

## Full Length Research Paper

# Antioxidant, hypolipidemic and preventive effect of Hawthorn (*Crataegus oxyacantha*) on alcoholic liver damage in rats

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The use of alcoholic beverages is more common and accepted by our society despite the health risks. Alcohol catabolism produces free radicals that cause oxidative stress and damage in liver principally. Hawthorn (*Crataegus oxyacantha*) is a medicinal plant that has been shown to have wide variety of polyphenolic compounds with antioxidant and hypolipidemic effect. The objective of this study was the evaluation of Hawthorn methanol extract as preventive treatment in alcoholic damage. A rat model of chronic alcoholic intake was generated with the administration of 3 g/kg/day in two times with 35 % ethanol for twelve weeks to evaluate the protective effect of 50 mg/kg/day for twelve weeks of Hawthorn administration by the determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT),  $\gamma$ -glutamyltranspeptidase ( $\gamma$ -GT), acid phosphatase (ACP), total bilirubin, liver glycogen, lipid peroxidation, serum total antioxidant capacity (TAC), total cholesterol, triglycerides, low density lipoproteins (LDL), and high density lipoproteins (HDL) levels in blood and hepatic tissues. Oxidative stress was evaluated by lipid peroxidation through MDA and TAC in the serum of animals. Lipid profile and glycogen was measured by LDL, HDL and glycogen concentration, respectively. Histological tissue cuts were visualized by hematoxylin eosin and Masson trichrome staining. Hawthorn treatment decreased AST, ALT,  $\gamma$ -GT and ACP activity in liver damage with a decrease of total bilirubin and an increase of liver glycogen stores in rats administrated with alcohol. Hawthorn showed an antioxidant and preventive effect decreasing liver lipid peroxidation levels and increasing serum TAC evidencing a hypolipidemic effect decreasing total cholesterol, triglycerides and LDL levels without affecting HDL levels. Our results indicate that Hawthorn exhibited a protective effect against liver damage in rats with chronic alcohol administration providing a possible alternative treatment for alcohol liver damage.

**Key words:** *Crataegus oxyacantha*, antioxidant, preventive treatment, hypolipidemic, alcoholic liver damage.

## INTRODUCTION

Today the use of alcoholic beverages is more common and accepted by our society despite the health risks. Acute and chronic alcohol intakes have shown to be a

big public health problem. Chronic alcohol consumption leads to various kinds of ailments as mainly liver and nervous system damage and other pathological conditions

produced by these diseases such as hepatitis, steatosis, cirrhosis, brain atrophy, psychological and social problems, accidents, alcohol dependence, violence, among other conditions (De Rick et al., 2009). The alcohol is metabolized in liver principally by alcohol dehydrogenase (ADH), an enzyme that converts alcohol into acetaldehyde. Later acetaldehyde is oxidized into acetic acid by aldehyde dehydrogenase (ALDH). The alcohol abuse conduces to ADH saturation and it is metabolized by other routes as P450 cytochrome (CYP2E1) and catalase releasing high levels of free radicals (FR) producing oxidative stress generating an imbalance between oxidant and antioxidant agents affecting cellular processes (Haseba, 2014; Schattenberg and Czaja, 2014). When cells remain exposed to oxidative stress the cell structures are damaged, including lipids, proteins and nucleic acids depending on exposure time. FR can act as secondary messengers in intracellular signaling cascades and the most serious damage to cells is the alteration of genetic information causing mutations in DNA and/or activation of the cell death pathway like apoptosis and necrosis (Albano, 2008; Djordjević et al., 2008). The FR causes lipid peroxidation, a reaction between FR and structural lipid from cell membranes. The malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are some of the principal products of lipid peroxidation (Long et al., 2006). The liver damage is very common during alcohol chronic consumption because liver is the principal organ responsible of metabolizing alcohol and it is one of the major organs affected for being in contact with FR production (Cederbaum et al., 2009).

Normally our organism can synthesize enzymatic antioxidants (e.g. superoxide dismutase [SOD], catalase, glutathione peroxidase and glutathione s-transferase) to neutralize FR and prevent oxidative stress generated in metabolic pathways. There are other antioxidants known as non-enzymatic antioxidants as glutathione and other functional antioxidant molecules (e.g. ubiquinol, uric acid and melatonin) (Hirst and Roessler, 2015; Michiels et al., 1994). Animals usually can get antioxidant compounds through daily diet to help their body to fight against oxidative stress fulfilling a function of "free radical scavengers" or exogenous antioxidants (e.g. vitamins A and E,  $\beta$ -carotene, phenolic acids, flavonoids and anthocyanidins) avoiding cellular oxidative damage in conjunction with endogenous antioxidants. The regulated endogenous antioxidants synthesis and exogenous antioxidant compounds intake balance are important to maintain homeostasis between oxidant and reducing agents. Plants are a major source of antioxidants and in many cases are related to its therapeutic effects from the

ethnopharmacological point of view. Hawthorn (*Crataegus oxyacantha* L, formerly known as *Crataegus laevigata*) is a fruit-bearing plant, member of Rosaceae family that grows mainly in Europe, Asia and North America. Hawthorn has proved be a potent antioxidant and medicinal plant because it has compounds as epicatechins, triterpene, saponins, oligomeric procyanidins, chlorogenic acid, flavonoids and flavon C-glycosides (isoquercitrin, hyperoside, orientin, isoorientin, quercetin, quercitrin, vitexin, isovitexin and rutin) (Konieczynski, 2015; Rigelsky and Sweet, 2002). Hawthorn has shown various therapeutic effects as mainly hypolipidemic, anti-inflammatory, immunomodulatory, digestive modulator, antimicrobial, anti-anxiety, antioxidant, cardiac stimulant and hypotensive agent (Benmalek et al., 2013; Elango and Devaraj, 2010; Vijayan et al., 2012). Most studies have focused on the use in cardiovascular diseases for their effectiveness in cardiac therapy. There are several studies that show a marked cardioprotective effect, reducing high blood pressure, lipid-lowering effect, cardiac arrhythmias, angina pectoris, myocardial infarction and an important use in congestive heart failure according to the New York Heart Association (Alp et al., 2015; Ammon and Händel, 1981; Jalaly et al., 2015; Rastogi et al., 2015; Wang et al., 2013). In alcoholism process, hyperlipidemic, inflammatory, and oxidant effects are general. Taking in count that Hawthorn has shown to reverse these effects we analyzed the antioxidant capacity, hypolipidemic and preventive effect of Hawthorn on alcoholic liver damage in rats.

## MATERIALS AND METHODS

### Plant extract

The leaves of Hawthorn were collected from cultivated plants in Tlalnepantla, México City, and were authenticated by *Nutra-Herbal de México Company*. Hawthorn extract was obtained by next procedure: Hawthorn's leaves were washed and dried at room temperature and then crushed and pulverized by a mill. Next we macerated it taking 50 g of powder per 500 mL of methanol. Subsequently distillation reflux was performed at 62°C for 2 h. After that the mixture was filtered in vacuum and it was bleached with 12.5 g of activated carbon (5 g of activated carbon per 20 g of plant) and newly filtered to obtain a light brown liquid. This extract was subjected to evaporation in a rotary evaporator system (Yamato, RE-51, CA, USA) at 70°C in order to concentrate the sample. The concentrate was transferred to a beaker with ice triple distilled water (30 mL) causing precipitation of the extract. Vacuum filtration was applied to allow drying at room temperature obtaining a light yellow, fresh filtrate. The filtrate was lyophilized at -60°C/1,333 Pa (Virtis SP SCIENTIFIC Sentry 2.0., PA, U.S.A.) and this powder was suspended in distilled water to 100 mg/ml.

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### **Animals and experimental protocols**

Male Wistar rats ( $n= 20$ ) weighing  $200 \pm 10$  g were randomly assigned to four groups of five rats each (control, liver damage, biosecurity and prevention group). Animals were treated for twelve weeks in a stable environment with controlled temperature ( $22 \pm 2^\circ\text{C}$ ), humidity and light (12 h light: Dark cycle) with free access to food (Harlan Teklad Global Diets) and water. All the experiments reported here are in accordance to university regulations on the care and handling of experimental animals and environment care (NOM-062-ZOO-1999 and NOM-087-ECOL-SSA1-2002 respectively). The control group was administrated orally with water. The liver damage group was administrated with a dose of 3 g/kg/day in two times (morning and night) with 35% ethanol. The biosafety group was administrated only with the Hawthorn extract in dose of 50 mg/kg/day; and finally the prevention group was administrated with ethanol (3 g/kg/day in two times) and Hawthorn extract in dose of 50 mg/kg/day (in the afternoon). At the end of the experimental study, animals were weighted and sacrificed under anesthesia in ether atmosphere to obtain total blood and serum by cardiac puncture in order to evaluate enzymatic and metabolic indicators. The liver tissue was removed to analyze glycogen, lipid peroxidation level and histological indicators.

### **Liver damage evaluation**

Liver damage was evaluated by measuring enzymatic and metabolic serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST),  $\gamma$ -glutamyltranspeptidase ( $\gamma$ -GT), acid phosphatase (ACP) and bilirubin by *BioSystems* kits in a spectrophotometer (Beckman DU-65, CA, U.S.A.).

### **Measure of glycogen concentration**

The Fong method (Fong et al., 1953) was used to perform liver glycogen concentration, which is based on the hydrolysis of glycogen to glucose units by potassium hydroxide in order to react with anthrone in sulfuric acid forming a green colored complex that is measured spectrophotometrically.

### **Lipid peroxidation**

Damage of tissues by oxidative stress induces the formation of oxidation products as MDA. This lipid peroxidation product was determined by measurement of thiobarbituric acid reactive substances (TBARS) described by Mihara and Uchiyama (Mihara and Uchiyama, 1978). 1,1,3,3-tetramethoxypropane (Aldrich, MO, USA) calibration curve was used to quantify MDA concentration.

### **Total antioxidant capacity (TAC)**

As an indicator of oxidative stress, we measured the total antioxidant capacity in the serum of animals. This indicator was analyzed by BioVision TAC colorimetric assay kit. For biological samples were taken 0.1  $\mu\text{L}$  of homogenized and 99.9  $\mu\text{L}$  of triple distilled water in each well of a microplate reader (STAT FAX 2100, FL, USA). A Tolox calibration curve was used to quantify TAC.

### **Lipidic profile**

These indicators were analyzed by *Bio Systems kits*. Lipidic profile was evaluated by measuring total cholesterol, triglycerides, low density lipoproteins (LDL) and high density lipoproteins (HDL).

### **Histological indicator**

Hematoxylin eosin (HE) and Masson trichrome staining (MTS) were used to identify cell damage with respect to the chronic administration of ethanol in liver tissue. Bencosme method was used to analyze this indicator (Bencosme, 1954) in a microscope Carl Zeiss Axiostar plus model 440950 CP ACHROMAT coupled with digital camera (Olympus, model C'7070 Wide Zoom).

### **Statistical analysis**

Data are presented as mean  $\pm$  standard deviation and were analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison. A discriminant classification analysis was made to observe the variability in the data obtained with respect to the measured indicators. We used STATGRAPHICS Centurion XV software for Windows. Differences were considered statistically significant at  $p < 0.05$ .

## **RESULTS**

### **Hawthorn decreases liver damage in rats with alcohol administration**

Rats with chronic alcohol administration were treated with 50 mg/kg/day of Hawthorn extract to evaluate its preventive effect in alcoholic liver damage. In present study several enzymatic indicators in liver damage as AST, ALT,  $\gamma$ -GT and ACP were analyzed. In case of ALT and AST, they are cytosolic enzymes. Their high serum levels indicate cell death as liver necrosis principally. In this study (Table 1), the liver damage group with administration of ethanol showed increases of 60.0% (1.74  $\mu\text{kat/L}$ ) and 72.3% (1.12  $\mu\text{kat/L}$ ) in AST and ALT respectively, compared to the average control value (0.65 and 1.09  $\mu\text{kat/L}$  respectively). Biosecurity groups proved no statistically significant differences compared to control groups, while treatment with Hawthorn evidenced a reduction of 18.4 and 7.0% in AST and ALT compared with liver damage group (1.42 and 1.04  $\mu\text{kat/L}$  respectively). The  $\gamma$ -GT and ACP enzymatic activity was analyzed too. The  $\gamma$ -GT is a membrane-bound enzyme in cells that have a secretory function as in liver and other secretory organs and ACP is a metabolic enzyme localized in high concentrations in liver, prostate, spleen and kidney lysosomes principally. The high serum levels of  $\gamma$ -GT and ACP can indicate damage in secretory organs as liver principally, therefore, enzymatic activity was analyzed in this work. The group of damage proved an increase of 82.1% (425.11 nkat/L) and 29.3% (525.88 nkat/L) in  $\gamma$ -GT and ACP respectively over the control group value (233.43 and 406.83 nkat/L respectively). In the case of prevention groups with Hawthorn administration, the groups evidenced a decrease of 38.5% (261.25 nkat/L) and 9.3% (477.1 nkat/L) in  $\gamma$ -GT and ACP respectively, compared with liver damage group. Biosecurity groups showed no statistically significant difference compared to control groups (Table 1). Glycogen is a polysaccharide formed in liver

**Table 1.** Hawthorn treatment diminishes enzymatic activity levels of AST, ALT,  $\gamma$ -GT and ACP in serum of rats with alcohol administration.

Groups	ALT ( $\mu$ kat/L)	AST ( $\mu$ kat/L)	$\gamma$ -GT (nkat/L)	ACP (nkat/L)
Control	0.65 $\pm$ 0.11 <sup>b</sup>	1.09 $\pm$ 0.12 <sup>b</sup>	233.43 $\pm$ 40.31 <sup>b</sup>	406.83 $\pm$ 23.29 <sup>b</sup>
Liver damage	1.12 $\pm$ 0.08 <sup>a</sup>	1.74 $\pm$ 0.10 <sup>a</sup>	425.11 $\pm$ 75.53 <sup>a</sup>	525.88 $\pm$ 73.62 <sup>a</sup>
Biosecurity	0.71 $\pm$ 0.11 <sup>b</sup>	1.12 $\pm$ 0.09 <sup>b</sup>	156.13 $\pm$ 33.42 <sup>b</sup>	397.46 $\pm$ 36.06 <sup>b</sup>
Prevention	1.04 $\pm$ 0.14 <sup>a</sup>	1.42 $\pm$ 0.10 <sup>a,b</sup>	261.25 $\pm$ 44.87 <sup>b</sup>	477.14 $\pm$ 50.09

<sup>a</sup>P<0.05 vs. control group; <sup>b</sup>P<0.05 vs. liver damage group. All data are expressed as mean  $\pm$  standard deviation (n=5 rats per group; experiment repeated three times). One way analysis of variance and least significant difference multiple-range test were used to determine the differences of means.

principally as glucose store and when there is liver damage these glycogen levels get low being an effective damage indicator. In this test (Figure 1A and C), the control group gave an average value of 5.4  $\mu$ g of glycogen per gram of liver tissue. The liver damage group for chronic ethanol administration showed a decrease in glycogen storage of 64.8% (1.9  $\mu$ g/g) compared to control group. Biosecurity group did not show significant difference compared to control group. With respect to prevention group with Hawthorn, it evidenced a decrease of 18.5% (4.4  $\mu$ g/g) compared to control group. Another indicator of liver injury is total serum bilirubin because the liver is responsible of its elimination and when there is damage in liver tissue, bilirubin uptake is low and accumulates in serum. Total bilirubin levels in control group showed an average value of 0.29 mg/dL, while liver damage group showed an increase of more than twice the value of the control group (0.68 mg/dL, that is, 2.3 times the control value). Biosecurity group proved no statistically significant difference compared to control group. The group administered with Hawthorn treatment reached a bilirubin increase of 55.2% (0.45 mg/dL) compared to control group (Figure 1B and C).

### Hawthorn shows an antioxidant effect

In order to evidence Hawthorn antioxidant property over oxidative stress produced by alcoholic liver damage in this model, we evaluated liver lipid peroxidation level and serum TAC. In these experiments (Figure 2A and C), the control group showed an average value of 13.0  $\mu$ mol/L, while liver damage group proved an increase of 90.1% (24.8  $\mu$ mol/L) compared to control group. The biosecurity group showed no statistically significant difference. Using Hawthorn as treatment, preventive group evidenced a decrease of 14.2% (11.1  $\mu$ mol/L) compared to control group. Furthermore, TAC is a test where we can evaluate samples antioxidant level given by reducing compounds obtained for feeding or/and administration of antioxidant treatments. The levels of TAC in the control group serum showed an average value of 5.37 nmol Trolox equivalent, while the group of damage showed a decrease of 19.2%

(4.34 nmol Trolox equivalent) with respect to control group. The biosecurity group with Hawthorn showed a high TAC value (11.6 nmol Trolox equivalent, that is, 2.2 times the control value). With respect to prevention treatment groups, Hawthorn proved an increase of 53.6% (8.26 nmol Trolox equivalent) compared to control group (Figure 2B and C).

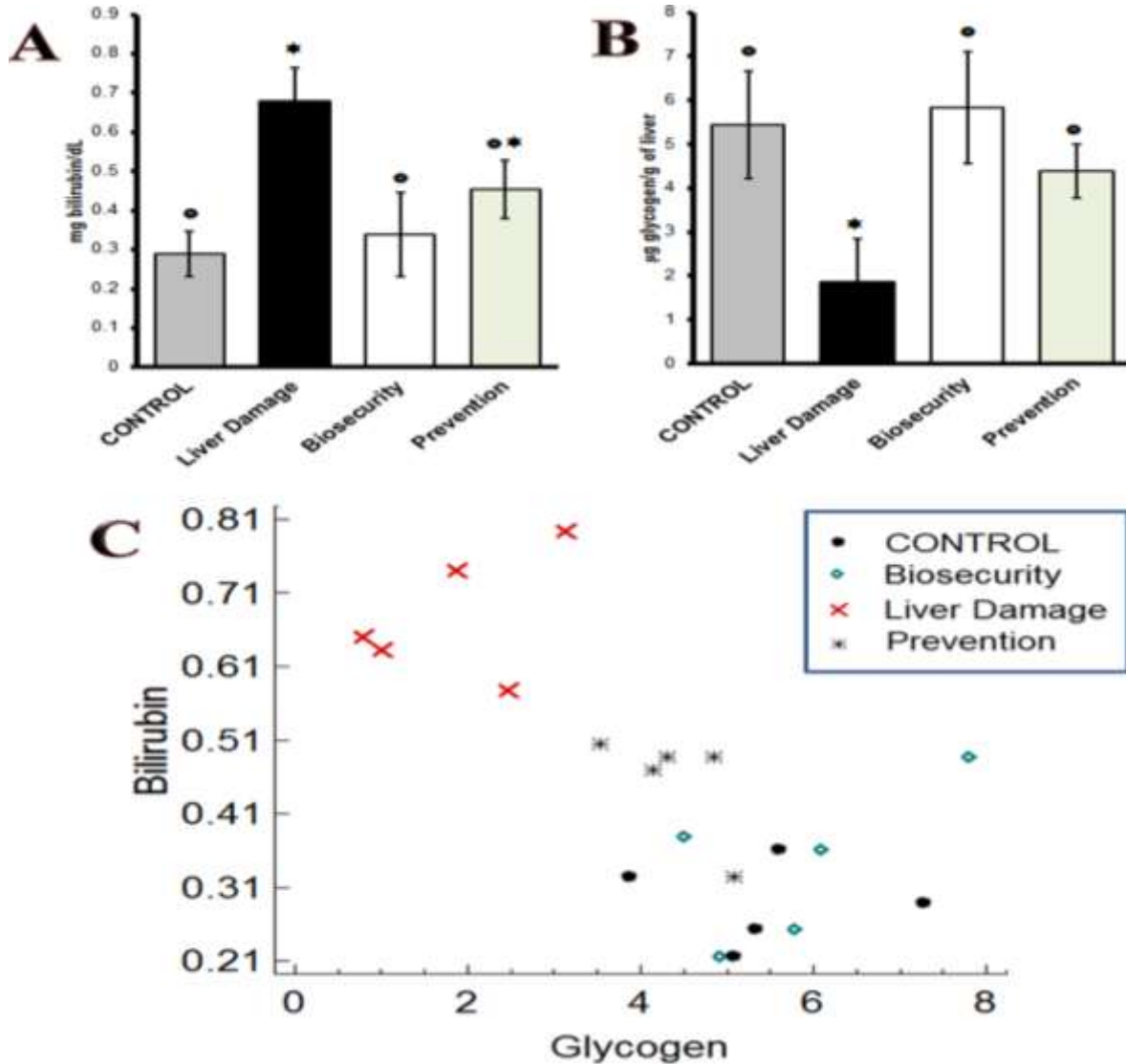
### Lipid levels in liver damage induced by chronic alcohol administration are regulated by Hawthorn treatment

One characteristic of alcoholic liver damage is the hyperlipidemia and steatosis for problems in metabolism and serum lipids uptake problems. Therefore we evaluated triglycerides, total cholesterol, LDL and HDL in serum samples. Triglycerides levels in control group showed an average value of 47.1 mg/dL, while liver damage group proved an increase of 54.5% (72.8 mg/dL). Biosecurity group gave values very similar to control group, while prevention group with Hawthorn evidenced a decrease in triglycerides of 38.0% (45.1 mg/dL) compared to liver damage group, normalizing triglyceride levels to normal value (Figure 3A and E).

The control gave an average value of 35.0 mg/dL of total cholesterol (Figure 3B and E) and liver damage group proved an increase of 67.3% (58.6 mg/dL) over the average control value. Biosecurity group average value did not show a significant difference compared to control value (42 mg/dL), while group of prevention with Hawthorn evidenced a slight increase in this indicator of 14.3% (40.0 mg/dL) compared to control group.

LDL indicator was analyzed (Figure 3C and E) and gave an average value of 33.1 mg/dL in control group, while the liver damage group showed a value of 54.1 mg/dL, that is, an increase of 63.4% compared to the control value. In biosecurity group, it proved a value of 36.5 mg/dL, while prevention group with Hawthorn evidenced an increase of 21.1% (40.1 mg/dL) compared to control group.

In HDL indicator (Figure 3D), control group showed an average value of 20.9 mg/dL, while liver damage group

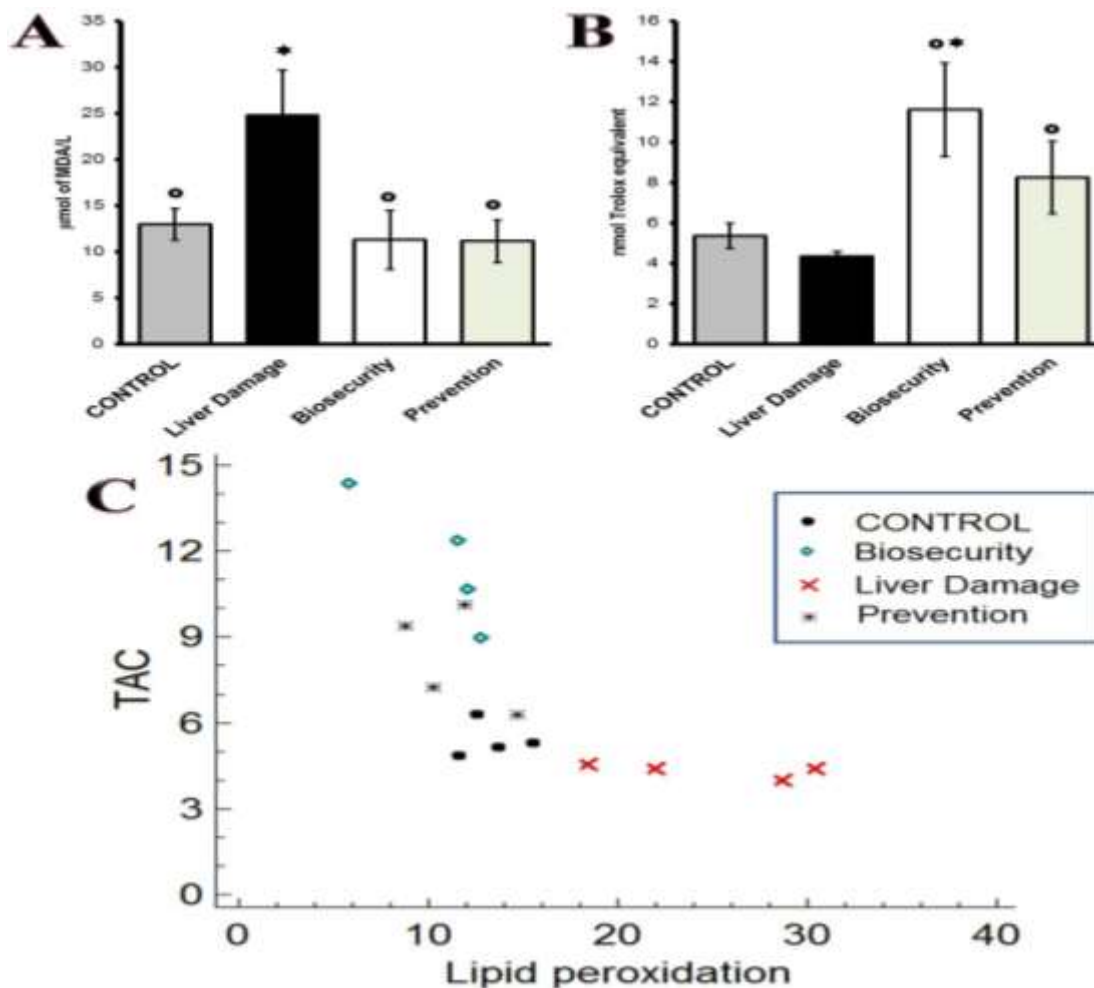


**Figure 1.** Hawthorn treatment increases liver glycogen stores and diminishes serum bilirubin levels in rats with alcohol administration. We analyzed liver glycogen concentrations and serum total bilirubin to evaluate liver cell damage and hawthorn preventive effect. (A) Liver damage group showed glycogen stores levels decrease of 64.8% while prevention group showed a decrease of 18.5% compared to control group. (B) Liver damage group showed high levels of serum total bilirubin compared to control group (2.3 times the control value) while co-administration of hawthorn as preventive treatment showed an increase of 55.2%. All biosecurity groups showed no statistically significant difference. (C) By a scatter diagram using a discriminant analysis we can see the relation between both indicators. Liver damage group shows isolated values compared to control and biosecurity group values indicating high levels of serum bilirubin and low glycogen levels in liver tissue demonstrating liver damage for chronic alcohol administration. Prevention group showed a dispersion of data values different to liver damage group data indicating an increase of liver glycogen and a decrease in serum total bilirubin compared to liver damage group demonstrating a preventive effect for the hawthorn co-administration. One way ANOVA and least significant difference multiple-range test were used to determine the differences of means. \* $P < 0.05$  vs. control group;  $\circ P < 0.05$  vs. liver damage group. All data are expressed as mean  $\pm$  standard deviation (n=5 rats per group).

gave an increase of 14.4% (23.9 mg/dL) compared to control group. Biosecurity group with Hawthorn showed a value of 22.4 mg/dL, while prevention group evidenced a value of 21.7 mg/dL, that is, an increase of 3.8% compared to control group (Figure 3).

#### Hawthorn prevents pathologic changes in liver tissue of rats with alcoholism

Figure 4 shows the liver structures in each experimental group. Control group (Figure 4A and B) showed a normal



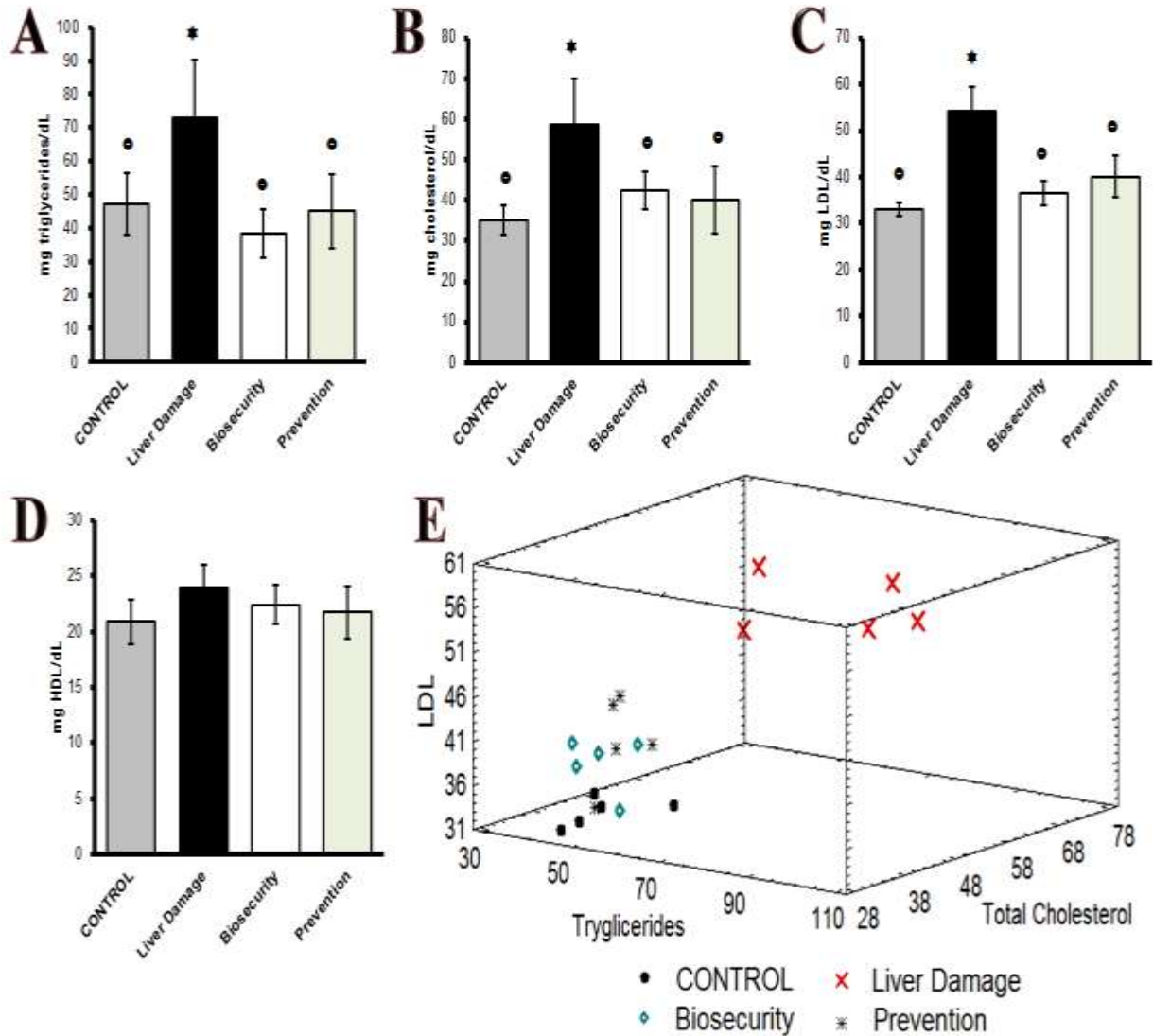
**Figure 2.** Hawthorn treatment diminishes liver lipid peroxidation level and increases serum TAC in rats with alcohol administration. We analyzed lipid peroxidation levels in liver tissue and TAC levels in serum to evaluate Hawthorn antioxidant property. (A) Hawthorn treatment showed a decrease of 55.1% in liver lipid peroxidation level compared to damage liver group. Hawthorn prevented oxidative stress caused by alcohol administration. (B) In TAC test, we can see an elevation of serum TAC when Hawthorn extract was administrated. Liver damage group showed a decrease of 19.2% compared to control group while prevention group showed an increase of 53.8%. In biosecurity group we can see a high elevation of TAC levels in more of two times control TAC value, indicating an efficient antioxidant effect. (C) Scatter diagram using a discriminant analysis shows the variability of each data in lipid peroxidation levels and serum TAC levels in the models. Graph shows a defined dispersion of each of the experimental groups. Liver damage group showed high oxidative damage and a low decrease of serum TAC compared to control group while prevention group indicates low oxidative damage (similar to control group) and high serum TAC levels. The results in lipid peroxidation levels were supported by TAC test. One way ANOVA and least significant difference multiple-range test were used to determine the differences of means. \* $P < 0.05$  vs. control group; ○ $P < 0.05$  vs. liver damage group. All data are expressed as mean  $\pm$  standard deviation ( $n = 5$  rats per group in lipid peroxidation indicator;  $n = 4$  rats per group in TAC test; discriminant analysis was done with  $n = 4$ ).

liver structure as it can be seen highly defined liver lobules around the central veins. The biosecurity group does not show pathologic changes in liver tissue indicating that hawthorn is a safe treatment to liver (Figure 4C and D). The liver damage group proved a loss of normal liver structure, cell congestion, cell necrosis, slight steatosis, liver fibrosis and sinusoidal distension (Figure 4E to I). Finally prevention group evidenced a liver

damage decrease. It proved slight cell congestion, necrosis and sinusoidal distension not showing fibrosis (Figure 4J to L).

## DISCUSSION

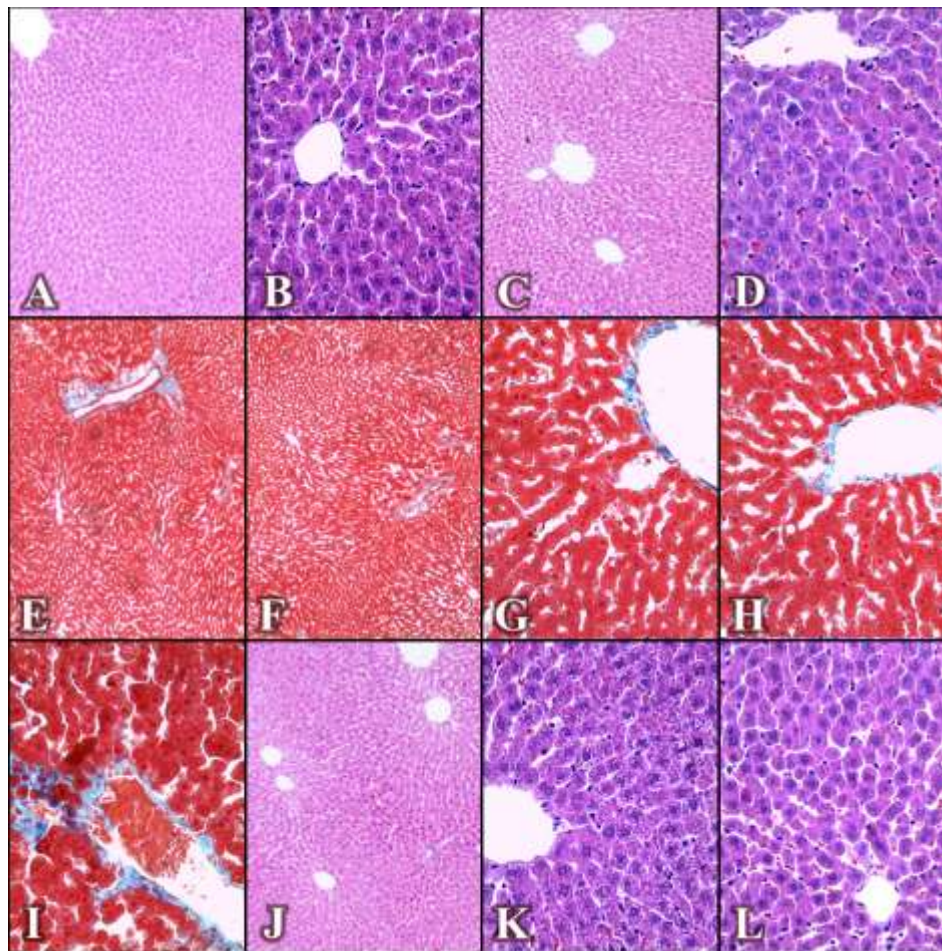
Hawthorn is a medicinal plant that has therapeutic



**Figure 3.** Hawthorn treatment shows a hypolipidemic effect in rats with alcohol administration. We analyzed serum lipid levels of triglycerides, cholesterol, LDL and HDL to evaluate liver damage and hawthorn preventive effect. (A) In triglycerides indicator, liver damage group showed an increase of 54.5% compared to control value while prevention group proved a decrease of 38.0% compared to liver damage group, normalizing to normal values. (B) Moreover high serum cholesterol levels were detected in liver damage group (increase of 67.3%) meanwhile prevention group showed a slight increase of 14.3%. (C) With respect to LDL indicator, liver damage group proved an increase of 63.4% compared to control value while prevention group showed an increase of 21.1%. (D) Finally in HDL indicator, the liver damage proved a no statistically significant difference of 14.4% compared to control group. Biosecurity and preventive groups did not show a significant change compared to control group HDL levels. (E) By a scatter diagram using a discriminant analysis it is shown the variability of each data in triglycerides, cholesterol and LDL levels in the models. Diagram shows that liver damage group has isolated values compared to control and biosecurity group data dispersion indicating high triglycerides, cholesterol and LDL serum lipid levels meanwhile preventive group proved similar values to control and biosecurity group. One way ANOVA and least significant difference multiple-range test were used to determine the differences of means. \* $P < 0.05$  vs. control group; \* $P < 0.05$  vs. liver damage group. All data are expressed as mean  $\pm$  standard deviation ( $n = 5$  rats per group).

properties and over time it has become an important part of traditional herbal. Hawthorn has a variety of phenolic compounds that have shown various therapeutic effects as epicatechins, triterpenes, saponins, oligomeric procyanidins and big diversity of flavonoids. Phenolic

compounds have attracted increasing attention from researchers. The antioxidants are necessary to maintain the balance between oxidant agents produced by metabolic pathways and antioxidants obtained in the diet and synthesized by our organism. The oxidative stress is



**Figure 4.** Hawthorn treatment diminishes tissue liver damage in rats with alcohol administration. We analyzed liver tissues to evaluate liver damage and hawthorn preventive effect. (A) Control group (10X, HE). Liver proved a normal structure with highly defined lobules around the central veins. (B) Control group (40X, HE). Histological image shows a normal liver structure with highly defined hepatocytes. (C) Biosecurity group (10X, HE). The Hawthorn administration does not show liver tissue damage. (D) Biosecurity group (40X, HE). Hawthorn treatment has proved a biosecure treatment for liver tissue showing a normal liver structure. (E and F) Liver damage group (10X, MTS). Liver proved cell congestion, fibrosis and high sinusoidal distension. (G, H and I) Liver damage group (40X, MTS). Liver showed a slight steatosis, necrosis and high sinusoidal distension. Moderate fibrosis was observed in certain areas of the liver lobule mainly near lobule central veins. (J) Prevention group (10X, HE). Liver proved a normal lobule structure with slight congestion and sinusoidal distension. (K and L) Prevention group (40X, MTS). Hawthorn reduces liver damage shown in liver damage group significantly. Liver proved in certain areas slight sinusoidal distension, cell congestion and necrosis not showing fibrosis.

a cell condition where there are high oxidant concentrations with respect to antioxidants promoting loss of cellular homeostasis and cell damage. The alcoholic liver damage is a pathology that presents FR formation by alcohol catabolism in the CYP2E1 route principally, generating oxidative stress and concluding to lipid peroxidation, inflammation, fibrosis, cell death and loss of parenchyma function (Cederbaum, 2003). Oxidative stress induces necrosis and apoptosis with a decrease in antioxidants as GSH and Vitamin E principally

(Loguercio and Federico, 2003). Taking in count that Hawthorn possesses an antioxidant effect, therefore it neutralizes FR and oxidative stress improving cell stability and avoiding and/or decreasing tissue damage, its therapeutic effect was evaluated. The Hawthorn hypolipidemic, antihypertensive and antioxidant properties reported by other studies are positive therapeutic effects in alcoholic liver disease.

In the present study, Hawthorn administration evidenced liver damage decrease for chronic alcohol



administration in a rat model. Hawthorn showed an AST and ALT serum activity reduction, indicating a cell preventive effect against oxidative stress, because normally aminotransferases levels are low on plasma and the presence of high ALT and AST levels in serum are related with cell damage, as in the case of liver and kidney damage principally. These aminotransferases are specific indicators to evaluate necrosis in various liver diseases (Gutierrez et al., 2010; Kozakova et al., 2012). The aminotransferases levels were corroborated with  $\gamma$ -GT serum activity test in order to analyze membrane stability.  $\gamma$ -GT is a membrane-bound enzyme present primarily in cells that have a secretory or absorptive function as liver and other organs as pancreas, prostate, brain and heart. It has been shown that  $\gamma$ -GT is considered a predictive biomarker of cellular antioxidant inadequacy and damage indicator in multiple diseases (Koenig and Seneff, 2015) similar to the present study. The injury produced by alcohol metabolism generates lipid peroxidation and cell lysis liberating in high concentrations this enzyme to plasma indicating cell damage. Hawthorn reduced  $\gamma$ -GT serum activity indicating a hepatoprotective effect. On the other hand, ACP is an enzyme localized mainly in lysosomes of prostate, kidney, spleen and liver and high concentration of ACP in serum indicates cell damage. Such effect could be related to cell destruction and entrance of cellular content to blood.

In this work it was shown an ACP level decrease demonstrating a preventive effect probably by inhibiting cell destruction. Interestingly, Hawthorn effect over AST, ALT,  $\gamma$ -GT and ACP serum activity levels were supported by liver function techniques as glycogen and bilirubin. As glycogen is a storage polysaccharide and it is in high concentration in liver its decrement is related to cell damage (Fong et al., 1953). Therefore, cell deaths or damaged cells have less glycogen levels in liver. Remarkably, the groups treated with Hawthorn showed normal glycogen level indicating a hepatoprotective effect of Hawthorn. Liver function improvement was also analyzed by measuring total bilirubin in serum. In this sense hyperbilirubinemia usually signifies severe parenchymal liver disease, anemia or renal failure and it could be used as prognostic indicator in cholestatic liver and hepatic failure. In contrast, other works suggest that high bilirubin levels are related with a significant antioxidant and preventive effect decreasing risk in diseases as hypertension by inactivating and inhibiting the synthesis of FR, (O'Malley et al., 2015; Wang and Bautista, 2015) but in the present investigation, taking in count the liver damage, a bilirubin antioxidant effect was not evidenced. Hawthorn shows a preventive effect in liver based on reduction of serum enzymatic activity indicators as the cytosolic enzymes as AST, ALT and ACP related with a decreased membrane-bound enzyme  $\gamma$ -GT indicating a reduction of cell lysis and cell death. These results could be related to a decrease of oxidative

stress by the Hawthorn antioxidant effect avoiding activation of cell death pathways. The antioxidant Hawthorn capacity was showed by liver MDA decrease and serum TAC increase. Hawthorn shows a variety of polyphenols, flavonol glycosides and C-glycosyl flavones which may relate to an antioxidant effect of this medicinal plant (Yang and Liu, 2012). However, Hawthorn could regulate gene expression, as it has being shown by the increase of mRNA and protein level of Nrf2-dependent genes as glutathione S-transferases (GSTA, GSTP, GSTM, GSTT), NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) in THLE-2 cells showing an antioxidant and detoxifying effect (Krajka-Kuźniak et al., 2014). For these therapeutic properties Hawthorn could prevent cell oxidative stress improving cell stability and decreasing cell damage for alcohol intake. The use of Hawthorn as preventive treatment in alcoholic liver disease shows a decrease in the progression of damage avoiding a significant pathologic change in the normal liver structure, fibrosis and steatosis.

Normally the alcoholic liver disease is accompanied by steatosis because ADH and ALDH cause the reduction of NAD into NADH. Reduction of NAD promotes fat accumulation in liver tissue for inhibition of metabolic pathways as gluconeogenesis and fatty acid  $\beta$ -oxidation, meanwhile lipid synthesis promotes liver tissue fat accumulation (Saheki et al., 2005). Other consequence of chronic ingest of alcohol is the increase of serum lipids as triglycerides, cholesterol and LDL with a respective decrease in HDL (Mantena et al., 2008). Hawthorn evidenced a regulator effect in serum lipids levels, decreasing LDL, triglycerides and total cholesterol without affecting HDL levels. Several works have shown that Hawthorn has an effect over LDL-receptors activity of rat liver plasma membrane isolated from atherogenic diet fed rats (Rajendran et al., 1996; Shanthi et al., 1994). The results obtained in  $\gamma$ -GT during chronic alcohol intake evidenced cell plasma membrane damage and this injury could be related with damage in LDL lipoproteins receptors, increasing its levels in plasma accompanied by high levels of triglycerides and cholesterol. In this study, it was proved that Hawthorn maintains normal levels of HDL; but a low increase of HDL level in liver damage group was observed. There are investigations about the effect of moderate alcohol intake that show an increase in HDL cholesterol levels by increasing the transport rate of apolipoproteins A-I and A-II (Silva et al., 2000).

## Conclusions

Hawthorn showed a preventive effect on liver damage caused by chronic ethanol intake. An antioxidant effect was detected, increasing serum TAC levels and decreasing lipid peroxidation in liver tissue, regulating glycogen levels and improving uptake and elimination of

bilirubin. This extract presented a hepatoprotective effect decreasing cell damage indicators as AST, ALT,  $\gamma$ -GT and ACP and avoiding loss of normal hepatic tissue structure, reducing fibrosis, steatosis, necrosis, congestion and sinusoidal distension. Hawthorn proved to be a therapeutic regulator of serum lipids as triglycerides, total cholesterol and LDL in alcoholic liver damage, decreasing high levels of these lipids without altering the levels of serum HDL. Hawthorn extract proved to be a safe treatment for healthy animals based on the indicators that we measured.

### Conflicts of interest

The authors declare no conflict of interest.

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