Protective effect of *Spirulina platensis* on fatty liver induced by a single sublethal dose of carbon tetrachloride in wistar rats

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Abstract: It has been reported that *Spirulina maxima* and other natural products are effective in attenuating hepatic damage. In this study were analyzed the effects of five days dietary *Spirulina platensis* (5%) in rats with fatty liver induced by CCl₄ (2 mL/kg b.w.). Animals were sacrificed at 24 and 48 h post-treatment. In the liver were evaluated total lipids by gravimetry and lipid profile by enzymatic-colorimetric methods, the concentration of thiobarbituric acid reactive substances and nitric oxide by chemical methods. In serum, alanine aminotransferase (kinetic method) and lipid profile were evaluated. The most important effects on the liver were: attenuation of lipid peroxidation, minimal variations on the total fatty acid methyl esters profile, and nitric oxide. These results suggest that *Spirulina platensis* could be used for fatty liver treatment as an alimentary supplement.

Keywords: TBARS, antioxidants, triacylglycerols, nitric oxide, cyanobateria, lipids.

Resumen: Se ha reportado que la *Spirulina maxima* y otros productos naturales son efectivos para atenuar el daño hepático. El objetivo del presente estudio fue evaluar los efectos de la *Spirulina platensis* dietaria (5%) durante cinco días en ratas con hígado graso inducido por CCl₄ (2 mL/kg p.c.). Los animales fueron sacrificados a las 24 y 48 h postratamiento. En el hígado se evaluaron los lípidos totales por gravimetria y el perfil de lípidos por métodos enzimático-colorimétricos, la concentración de sustancias reactivas al ácido tiobarbitúrico y óxido nítrico por métodos químicos. En suero fueron evaluados alanina aminotransferasa (método cinético) y perfil de lípidos. Los principales efectos sobre el hígado fueron: la atenuación de la lipoperoxidación, variaciones mínimas en el perfil de metil ésteres de ácidos grasos totales y del óxido nítrico. Estos resultados sugieren que la Spirulina platensis podría ser utilizada como suplemento alimenticio en el tratamiento de hígado graso.

Palabras clave: TBARS, antioxidantes, triacilglicerolos, óxido nítrico, cianobacteria, lipidos.
LIST OF ABBREVIATIONS
ALT: Alanine aminotransferase; AOAC: Association of Official Analytical Chemists; BHT: Butylated hydroxytoluene; CCl₄: carbon tetrachloride; FAMES: Fatty acids methyl esters; GC-MS: Gas chromatography-mass spectroscopy; NAFLD: Nonalcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; NO: Nitric oxide; Sp: Spirulina platensis; Sm: Spirulina maxima; TAG: Triacylglycerols; TBA: Thiobarbituric Acid; TBARs: Thiobarbituric acid reactive substances.

INTRODUCTION
Nonalcoholic fatty liver disease (NAFLD) includes a group of diseases ranging from hepatic steatosis until cirrhosis and liver failure (Obika and Noguchi, 2012). Nowadays it is recognized as a metabolic disorder characterized by fatty accumulation in the liver without alcohol consumption and it has been linked to metabolic syndrome (consisting of central obesity, hyperglycemia, dyslipidemia and hypertension) (Bogdanova et al., 2006; Cho, 2011). Although, the pathogenesis of non-alcoholic steatohepatitis (NASH) is multifactorial (Rolo et al., 2012), strong evidence from human and animal models indicates that inflammatory activation clearly plays a pivotal role in the progression of this disease (Braunersreuther et al., 2012; Farrell et al., 2012). Other proposed mechanisms in NAFLD pathophysiology include: increased oxidative stress, cytokine production, lipotoxicity and autoimmunity (Otogawa et al., 2007; Anderson and Borlak, 2008; Fon Tacer and Rozman, 2011; Rolo et al., 2012).

Although isolated fatty liver is thought to have a relatively benign natural history in reference to histopathologic progression of disease, NASH may progress to cirrhosis and finally to liver failure (Mirza, 2011). There are not sufficient epidemiological data about the prevalence of NAFLD in Mexico even liver diseases are leading causes of death in this country (Ferreira-Hermosillo et al., 2010). Therefore, effective treatments are needed.

Among others, weight reduction, ursodeoxycholic acid, vitamin E, metformin, and betaine have been used as modalities of treatment, none of them are totally effective (Akcam et al., 2011; Mukherjee, 2011; Ratziu et al., 2011; Caporaso et al., 2012; Enjoji et al., 2012; Farrell et al., 2012; Shargorodsky et al., 2012).

Natural products are growing up as an alternative treatment for hyperlipidemia, obesity and metabolic syndrome, since its minimal side effects and multiple ways to control lipid metabolism (Sakane, 2011; Molloy et al., 2012; Park et al., 2012). It has been demonstrated that Spirulina maxima (Sm) is effective in to ameliorate NAFLD induced by CCl₄ in animals (Torres-Duran et al., 2006), and NASH in human beings (Ferreira-Hermosillo et al., 2010), mainly due to its hypolipidemic (Torres-Duran et al., 2007) and antioxidant properties (Ponce-Canchihuaman et al., 2010).

Spirulina platensis (Sp) is a cyanobacterium, which grows naturally in alkaline lakes. Because of their high content of protein (60-70%), high concentration of essential aminoacids, and other nutritional elements, including B complex vitamins, vitamin E, manganese, zinc, copper, iron and selenium (Chamorro et al., 2002; Torres-Duran et al., 2007) Sm and Sp have been grown on non-natural conditions for production of commercial food supplements. Sm and Sp are excellent food supplements, recently assigned as a class A product by the Dietary Supplements Information Expert Committee (DSI-EC) of the United States Pharmacopeial Convention (USP) (Marles et al., 2011).

It has been demonstrated that Sp is an important candidate for Se enrichment (Kravchenko et al., 2008). Also, it was reported that Sp is a promising source for dietary Se supplementation (Kravchenko et al., 2008). Furthermore, Sp is an important source of pigments like β-carotene and other carotenoids, phycocyanine and chlorophyll (Jassey et al., 1971; Ciferri, 1983). Sp has essential fatty acids like ω-3 and ω-6 fatty acids (Colla et al., 2004); precursors of important metabolites like prostaglandins and leukotrienes.

Sp and Sm have several biological effects such as hypcholesterolemic effects, decreased cancer risk (Nakaya et al., 1988; Byers, 1992), and an attenuation of fatty liver in experimental models (Torres-Duran et al., 1998; Torres-Duran et al., 2006) and clinically assessed in humans (Ferreira-Hermosillo et al., 2010).

The aim of this study was to analyze the potential effects of Sp supplementation to decrease liver lipoperoxidation induced by CCl₄. It was measured lipid profile and fatty acid composition in the injured rat liver with the purpose to elucidate the possible mechanisms involved in the NAFLD development.
MATERIALS AND METHODS

Chemicals and reagents
The spray-dried powder of Sp was purchased from Genix (Empresa de producción y comercialización de microalgas y sus derivados; La Habana, Cuba). Purified diet AIN-76a (American Institute of Nutrition -76a) was purchased from ICN Pharmaceuticals (Mexico). Carbon tetrachloride, organic solvents and typical reagents were purchased from Merck (Mexico). Thiobarbituric Acid (TBA) was purchased from Sigma (St. Louis, MO). Cholesterol and triacylglycerols were measured using commercial enzymatic-colorimetric kits purchased from Jas (Mexico). Alanine aminotransferase activity kits (ALT) were purchased from Jas (Mexico).

Proximate analysis of Sp
A sample of Sp was analyzed by proximate analysis methods according to the Association of Official Analytical Chemists (AOAC): moisture, ether extract, protein, carbohydrates, crude fiber and ash. In addition, total lipids were extracted with Folch solvent (chloroform/methanol, 3:1, v/v).

Animals and treatments
Sixty male Wistar rats, weighing 190 - 250 g (purchased and bred in the Animal Care and Breeding Unit of the Facultad de Medicina, UNAM, Mexico City), were randomly allocated in two groups according to their diet: Control Group (control purified AIN-76a diet without Sp) and Sp group (experimental purified diet, AIN-76a diet with 5% Sp) according to previously dose used with Sm (Gonzalez de Rivera et al., 1993).

The distribution of the groups according to the diet and treatment is shown in Table 1. Animals were housed (groups of 2 or 3 rats per cage) in a room with controlled temperature (20 - 25° C) and light exposure (07:00 - 19:00 h) for five days before CCl₄ or vehicle (corn oil) treatment. The rats were fed on AIN-76 a diet (20 g of purified diet/day/per rat) with or without Sp throughout the experimental period. Water was provided ad libitum.

On fifth day of feeding respective diet, animals (12 h fasting) were treated either with a single intraperitoneal injection of CCl₄, 2 mL/kg of body weight with corn oil as vehicle (1:1, v/v) to induce fatty liver (Torres-Duran et al., 2006). Afterwards, 24 or 48 h after treatment, the animals were killed by cervical dislocation, after have been anesthetized with diethylether, this procedure according to the “Guiding Principles in the Use of Animals in toxicology”:

Table 1

<table>
<thead>
<tr>
<th>Diet/time after treatment</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN</td>
<td>Control</td>
<td>Control</td>
<td>CCl₄</td>
</tr>
<tr>
<td>AIN+ 5% Sp</td>
<td>Control + Sp</td>
<td>Control + Sp</td>
<td>CCl₄ + Sp</td>
</tr>
</tbody>
</table>

Diet was provided throughout the experimental period (5 days pre-treatment plus 1 or 2 days after treatment). Control groups at 24 and 48 h were treated with the vehicle. The animals were conformed in ten groups with six rats each one.

Serum was obtained from blood centrifugation and stored at -78° C until use. Livers were carefully excised, weighed and stored at -78° C, adding 0.025 % of butylated hydroxytoluene (BHT) as antioxidant, until lipid analyses were performed.

Ethical statement
All procedures were performed in strictly observing the international (Animal Research: Reporting In Vivo Experimental, ARRIVE guidelines) (Kilkenny et al., 2012) and national guidelines for care and use of experimental animals (Laboratory Animals and Official Mexican Norms, NOM-062-ZOO-1999) (de Aluja, 2002). The project was evaluated and approved (registration project number 111-2008) by the Ethical and Research Commission, a dependence of Research Coordination of Facultad de Medicina, UNAM.

Liver total lipids
Total lipids were extracted with chloroform-methanol by a modified version of Folch’s method (Folch et al., 1957). For liver samples, 1 g of tissue was homogenized in 4 volumes of 0.05 M phosphate buffer, pH 7.2. Then, pH was adjusted to 6.0 by addition of HCl. This suspension was extracted three
times with 20 volumes of chloroform/methanol (3:1, v/v) each. The extract was washed with 10 mL of water; the organic fraction was evaporated under a nitrogen stream, then analytically weighed (gravimetric method), and stored at -78º C until total cholesterol, triacylglycerols (TAG), thiobarbituric acid reactive substances (TBARs), and gas chromatography-mass spectroscopy (GC-MS) of fatty acid methyl esters (FAMEs) analyses were performed.

Lipid peroxidation
Analysis was performed on total lipids according to TBARs reaction, a previous technique performed in our laboratory (Torres-Duran et al., 1998).

Nitric Oxide Production
The nitric oxide (NO) production was assayed measuring its stable derivative, nitrates. Samples of each liver (24 h CCl₄ groups, with or without Sp) were homogenized (1 g wet weight) by duplicate in 4 volumes of deionized water. Proteins in the homogenate were precipitated using 0.1 volume 28% trichloroacetic acid, mixed vigorously during 1 minute, after 10 min they were centrifuged at 8,000 x g during 8 min. The supernatant was adjusted at pH 7.0, then lyophilized and was 2.5 times concentrated from the original volume. Nitrates were analyzed as recommended by the supplier (ROCHE, Mexico). Briefly, nitrates were reduced by nitrate reductase in presence of non-limiting concentrations of NADPH and FAD incubated at 37º C during 30 min. The Griess reactive was added to the sample and incubated at 37º C during 10 min. The sample absorbance was read at 540 nm. The developed color was stable at least 1h at room temperature. Concentration of NO derivatives (nitrates) was normalized by protein content. The total protein concentration was performed in aliquots of the homogenate, using the Lowry method (Lowry et al., 1951).

Determination of serum and liver lipids
Serum and liver contents of TAG and TC were measured using commercial enzymatic-colorimetric kits (Jas, Mèxico).

Determination of serum ALT
Serum ALT enzyme activity was measured using commercial kits (Jas, Mèxico).

Fatty acids methyl esters (FAMEs)
Fatty acid methyl esters from liver lipids were prepared with anhydrous methanol using a concentrate of sulfuric acid, and recovered in hexane, as reported previously (Torres-Duran et al., 2006).

Gas chromatography-mass spectrometry analysis
We used GC-MS for specific determination of FAMEs, as previously described (Torres-Duran et al., 2006). For each liver an aliquot of lipid extract was analyzed by gas chromatography in a Hewlett Packard (HP 5890) gas chromatograph coupled to a mass selective detector (HP model 5972).

Statistical analysis
Results were evaluated by ANOVA for multiple comparison tests with a post hoc Tuckey test, according to data distribution using SPSS version 17.0 as statistical package. A p value of 0.05 or lower was considered significant.

RESULTS
The proximate analysis of Sp showed the following values, expressed as % dry base: 60% protein, 9% ash, 17% carbohydrates (including 3.2% crude fiber), and 14% total lipids extracted with chloroform/methanol (3:1, v/v), where 1.8% of total lipids were neutral lipids (diethyl ether extract).

In the present study, it was tested the hepatoprotective effects of Sp against oxidative stress on acute liver injury induced by CCl₄ in rats. Administration of CCl₄ to rats caused severe hepatic damage, as demonstrated by the significant increase of ALT activity in serum at 24 and 48 h post CCl₄ treatment (Figure 1); however, at 48 h a significant attenuation in rats fed on Sp was observed compared to rats fed without Sp (p < 0.01).

Total lipids from the liver showed an increasing trend in groups treated with CCl₄; however the differences were not significant.

After CCl₄ treatment, as expected, liver content of triacylglycerols was increased in both groups (Figure 2A); however, these lipids were significantly lower at 48 h in rats fed on Sp diet than in rats without Sp in their diet. In a similar way, the serum TAG values were higher at 48 h in both treated groups compared with the control group (Figure 2B), which coincides with the induction of fatty liver, as well as with the increase of ALT activity (Figure 1). Furthermore, in animals that were not treated with CCl₄, the serum TAG concentration was significantly lower in rats fed on Sp diet than in animals without Sp in their diet (Figure 2B).

On the other hand, liver total cholesterol content was decreased at 24 h post-treatment,
returning to the basal values at 48 h (Figure 2C). These findings were not associated with the serum TC since there were no significant changes vs. their respective control groups, although the cholesterol value was lower in the groups fed on Sp diet (75 ± 19.6 mg/dL) than in the groups fed on diet without Sp (99 ± 16.5 mg/dL), (n = 15, p < 0.01).

In addition, the liver levels of oxidative stress indicators after CCl₄ treatment were increased in the animals fed on diet without Sp (Figure 3). TBARs levels increased at 24 h and 48 h in groups fed without Sp (Figure 3A). In contrast, its concentration did not change in rats fed on 5% Sp at 24 h and 48 h (p < 0.01). As for nitric oxide production (Figure 3B), the results showed that the control groups had values between 2.5 and 5.0 nmol/mg protein (without or with Sp), while in the groups treated with CCl₄ and fed on diet without Sp at 24 h showed higher concentrations than those in Sp-fed groups (p < 0.01).

**DISCUSSION**

The proximate analysis in this study showed a protein level lower than the one obtained in another study where 70% protein content was observed (Ciferri, 1983). Other minimal differences were in ash content (5%), and total carbohydrates (19%) (Ciferri, 1983). The differences could be due to the different environment where Sp was cultured, since cyanobacteria may receive different nutrients and be influenced by the season of the year or inclination of sunlight (Ciferri, 1983). However, the values obtained in this study are similar to those obtained for Sm analysis in our study (Torres-Duran et al., 2006), where 60% protein content was found. Only crude fiber was higher in Sp than in Sm. Therefore, as explained for Sm, these very low values of dietary fiber could hardly explain the effects on lipid concentrations observed in the present study.
Effects of *Spirulina platensis* on serum and liver lipids. Liver (A) and serum (B) triacylglycerol levels, as well as total cholesterol concentration in the liver (C) were analyzed 24 and 48 h after CCl₄ treatment. Results are expressed as mean ± SD of n = 6 rats. Although liver TAG levels were significantly increased in all the CCl₄ treated groups, the p value is omitted by simplicity. Then, the difference between Control + CCl₄ vs. Sp + CCl₄ groups at 48 h is shown. Statistical analysis was carried out with one-way ANOVA and Tuckey post hoc test.
In the present study, we have demonstrated the hepatoprotective effect of Sp in the liver injury induced by CCl\(_4\). The administration of this hepatotoxin causes severe acute liver damage in rats, as demonstrated by the significant elevation of serum ALT activity, similar to AST (Aspartate aminotransferase) elevation seen in a previous work (Torres-Duran et al., 2006). The hepatoprotective effect was similar to the one obtained using Sm (Torres-Duran et al., 2006). Similarly, and according to our results, Lu et al., (2010), demonstrated that AST and ALT concentration were attenuated in rats fed on a diet with 6% Sp after treatment with acetaminophen and D-galactosamine like injury liver inductors. Additionally, they found that MDA concentration in liver, IL-18 mRNA expression and IL-18 serum levels decreased after Sp treatment, which could mean an effective protection against liver injuries through a decrease on lipoperoxidation and inflammation. Studies with different Spirulina species have demonstrated that it has hypolipidemic activity in rats with and without toxic substances (Iwata et al., 1990; Ble-Castillo et al., 2002). In the same way, in this study, the main observed effects on lipids were the decrease of TAG in liver and a transient decrease of cholesterol levels in liver.

**Figure 3**

Effects of *Spirulina platensis* on liver lipoperoxidation and nitric oxide production.

Thiobarbituric acid reactive substances concentration in total lipid extract (A), and Nitric oxide concentration in liver homogenate (B) were analyzed after CCl\(_4\) treatment. The results are expressed as mean ± SD of n = 6 rats. Statistical analysis was carried out with one-way ANOVA and Tuckey post hoc test.
After CCl₄ treatment, as expected, liver triacylglycerols were increased in both groups; however, lipids were significantly lower at 48 h in rats fed on a diet with Sp than in rats without Sp in their diet. The serum TAG concentration, at 48 h, was increased in both treated groups compared with their respective control groups, which coincides with the induction of fatty liver. Furthermore, serum TAG concentration in animals fed on diet with Sp showed a significant decrease compared to animals fed on normal diet (both groups without CCl₄ treatment). This hypolipidemic effect has been previously observed in rats (Torres-Duran et al., 2006), in hypertriglyceridemic individuals (Torres-Duran et al., 2007), and in carriers of steatohepatitis (Ferreira-Hermosillo, 2011).

On the other hand, liver total cholesterol concentration was decreased at 24 h, returning to the basal values at 48 h, but in contrast to the behavior observed with TAG, the cholesterol values were not associated to their serum levels; this observation has been found in other studies, in which it was attributed to the duration of the study because cholesterol clearance is slower compared to other metabolites (Nakaya et al., 1988) however, we are unable to present any other explanation. The TBARS content in groups treated with the hepatotoxin and fed on diet without Sp was significantly increased at 24 and 48 h; however, its concentration did not change in rats fed on 5% Sp. These effects are similar to those observed using the Sm (Torres-Duran et al., 2006) and confirm the hepatoprotective properties of genus Spirulina (Ferreira-Hermosillo et al., 2011).

<table>
<thead>
<tr>
<th>FAME (%)</th>
<th>14:0</th>
<th>16:0</th>
<th>18:0</th>
<th>SAT</th>
<th>16:1</th>
<th>18:2*</th>
<th>18:1</th>
<th>20:4</th>
<th>UNSAT</th>
<th>Ratio SAT/UNSAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>0.46 ± 0.29</td>
<td>39.26 ± 16.8</td>
<td>23.02 ± 8.1</td>
<td>62.75 ± 8.4</td>
<td>1.82 ± 2.2</td>
<td>13.56 ± 6.4</td>
<td>11.25 ± 6.4</td>
<td>14.10 ± 5.0</td>
<td>40.73 ± 5.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Control + Sp</td>
<td>34.75 ± 9.9</td>
<td>29.27 ± 6.4</td>
<td>64.03 ± 8.1</td>
<td>1.48 ± 1.1</td>
<td>25.74 ± 7.1</td>
<td>10.99 ± 5.2</td>
<td>8.62 ± 6.8</td>
<td>46.83 ± 5.0</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>24h CCl₄</td>
<td>1.49 ± 1.0</td>
<td>47.62 ± 112</td>
<td>17.40 ± 10.9</td>
<td>66.51 ± 7.7</td>
<td>1.92 ± 1.2</td>
<td>22.51 ± 13.0</td>
<td>20.16 ± 17.2</td>
<td>3.20 ± 1.7</td>
<td>47.80 ± 8.3</td>
<td>1.4</td>
</tr>
<tr>
<td>24h CCl₄ + Sp</td>
<td>0.79 ± 0.6</td>
<td>42.42 ± 6.5</td>
<td>11.45 ± 4.6</td>
<td>54.66 ± 3.9</td>
<td>2.34 ± 1.5</td>
<td>33.58 ± 9.9</td>
<td>2.66 ± 2.4</td>
<td>38.58 ± 4.6</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>48h CCl₄</td>
<td>0.64 ± 0.2</td>
<td>36.51 ± 2.9</td>
<td>12.73 ± 2.0</td>
<td>49.87 ± 1.7</td>
<td>1.03 ± 0.7</td>
<td>28.38 ± 2.1</td>
<td>25.25 ± 10.6</td>
<td>5.43 ± 3.4</td>
<td>60.09 ± 4.2</td>
<td>0.8</td>
</tr>
<tr>
<td>48h CCl₄ + Sp</td>
<td>52.09 ± 18.7</td>
<td>9.21 ± 1.9</td>
<td>61.30 ± 10.3</td>
<td>1.47 ± 0.8</td>
<td>13.53 ± 11.9</td>
<td>21.56 ± 7.4</td>
<td>36.56 ± 6.7</td>
<td>1.7</td>
<td></td>
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</tr>
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</table>

On the other hand, in brown adipose tissue, inhibition of the constitutive NO synthase by Nω-nitro-L-arginine methyl ester (L-NAME) causes a significant increase in the unsaturation index (Saha et al., 1997), whereas Zheng et al. (2002) reported that in rats, hepatic steatosis induced by total parenteral...
nutrition was protected by NO. Hence, it is plausible that the hepatoprotective action of dietary \( Sp \) in this study is related to its ability to increase the basal synthesis/release of NO, preventing the inducible overproduction (see Figure 3B), as reported for \( Sm \) specie (Paredes-Carbajal et al., 1997; Paredes-Carbajal, 1998; Paredes-Carbajal et al., 2001). Nitric oxide production was only analyzed at 24 h post-treatment because of the biological material was not enough at 48 h.

This is also in accordance with previous studies that provide strong evidence of the beneficial use of natural antioxidants in the treatment of NAFLD. Recently, the prevalence of non-alcoholic fatty liver disease (NAFLD) has been increasing in the world. Non-alcoholic steatohepatitis (NASH), one kind of NAFLD, was first reported by Ludwig et al. (1997). It is considered that fatty liver progresses further to NASH. This condition is more difficult to treat than alcoholic steatohepatitis, and can lead to liver cirrhosis or cancer. The successful therapeutic application of \( Sm \) has already been reported by Ferreira Hermosillo et al. (2010).

\( Sp \) contains high concentrations of antioxidants such as phycocyanine, xanthophylls, chlorophylls, carotenoids, and vitamin E, among others; therefore, it is possible that its protective effects may be related to these molecules (Kay, 1991; Chamorro et al., 2002).

**CONCLUSION**

Administration of \( Sp \) improves liver damage induced by CCl\(_4\) in Wistar rats, due to its antioxidant and hypolipidemic effects. It could be effective as a supplement in NASH treatment or hepatic diseases, since \( Sp \) has high concentrations of antioxidants.

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