Artículo original

Hepatoprotective effect of *Morus nigra* L. leaves on acetaminophen induced liver damage in rats

Efecto hepatoprotector de hojas de *Morus nigra L.* sobre daño hepático en ratas inducido por acetaminofeno

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ABSTRACT

Introduction: Acetaminophen, one of the most commonly used analgesic and antipyretic drugs, is characterized by its hepatotoxic effects after prolonged administration, due to the excessive formation of intermediate N-acetyl-p-benzoquinone imine, a product of phase I metabolism. Because of their chemical composition and antioxidant activity, *Morus nigra* L. leaves display therapeutic activity.

Objectives: Evaluate the hepatoprotective effect of *Morus nigra* L. hydroalcoholic extract on acetaminophen-induced liver damage in rats.

Methods: Twenty rats (*Rattus norvergicus albinus*) were distributed into four groups: control, acetaminophen (250 mg/kg) as hepatotoxicity control group, acetaminophen + silymarin (100 mg/kg) and acetaminophen + *Morus nigra* L. (250 mg/kg). Liver function enzymes ALT and AST were measured on days 1, 6, 12 and 21. Additionally, a histopathological study was conducted of liver sections.

Results: Acetaminophen raised ALT and AST levels, which remained high after administration of distilled water as placebo until day 21. Silymarin and *Morus nigra* L. leaf extract lowered ALT and AST levels to values similar to the control (baseline).

Conclusions: Results show that administration of *Morus nigra* L. improves the hepatic lesion caused by acetaminophen with an effect significantly similar to that of silymarin.

Keywords: alanine aminotransferase; aspartate aminotransferase; hepatoprotective; histopathology.

RESUMEN

Introducción: El acetaminofeno es uno de los fármacos analgésicos y antipiréticos más utilizados. Se caracteriza por los efectos hepatotóxicos que produce luego de una administración prolongada por la formación excesiva de N-acetil-p-benzoquinoneimina intermedia, producto del metabolismo en fase I. Las hojas de *Morus nigra* L. presentan actividad terapéutica debido a su composición química y capacidad antioxidante.

Objetivos: Evaluar el efecto hepatoprotector del extracto hidroalcohólico de *Morus nigra L.* en ratas con daño hepático inducido por acetaminofeno.

Métodos: Veinte ratas (*Rattus norvergicus albinus*) se dividieron en cuatro grupos: control, acetaminofeno (250 mg/kg) como grupo control de hepatotoxicidad, acetaminofeno + silimarina (100 mg/kg) y acetaminofeno + *Morus nigra* L. (250 mg/kg). Las enzimas de función hepática ALT y AST fueron medidas los días 1, 6, 12 y 21. Además, se realizó un estudio histopatológico en secciones de hígado.

Resultados: El acetaminofeno aumentó los niveles de ALT y AST, lo cuales se mantuvieron elevados luego de la administración de agua destilada como placebo hasta el día 21. La silimarina y el extracto de hojas *Morus nigra* L disminuyeron los niveles de ALT y AST hasta niveles similares del control (basal).

Conclusiones: Los resultados del presente estudio demostraron que la administración de *Morus nigra* L mejora la lesión hepática producida por el acetaminofeno con efecto significativamente similar al de la silimarina.

Palabras clave: alanina aminotransferasa; aspartato aminotransferasa; hepatoprotector; histopatología.

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Introduction

Liver diseases account for approximately 3.5% of all deaths per year worldwide. The main causes are complications of cirrhosis, viral hepatitis and hepatocellular carcinoma.[1] Liver damage arises from decreased metabolic functions of the liver caused by xenobiotics,[2] antibiotics,[3] chemicals or toxins,[4] virus,[5] alcohol,[6] among others. Studies on hepatoprotective effect generally use the induction of liver damage using acetaminophen (N-acetyl-p-amino-phenol, APAP) as an experimental model.[7] APAP is a highly effective analgesic, however at high or prolonged doses it can have negative effects, from acute liver toxicity to liver failure.[8] This is because at therapeutic doses APAP is metabolized by phase II reactions and it is excreted conjugated with glucuronic acid and sulfate, however, when the dose is high a part is metabolized by phase I reactions producing intermediate N-acetyl-p-benzoquinoneimine (NAPQI), that is normally detoxified through the interaction with cellular glutathione (GSH).

When GSH is depleted by the overproduction of NAPQI caused by the saturation of conjugation pathways in high doses, NAPQI binds to cellular macromolecules leading to oxidative stress, necrosis and cell death.[9,10,11]

A comparison pattern in studies of the hepatoprotective effect is silymarin (SN), which is a flavonolignan, obtained from seeds of *Silybum marianum* L., that has antioxidant, hepatoprotective, antidiabetic, antiinflammatory, antifibrotic and cytoprotective effects. The hepatoprotective mechanism of the SN is explained by an increase in the regeneration of hepatocytes, increasing the reduced level of glutathione in the liver, hence leading to a decrease in the binding of hepatotoxins to receptor sites in the hepatocyte.[12,13]

On the other hand, genus Morus are plants that belong to the Moraceae family, which are used in traditional medicine due to the presence of secondary metabolites that have shown bioactive effects, such as anti-inflammatory, antioxidant, antimicrobial, etc.[14]

Morus nigra L. (MN) is known as black mulberry and its leaves are used in traditional medicine to treat various conditions during menopause, for example. Likewise, several studies have demonstrated its therapeutic benefits, such as its antiobesity, antihyperlipidemic, antidepressant, neuroprotective,[15,16] antimicrobial,[17] cicatrizing,[18] antidiabetic,[19,20] antinociceptive,[21] antiinflamatory,[22] antispasmodic, analgesico, hipotensive,[23] hypocholesterolemic,[24] antiparkinsonian,[25] and anticarcinogenic effects,[26,27] its capacity as a natural origin bleach for to reduce hyperpigmentation of the skin[28] and its, also proved, use in the complementary treatment of snake bites.[29]

The biological properties of *Morus nigra* L. are potentially related to its antioxidant capacity, however, it has been shown that climate seasonal variations affects the performance of phytocomposites after the extraction process.[30] Various parameters, such as variety, geographical region, climate and stress, affect this performance, as well as, operational parameters such as the type of solvent, temperature, pressure, among others.[31] These parameters determine in most cases the composition of the extract and therefore the therapeutic activity.

The objective of this research was to evaluate the hepatoprotective effect of the hydroalcoholic extract of *Morus nigra* L. in rats with liver damage induced with acetaminophen.

Methods

Experimental animals

Twenty three month old male rats (*Rattus norvergicus albinus*) weighing 280±20 g were used in the study. The environmental conditions were kept constant: temperature was kept at 22±1°C, humidity at 55%, with a light/dark cycle of 12 h.[32] The rats were fed a standard diet for rodents with tap water ad libitum. The research was carried out in accordance with internationally accepted principles for the use and care of laboratory animals (1986 EEC Directive; 86/609/EEC). The procedures developed herein have been approved by the ethics committee of the Private University Autonomous of South.

Reagents

The acetaminophen and silymarin for the experiences were obtained from Sigma Aldrich and the ethanol for the extract from JT Baker. All the reagents used were analytical grade.

Extract

The leaves of *Morus nigra* L. (MN) were collected, and then taxonomically identified at the National University of San Agustín of Arequipa with code 062-2019-CIDEC-UNSA. The plants were cleaned dust and other debris were removed using deionized water[33] and then dried at room temperature for 72 h. To prepare the hydroalcoholic extract of MN, 70% ethanol and 20 g of powdered leaves were used, and the extraction method was

Soxhlet's. The solvent was completely evaporated under reduced pressure using a rotary evaporator at 60°C. Finally, the dried concentrated extract was stored at -20°C until the experiments were carried out.

Experimental design

The rats were divided into four groups containing 5 rats per group.

-Group 1. Control group, received 5 ml/kg of distilled water[34] by orogastric route during the 21 days of the study.

-Group 2. Liver damage induced by the administration of 250 mg/kg of acetaminophen[35,36] during 5 days. From day 6 to 21 the rats received a placebo instead of the acetaminophen treatment consisting of distilled water by orogastric route.

-Group 3. Liver damage induced by the administration of 250 mg/kg of acetaminophen during 5 days. From day 6 to 21, 100 mg/kg of silymarin[37,38,39] was administered by orogastric route.

-Group 4. Liver damage induced by the administration of 250 mg/kg of acetaminophen during 5 days. From day 6 to 21, 250 mg/kg of *Morus nigra* L.[40] was administered by orogastric route.

The liver samples were collected 21 days after the treatments. CO_2 was used as a euthanasia agent for laboratory rats.

Laboratory analysis

Before the administration of treatments, the basal levels of the liver profile were determined (day 1). For this purpose, after 12-hours fasting, a blood sample was taken from the tail of the experimental units using Archer's method : the tail of the rat was cleaned with pure alcohol and cotton, and then heated using an infrared radiation lamp to cause the vasodilation of the caudal vein, after which, the blood was extracted making peristaltic movements and finally, stored in capillaries, to determine the hepatic profile. Blood samples were taken using the same procedure on days 6, 12 and 21.

Biochemical analysis of the enzymatic activity

To evaluate the biochemical markers in liver lesions, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using the colorimetric method of Reitman and Frankel.[38,41]

Histological examination

The necropsy and the extraction of the liver were performed to observe its microscopic characteristics. The histological diagnosis of the samples obtained from the livers was made in the Pathological Anatomy laboratory of the National University of San Agustín in Arequipa through Hematoxylin Eosin (H&E) staining in a CAR-ZEIZ binocular optical microscope.

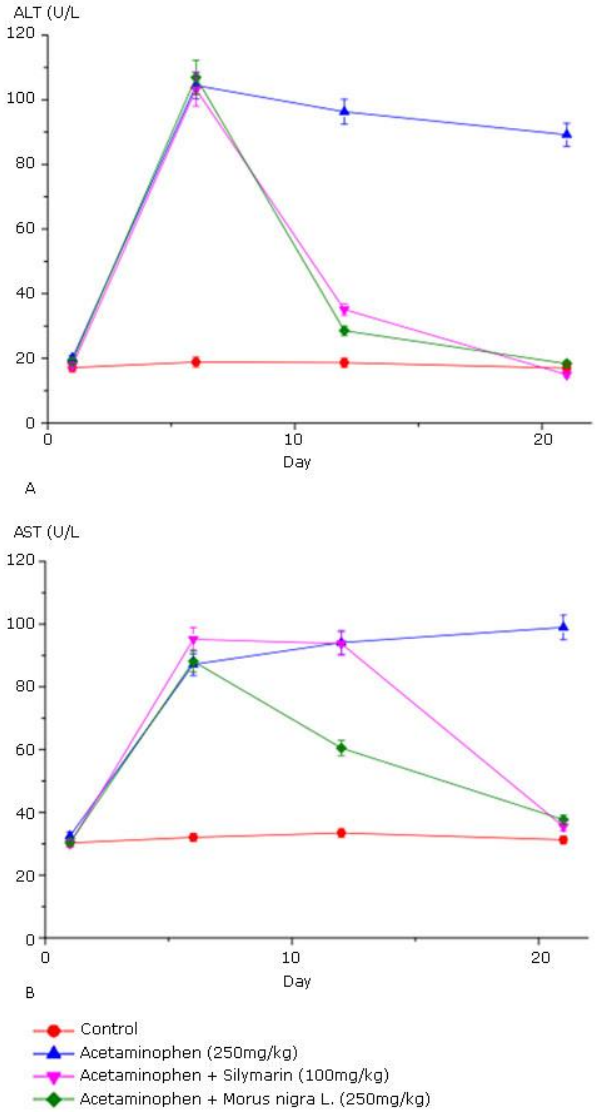
Statistical analysis

Statistical analysis was performed using OriginPro 9.0. The data were expressed as the mean±standard deviation (SD). Subsequently, multiple comparisons were analyzed using a one-way analysis of variance (ANOVA) at the end of the treatment (day 21). The Tukey test was used as a post hoc test[38] a value of $p < 0.05$ was considered as the existence of a statistically significant difference.

Results

Effect of *Morus nigra* L. on serum levels of ALT and AST

The results of the levels of ALT and AST evaluated are shown in Table 1. The levels of ALT and AST in rats raised after the induction of liver damage with 250 mg/kg of acetaminophen. After the administration of acetaminophen to rats in group 3 who were treated with a dose of 100 mg/kg of silymarin, they showed a significant decrease in ALT levels (Fig 1A.), however, AST levels decreased below the control group at day 21 (Fig. 1B.). On the other hand, rats treated with 250 mg/kg of *Morus nigra* L. after the induction of liver damage with acetaminophen, also showed a significant decrease in AST and ALT levels similar to the one attained using silymarin (Fig. 1A and B.).



The data presented are means±SD during days 1, 6, 12 and 21. They denote significant difference ($p < 0.05$) in at least one group at the end of treatment (day 21).

Fig. 1 - Effect of *Morus nigra* L. on ALT and AST levels after liver damage induced with acetaminophen.

Table 1 - Effect of *Morus nigra* L. AST and ALT levels on serum after liver lesions induced with acetaminophen in rats

Groups	ALT (U/L)				AST (U/L)			
	Day 1	Day 6	Day 12	Day 21	Day 1	Day 6	Day 12	Day 21
Control	17.13±1.23	18.84±1.26	18.64±1.33	16.94±1.49	30.25±1.32	32.05±1.49	33.45±2.27	31.25±1.30
Acetaminophen (250 mg/kg)	20.04±4.59	104.43±10.18	96.30±6.36	89.19±6.69	32.45±1.25	87.14±2.15	94.11±2.30	98.98±3.24
Acetaminophen + Silymarin (100 mg/kg)	19.04±2.49	106.94±12.10	28.55±1.90	18.34±1.73	30.46±2.19	88.16±2.52	60.51±2.56	37.68±3.16
Acetaminophen + *Morus nigra* L. (250 mg/kg)	17.74±1.23	103.22±8.09	35.09±6.07	14.93±0.82	30.06±1.62	95.17±3.76	93.77±2.01	35.47±1.36

The data presented are means ± SD during days 1, 6, 12 and 21.

The one-way analysis of variance (ANOVA) shows significant differences in at least one group at the end of the treatment (day 21) concerning levels of ALT and AST (p<0.05). Concerning the levels of ALT at day 21, data shows that there is no significant difference between the effect of *Morus nigra* L. and silymarin (p<0.05); *Morus nigra* L. and the control group (p <0.05); and *Morus nigra* L. and the control group (p<0.05), according to the Tukey's test. On the other hand, concerning AST levels at day 21, data shows that there is no significant difference between the effect of *Morus nigra* L. and silymarin (p <0.05), and *Morus nigra* L. and the control group (p<0.05). The one-way ANOVA of the levels of ALT and AST on day 21 resulted in values of p<0.05 for both cases, meaning that there was a significant difference within the groups. Subsequently, the post hoc analysis (Tukey's test) at 95% confidence shown in Fig 2 and 3 indicates that, regarding the final levels of ALT and AST (day 21) there is no significant difference between the hepatoprotective effect (HE) of the leaves of *Morus nigra* L. (250 mg/kg) and the effect of silymarin (100 mg/kg). On the other hand, the HE of the leaves of *Morus nigra* L. (250 mg/kg) allows the reduction of the levels of ALT to baseline levels, since there is no significant difference with the levels of the ALT of the control group at the end of the treatment (p>0.05), which is not observed for AST levels. However, the decrease for the AST enzyme marker is also significant.

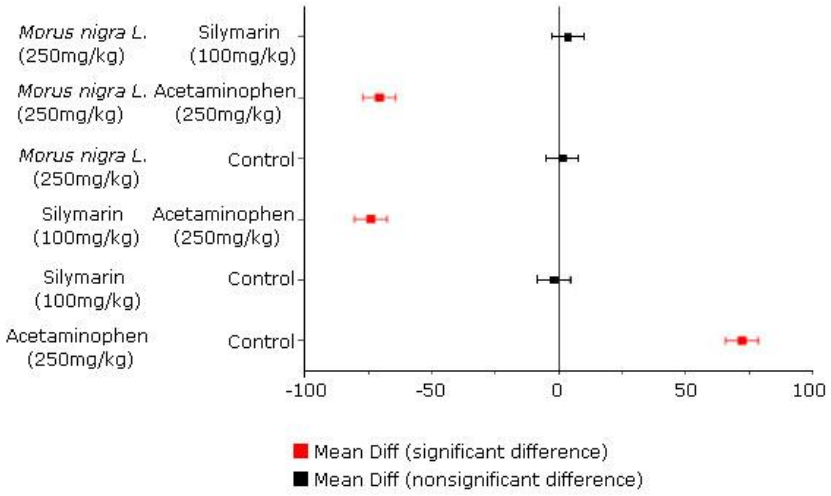


Fig. 2 - Means comparison of the ALT levels of the groups studied at the end of treatment (day 21) using Tukey's test.

If the difference approaches zero then there is no significant difference. The data presented are means ± SD, it shows that there is no significant difference between the hepatoprotective effect of *Morus nigra* L. and silymarin (p<0.05), *Morus nigra* L. and the

control group (p<0.05), and *Morus nigra* L. and the control group (p<0.05). On the other hand, the data shows significant difference between *Morus nigra* L. and the group with acetaminophen (p>0.05), silymarin and the group with acetaminophen (p>0.05), and acetaminophen and the control group (p>0.05).

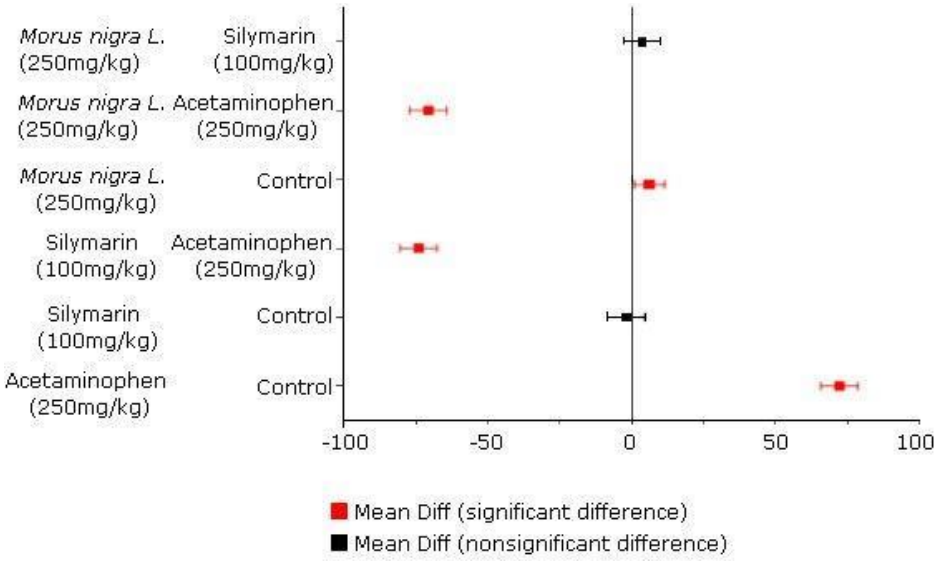


Fig. 3 - Means comparison of the AST levels of the groups studied at the end of the treatment (day 21) using Tukey's test.

If the difference approaches zero then there is no significant difference. The data presented are means ± SD and it shows that there is no significant difference between the hepatoprotective effect of *Morus nigra* L. and silymarin (p<0.05) and between *Morus nigra* L. and the control group (p<0.05). On the other hand, data shows a significant difference between *Morus nigra* L. and the group with acetaminophen (p>0.05), *Morus nigra* L. and the control group (p<0.05), silymarin and the group with acetaminophen (p>0.05); and acetaminophen and the control group (p>0.05).

Effect of *Morus nigra* L. on histopathological changes

The histopathological analysis performed by H&E staining on the studied groups is shown in Figure 4. In the histological sections of the livers of the control group rats, there were no histological alterations given that there are no signs of necrosis and the arrangement of hepatocytes is normal (Fig. 4A.). The hepatotoxicity of acetaminophen is observed in Fig. 4B. This group only received distilled water until day 21. The liver

photomicrograph shows signs of severe hepatocellular necrosis, as well as nuclear debris and an increase of Kupffer cells.

The histological cuts of the livers from the group of rats that received 100 mg/kg silymarin treatment of after induction of liver damage with acetaminophen shows signs of a slight necrosis, normal portal space, normal Kuffer cells, as well as a probable binucleate hepatocyte regeneration (Fig. 4C.). The 250 mg/kg treatment with *Morus nigra* L. clearly showed a histopathological improvement, since in Fig. 4D. signs of moderate necrosis are observed with a recovery of the hepatocyte arrangement and apparently normal Kupffer cells.

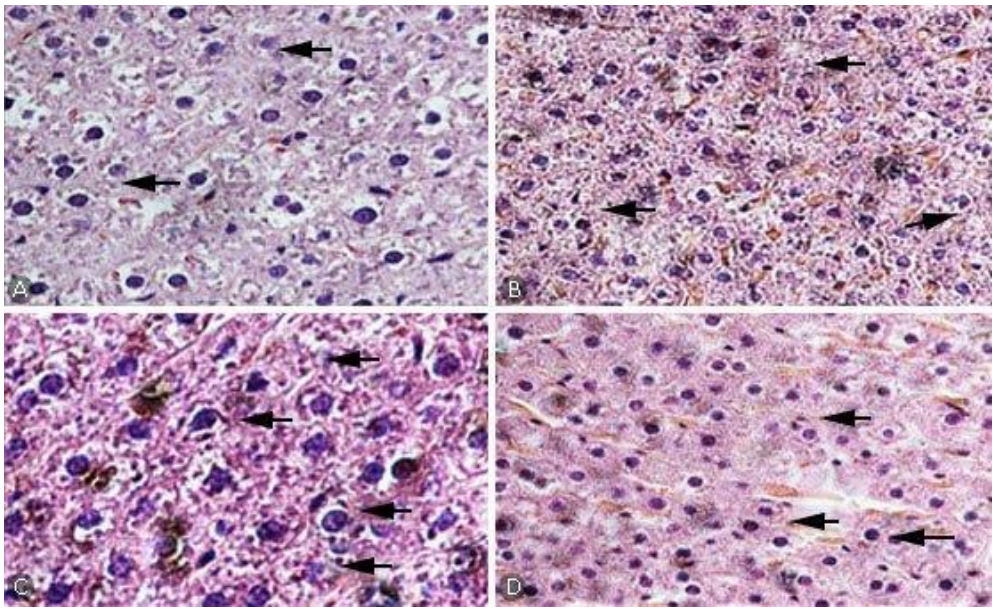


Fig. 4 - Microphotographs of liver sections stained with hematoxylin and eosin (H&E). **A.** Control group rat liver: there are no signs of necrosis. **B.** Rat liver lesioned with acetaminophen (250 mg/kg) and distilled water as a placebo: signs of severe hepatocellular necrosis, nuclear debris and increased Kupffer cells. **C.** Rat liver of the acetaminophen + silymarin group (100 mg/kg): signs of light necrosis, normal portal space, normal Kuffer cells. **D**. Rat liver of the acetaminophen group + *Morus nigra* L. (250 mg/kg): signs of moderate necrosis with recovery of hepatocytes arrangement and apparently normal Kupffer cells.

Discussion

Currently, acetaminophen or N-acetyl-p-aminophenol (APAP) is used to relieve pain, inflammations and as an analgesic, however, its prolonged use triggers the production of N-acetyl-p-benzoquinoneimine (NAPQI), that causes the death of liver cells, which is

why it was used to provoke hepatotoxicity in the present investigation. The APAP toxicity allows to observe specific aspects of acute liver failure in humans in order to propose new therapies, since APAP hepatotoxicity models closely mimic the entire process observed in patients, unlike other models that only simulate some aspects of the human disease.[42] Silymarin is a hepatoprotective agent widely used to treat liver lesions of different origin,[43] therefore, it was used as a standard for comparison in a 100 mg/kg dose.

A study evaluated the seasonal phytochemical profile and the antioxidant effect of the leaves of *Morus nigra* L. finding that the highest concentration of total phenols was observed in summer, flavonoids and carotenoids in spring, while ascorbic acid was more abundant during fall.[30] Another study reports variability between morphological and chemical characteristics of MN which influences directly the variability of total phenols.[44] These studies motivate a constant study of the pharmacological activity of the leaves of MN, because due to various factors, already exposed, the properties of MN could be related to the provenance of the species.

The consumption of substances with hepatoprotective effect is essential. The present study evaluated the benefits of the leaves of *Morus nigra* L. as a hepatoprotector after the administration of acetaminophen, since a previous study had shown that the ethanolic extract of MN did not have significant toxic effects when administered orally in rats under acute treatment. The extract was classified as safe, even showing a decrease in AST in males at doses of 750 and 1000 mg/kg and in females at 1000 mg/kg doses. It also produced a reduction in total cholesterol in female rats at doses of 750 and 1000 mg/kg.

Likewise, the study found quercetin and caffeic acid in the chemical composition of MN,[24] which could be related to the decrease in AST. The ethanolic extracts (EE) of MN caused a significant decrease in blood glucose level on fasting rats at two different doses (250 and 500 mg/kg) for the streptozotocin-induced diabetic rats model.[19] A treatment with 500 mg/kg of MN ethanol extract from showed a moderate improvement in the protection of liver cells with methotrexate (MTX) injury, so the study indicated that the joint administration of MN and MTX may prevent hepato-cytotoxicity caused by MTX.[45] On account of this study, a dose of MN of 250 mg/kg was used in the present research.

Blackberry leaves have proved to have various secondary metabolites. In the EE, chlorogenic acid, rutin, isoquercitrin,[28] anthocyanins (7.3 mg/100 g of dry matter),[46] β-sitosterol, quercetin-3 -O-glucopyranoside and kaempferol-3-O-glucopyranoside, rutin

and quercetin were found. The latter, related to the prevention of edema caused by serotonin and bradykinin.[29]

Concerning the hepatoprotective effect, studies have been performed administrating 250 and 500 mg/kg doses of methanolic extract (ME) of MN leaves before the administration of acetaminophen. Their results indicate that the MN extract reduces the levels of ALT and AST to levels comparable to those attained by silymarin, which is confirmed with the histopathological examination. This effect was related to the presence of luteolin, quercetin, and isorhamnetin.[40] In the present investigation with MN EE, a decrease in ALT and AST levels after liver damage with acetaminophen was also found. These effects were statistically similar to the effect of silymarin. In addition, the improvements were also noted in the analyzed histological sections. The results of the present study could suggest a similarity in the hepatoprotective effects of MN extracts obtained by maceration with methanol and by Soxhlet with ethanol.

Another study reports that the benefits of MN leaves are mainly due to their chemical composition. They have found total phenolic compounds (TPC) between 16.21-24.37 mg of gallic acid equivalent (GAE)/g, total flavonoids (TF) 26.41-31.28 mg of routine equivalent (RE)/g, 0.97-1.49 mg of ascorbic acid (AA)/g and an antioxidant capacity of 6.12-9.89 mM of Trolox equivalent/g of dried leaves, in the aqueous ME.[47]

Other studies on ME found that the pharmacological effects were related to components of MN such as betulinic acid, β-sitosterol, germanicol,[22] gallic acid, protocatechuic acid, p-hydroxybenzoic, vanyl, chlorogenic, syringic, p-coumaric acid, ferulic and m-coumaric,[48] morusin, U kuwanon, E kuwanon, P moracin, O moracin, A albanol, B albanol,[23] cyanidine 3-glucoside and cyanidine 3-rutinoside, quercetin 3-glycoside, rutin, caffeic acid and other derivatives of hydroxycinnamic and ellagic acid,[49] flavonol, mainly glycosylated forms of quercetin, kaempferol, caffeylquinic acids, simple phenolic acids and some organic acids.[50] Studies conducted to date show that the study of compositional properties of MN should be continued, since new compounds present in MN are still being discovered.[51,52]

From the present study it can be concluded that, according to the experimental hepatotoxic model of acetaminophen in rats, the hydroalcoholic extract of *Morus nigra* L. leaves has an hepatoprotective effect at the studied 250 mg/kg dose, which is comparable to silymarin. The hepatoprotective activity was evidenced through the levels of ALT and AST, and the histological examination.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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