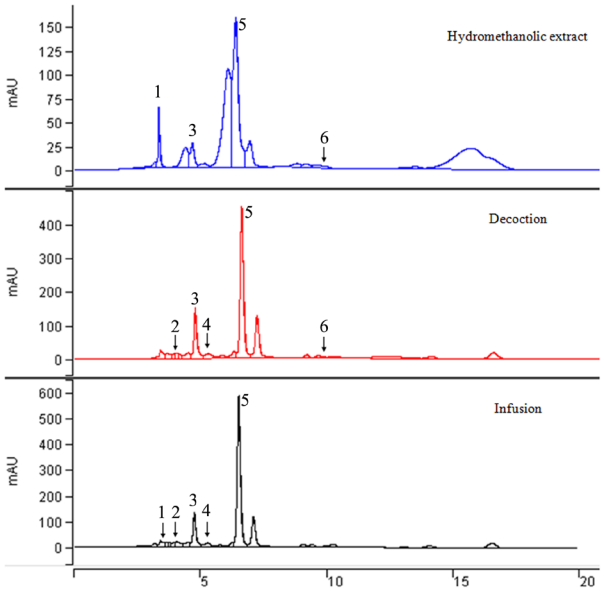

Figure 1 - Chromatogram obtained by RP-HPLC (330 nm) of phenolic compounds of hydromethanolic, decoction and infusion extracts from amoreira-preta leaves (*Morus nigra* L.). Peaks: (1) quercetin, (2) rutin, (3) gallic acid, (4) catechin, (5) chlorogenic acid and (6) caffeic acid.

at 330 nm to perform a comparison among extracts is a possible limitation of this study. In addition, further approaches needs perform co-injections and quantification of the compounds.

EFFECT OF *Morus nigra* INFUSION EXTRACT (MN) ON SERUM LIPID PROFILE IN TRITON WR-1339-INDUCED HYPERLIPIDEMIC RATS

According to Schurr et al. (1972), Zarzecki et al. (2014) and Rony et al. (2014) the Triton WR-1339 is widely used to screening natural and chemical hypolipidemic drugs producing a hyperlipidemic condition as showed here compared to the control group (Figure 2). In agreement with Cruz et al. (2016) it increased TC, TG, LDL-c and VLDL-c and, decreased HDL-c levels (p<0.001). On the other hand, MN, the selected extract in the phytochemical step, or fenofibrate treatment totally suppressed augmentation of TC and TG in

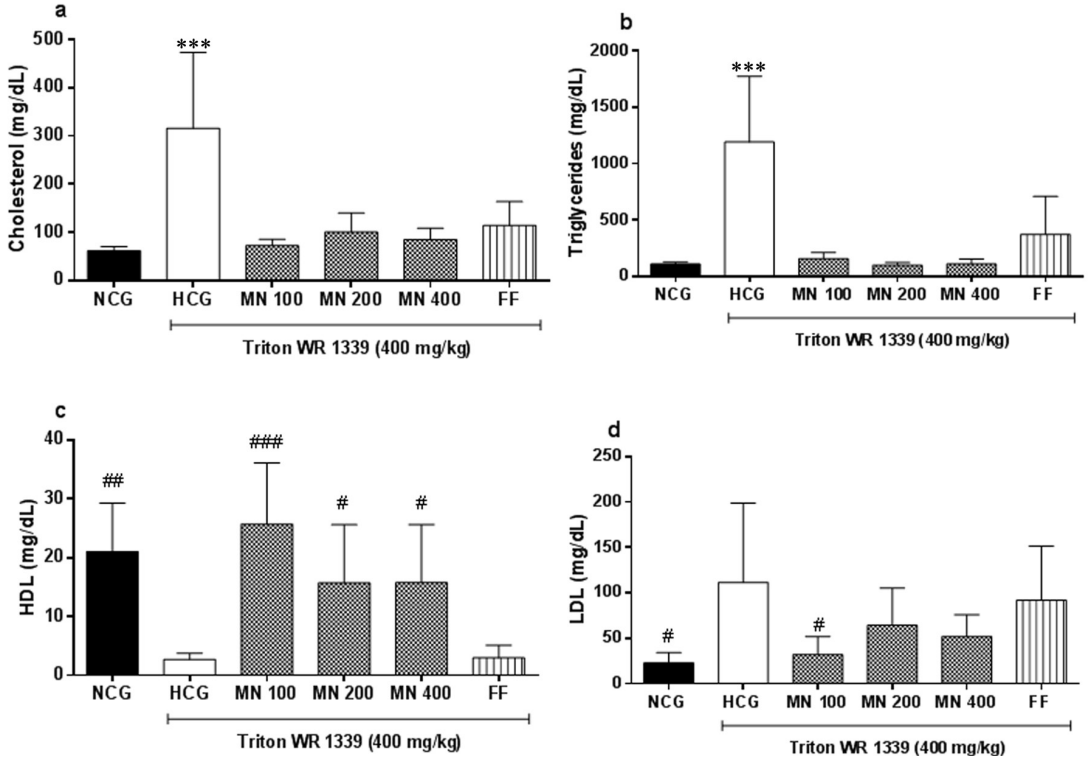

Figure 2 - Effect of *Morus nigra* infusion extracts (MN) or fenofibrate (FF) on serum total cholesterol (**a**), triglycerides (**b**), HDL (**c**) and LDL (**d**) levels in Triton WR-1339-induced hyperlipemic rats (HCG). Values are expressed as means ± SEM (n=6-8). ***p<0.001 HCG compared with other groups and ###p<0.001 or #p<0.05 compared with HCG group.

hyperlipidemic rats (p<0.001). Oliveira et al. (2013) after treating rats during 30 days with *Morus nigra* extract did not find alterations in hematological and biochemical parameters including TC and TG levels considering the extract of low toxicity.

As also depicted in Figure 2, only the group MN 100 demonstrated a significant decrement in LDL (p<0.05) although all doses of MN were capable to augment significantly HDL level, MN100 (p<0.001), MN200 and MN400 (p<0.05). However, FF group did not alter HDL level comparing with hyperlipidemic group (p>0.05). Finally, VLDL content (data not shown) decreased in serum of hyperlipidemic rats treated with MN extracts or FF groups (p<0.001). Previously, Volpato et al. (2011) showed decrease of TC, TG and VLDL levels in diabetic pregnant rats. To the best of our knowledge is the first time that *Morus nigra* leaves demonstrated capability to diminish TC, TG, VLDL and LDL with augmentation of HDL levels in hyperlipidemic rats. Nonetheless, popular hypolipidemic effect has been claimed for *Morus nigra* leaves (Volpato et al. 2011).

In this study, we identified great amounts of chlorogenic acid and quercetin in leaves of MN that could be sharing the responsibility for the hypolipidemic effect found here. Reinforcing this notion, some studies performed by Li et al. (2009) and Wan et al. (2013) reported the modulation of lipid metabolism by chlorogenic acid decreasing TC, TG and LDL but not increasing HDL levels through up-regulating the expression of hepatic peroxissome proliferator-activated receptor (PPAR-α). Although, Nishi and Kumar (2013) showed increment in HDL-c level besides, the improvement on the lipid profile. Several studies claimed the positive effects of quercetin in lipid metabolism, including TC and TG reductions (Ricardo et al. 2001, Kamada et al. 2005, Jung et al. 2013, Gnoni et al. 2009). Furthermore, Padma et al. (2012) demonstrated reduction in lipid levels and increasing in HDL-c in lindane-induced hyperlipidemia in rats by quercetin.

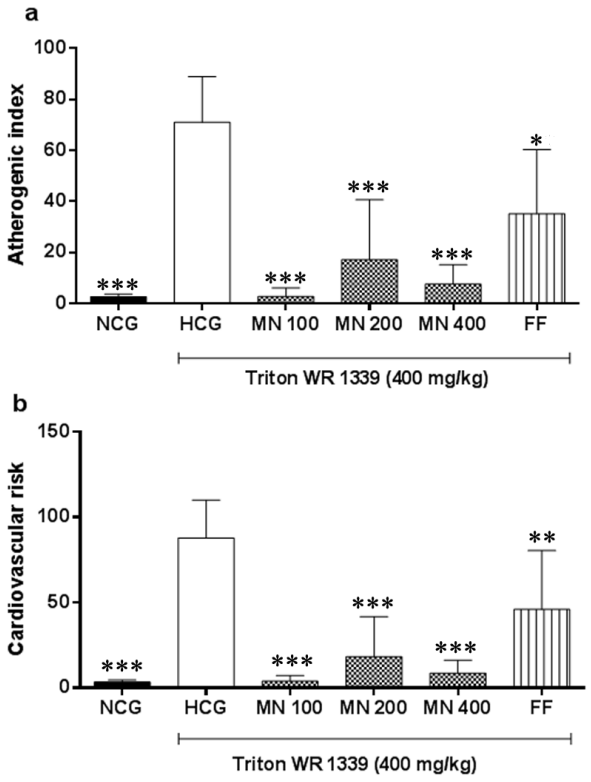

Figure 3 - The atherogenic index (**a**) and cardiac risk factor (**b**) of Triton WR-1339-induced hyperlipemic rats (HCG) treated with *Morus nigra* infusion extract (MN) or fenofibrate (FF). Values are expressed as means ± SEM (n=6-8). ***p<0.001 and **p<0.01compared with HCG group.

In this research, both atherogenic index (AT) and cardiac risk factor (CR), which express the risk of cardiovascular diseases, were markedly elevated in the HCG compared to NCG group. The MN was capable to decrease AT and CR (p<0.001) parameters compared with the HCG groups (Figure 3) without statistic difference among MN groups.

The effect of Triton WR-1339 on lipid peroxidation in the animal model tested showed that the argumentation of serum lipids was accompanied by increase on the TBARS levels in liver, kidney, cerebral cortex and hippocampus of rats (p<0.05 and p<0.001). Also Zarzecki et al. (2014) showed increase of TBARS levels in liver of rats treated with Triton WR-1339. The treatment with MN extracts and FF were capable to counteract the lipid

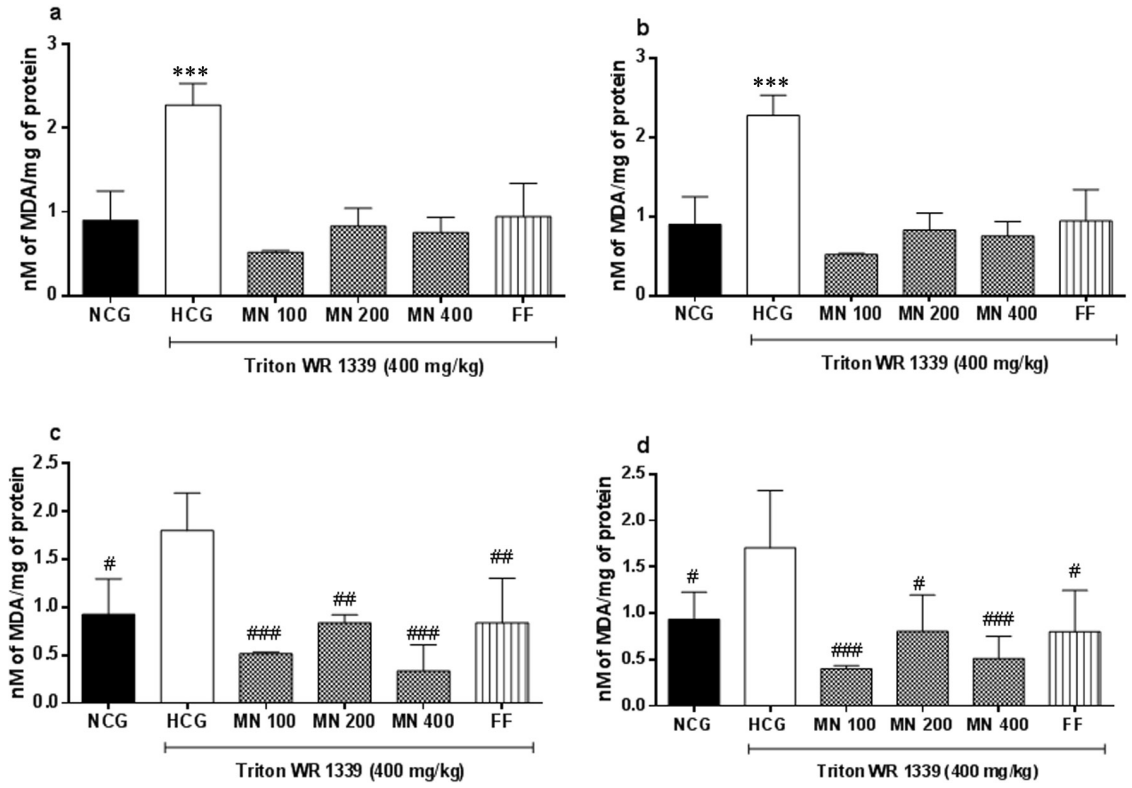

Figure 4 - Effect of *Morus nigra* infusion extract (MN) or fenofibrate (FF) on lipid peroxidation in the liver (**a**), kidney (**b**), cerebral cortex (**c**) and hippocampus (**d**) of Triton WR-1339-induced hyperlipidemic rats (HCG). Values are shown as means ± SEM (n=5). ***p<0.001 HCG compared with other groups, ###p<0.001, ##p<0.01 or #p<0.05 compared with HCG group.

peroxidation in all structures tested significantly (Figure 4a, b, c and d). Therefore, chlorogenic acid and quercetin, both identified in the infusion of *M. nigra,* were capable to decrease MDA levels in serum, erythrocytes, cerebral cortex, hippocampus and liver (Jung et al. 2009, Meng et al. 2013, Stefanello et al. 2014, Xia et al. 2015, Imessaouedene et al. 2016).

CONCLUSIONS

Herein, the content of phytochemicals in *Morus nigra* extracts was influenced by the extraction form, therefore, revealing that the infusion is a promissory rich fount of 05 known antioxidant phenolics identified. To our knowledge, this is the first study reporting the comparison among different extracts from *Morus nigra* leaves, mainly aqueous and a lipid-lowering effect exhibiting an increase of HDL level, an essential and difficult parameter to rise on lipid profile. Besides, we suggest that both chlorogenic acid and quercetin, at least in part, could be responsible for the MN hypolipidemic effect. In conclusion, we demonstrated the hypolipidemic effect popularly claimed for *Morus nigra* leaves and we suggested a therapeutic potential usage of the infusion in dislipidemic conditions.

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