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

[Efecto protector de la *Spirulina platensis* sobre el hígado graso inducido con tetracloruro de carbono, a una dosis subletal, en ratas Wistar]

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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 CCl_4 (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 in 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 CCl_4 (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 gravimetría 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, triacilgliceroles, óxido nítrico, cianobacteria y lípidos.

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LIST OF ABBREVIATIONS

ALT: Alanine aminotransferase; AOAC: Asociation of Official Analytical Chemists; BHT: Butylated hydroxytoluene; CCl_4: 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 CCl_4 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 hypocholesterolemic 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 CCl_4. 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 CCl_4 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 CCl_4, 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
Experimental design and distribution of the groups

Diet/time after treatment	0 h	24 h		48 h	
AIN	Control	Control	CCl_4	Control	CCl_4
AIN+ 5% *Sp*	Control + *Sp*	Control + *Sp*	CCl_4 + *Sp*	Control + *Sp*	CCl_4 + *Sp*

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 -78º C until use. Livers were carefully excised, weighed and stored at -78º 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, nitrites. Samples of each liver (24 h CCl_4 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. Nitrites 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 (nitrites) 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, México).

Determination of serum ALT

Serum ALT enzyme activity was measured using commercial kits (Jas, México).

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 CCl_4 in rats. Administration of CCl_4 to rats caused severe hepatic damage, as demonstrated by the significant increase of ALT activity in serum at 24 and 48 h post CCl_4 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 CCl_4; however the differences were not significant.

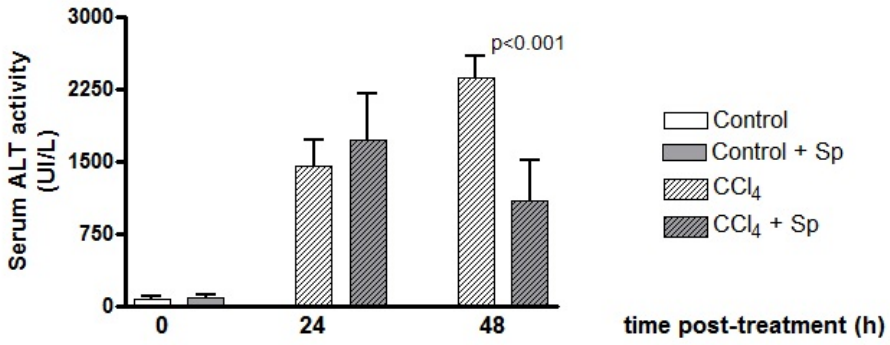
After CCl_4 treatment, as expected, liver content of triacylglycerols was increased in both groups (Figure 2A); however, these lipids were significantly lower at 48 h in rats fed on *Sp* diet than in rats without *Sp* in their diet. In a similar way, the serum TAG values were higher at 48 h in both treated 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 CCl_4, 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 CCl_4 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 CCl_4 and fed on diet without *Sp* at 24 h showed higher concentrations than those in *Sp*-fed groups ($p < 0.01$).

Figure 1
Serum alanine aminotransferase activity after CCl_4 treatment in rats.



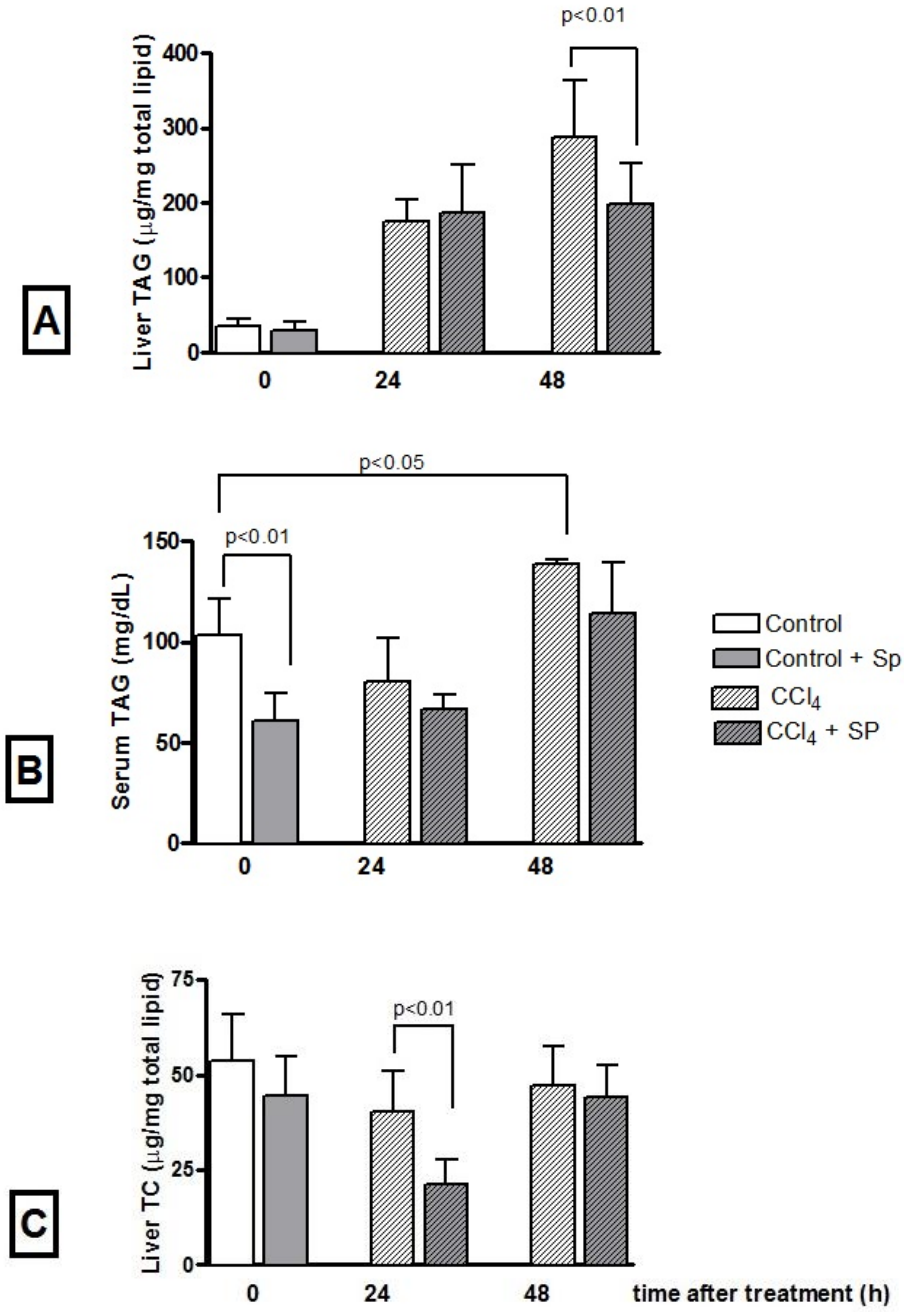
Values are expressed as U/L (mean ± SD of n = 6 rats). Statistical analysis was carried out with one-way ANOVA and Tuckey post hoc test. Although ALT activity was significantly increased in all the CCl_4 treated groups, the p value is omitted by simplicity. Then, the difference between Control + CCl_4 vs. Sp + CCl_4 groups at 48 h is shown.

The relative abundance (%) of the unsaturated and saturated fatty acids in control groups without CCl_4 treatment showed that saturated fatty acids explain almost 60% of the total identified FAMEs (16:0 > 18:0), whereas the abundance of unsaturated fatty acids was a little higher than 40% (18:1, 18:2, 18:3, 20:4, were present in about the same proportion). After CCl_4 treatment the saturated/unsaturated ratio was lower in animals fed on diet without *Sp* than those fed on diet with *Sp*, this change was in a inversed way than the one observed in control animals (see control group vs 48 h CCl_4 group in Table 2).

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.

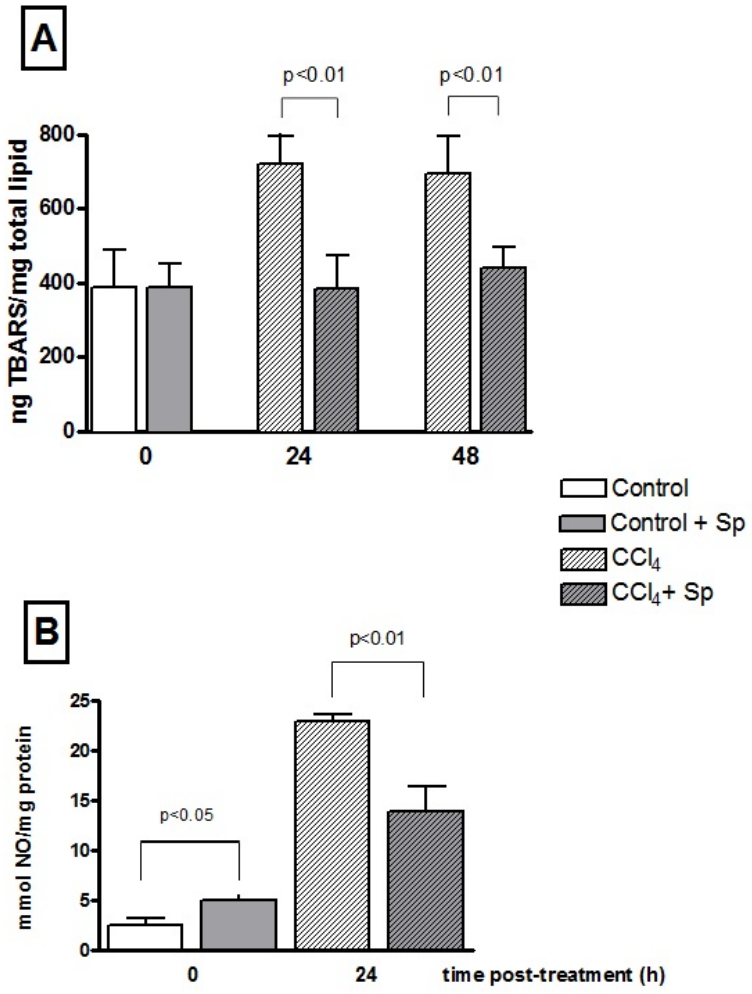
Figure 2



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 CCl_4 treatment. Results are expressed as mean ± SD of n = 6 rats. Although liver TAG levels were significantly increased in all the CCl_4 treated groups, the p value is omitted by simplicity. Then, the difference between Control + CCl_4 vs. Sp + CCl_4 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 CCl_4 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 CCl_4 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 2
Profile of fatty acid methyl esters (FAME) in total lipids of the liver.

FAME (%) / Group	14:0	16:0	18:0	SAT	16:1	18:2[a]	18:1	20:4	UNSAT	Ratio SAT/UNSAT
Control	0.46 ± 0.29	39.26 ± 16.8	23.02 ± 8.1	62.75 ± 8.4	1.82 ± 2.2	13.56 ± 6.4	11.25 ± 6.4	14.10 ± 5.0	40.73 ± 5.0	1.5
Control + *Sp*		34.75 ± 9.9	29.27 ± 6.4	64.03 ± 8.1	1.48 ± 1.1	25.74 ± 7.1	10.99 ± 5.2	8.62 ± 6.8	46.83 ± 5.0	1.4
24h CCl_4	1.49 ± 1.0	47.62 ± 11.2	17.40 ± 10.9	66.51 ± 7.7	1.92 ± 1.2	22.51 ± 13.0	20.16 ± 17.2	3.20 ± 1.7	47.80 ± 8.3	1.4
24h CCl_4 + *Sp*	0.79 ± 0.6	42.42 ± 6.5	11.45 ± 4.6	54.66 ± 3.9	2.34 ± 1.5		33.58 ± 9.9	2.66 ± 2.4	38.58 ± 4.6	1.4
48h CCl_4	0.64 ± 0.2	36.51 ± 2.9	12.73 ± 2.0	49.87 ± 1.7	1.03 ± 0.7	28.38 ± 2.1	25.25 ± 10.6	5.43 ± 3.4	60.09 ± 4.2	0.8
48h CCl_4 + *Sp*		52.09 ± 18.7	9.21 ± 1.9	61.30 ± 10.3	1.47 ± 0.8	13.53 ± 11.9	21.56 ± 7.4		36.56 ± 6.7	1.7

On the other hand, in the present study an increase of unsaturated fatty acid content was observed after CCl_4 treatment. The largest increase was found in the group fed on diet without *Sp* at 48 h post-treatment (see Table 2). It is known that CCl_4 treatment releases trichloromethyl radicals, causing cellular injury, and lipid peroxidation (Torres-Duran *et al.*, 2006); a second process involved in liver damage is the liberation of inflammatory mediators by macrophages and damaged hepatocytes, including nitric oxide; which is synthesized by both constitutive and inducible NO synthase; however, distinct roles has been proposed for nitric oxide concentrations in acute liver injury (Carnovale *et al.*, 2000; Morio *et al.*, 2001).

The antioxidant effect of dietary *Sp* is important for its hepatoprotective effects, keeping the saturated/unsaturated fatty acid ratio under minor changes (see Table 2). Higher concentrations of unsaturated fatty acids have been reported also in other conditions, in which there is liver damage, i.e., non-alcoholic steatohepatitis (Ghebremeskel *et al.*, 2002; Sato *et al.*, 2004; Torres-Duran *et al.*, 2006) and partial hepatectomy (Kishino *et al.*, 2000).

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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REFERENCES

Akcam M, Boyaci A, Pirgon O, Kaya S, Uysal S, Dundar B. 2011. Therapeutic effect of metformin and vitamin e versus prescriptive diet in obese adolescents with Fatty liver. **Int J Vitam Nutr Res** 81: 398 - 406.

Anderson N, Borlak J. 2008. Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. **Pharmacol Rev** 60: 311 - 357.

Ble-Castillo JL, Rodriguez-Hernandez A, Miranda-Zamora R, Juarez-Oropeza MA, Diaz-Zagoya JC. 2002. Arthrospira maxima prevents the acute fatty liver induced by the administration of simvastatin, ethanol and a hypercholesterolemic diet to mice. **Life Sci** 70: 2665 - 2673.

Bogdanova K, Poczatkova H, Uherkova L, Riegrova D, Rypka M, Feher J, Marchesini G, Vesely J. 2006. Non-alcoholic fatty liver disease (NAFLD)--a novel common aspect of the metabolic syndrome. **Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub** 150: 101 - 104.

Braunersreuther V, Viviani GL, Mach F, Montecucco F. 2012. Role of cytokines and chemokines in non-alcoholic fatty liver disease. **World J Gastroenterol** 18: 727 - 735.

Byers TPG. 1992. Dietary carotenes, vitamin C and Vitamin E as protective antioxidant in human cancer. **Rev Nut Ann** 12: 139 -159.

Caporaso N, Morisco F, Camera S, Graziani G, Donnarumma L, Ritieni A. 2012. Dietary approach in the prevention and treatment of NAFLD. **Front Biosci** 17: 2259 - 2268.

Carnovale CE, Scapini C, Alvarez ML, Favre C, Monti J, Carrillo MC. 2000. Nitric oxide release and enhancement of lipid peroxidation in regenerating rat liver. **J Hepatol** 32: 798 - 804.

Ciferri O. 1983. Spirulina, the edible microorganism. **Microbiol Rev** 47: 551 - 578.

Colla LM, Bertolin TE, Costa JA. 2004. Fatty acids profile of Spirulina platensis grown under different temperatures and nitrogen concentrations. **Z Naturforsch C** 59: 55 - 59.

Chamorro G, Salazar M, Araujo KG, dos Santos CP, Ceballos G, Castillo LF. 2002. Update on the pharmacology of Spirulina (Arthrospira), an unconventional food. **Arch Latinoam Nutr** 52: 232 - 240.

Cho LW. 2011. Metabolic syndrome. **Singapore Med J** 52: 779 - 785.

de Aluja AS. 2002. [Laboratory animals and official Mexican norms (NOM-062-ZOO-1999)]. **Gac Med Mex** 138: 295 - 298.

Enjoji M, Yasutake K, Kohjima M, Nakamuta M. 2012. Nutrition and nonalcoholic Fatty liver

disease: the significance of cholesterol. **Int J Hepatol** 2012: 925807.

Farrell GC, van Rooyen D, Gan L, Chitturi S. 2012. NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications. **Gut Liver** 6:149-171.

Ferreira-Hermosillo A, Torres-Duran PV, Juarez-Oropeza MA. 2010. Hepatoprotective effects of Spirulina maxima in patients with non-alcoholic fatty liver disease: a case series. **J Med Case Rep** 4: 103.

Ferreira-Hermosillo A, Torres-Durán, PV, Shamosh-Halabe, S, Juárez-Oropeza, MA. 2011. Biological effects of Spirulina and current research on its antioxidant activity. **Toctli RICTB** 2: 1 - 13.

Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. **J Biol Chem** 226: 497 - 509.

Fon Tacer K, Rozman D. 2011. Nonalcoholic Fatty liver disease: focus on lipoprotein and lipid deregulation. **J Lipids** 2011: 783976.

Ghebremeskel K, Bitsanis D, Koukkou E, Lowy C, Poston L, Crawford MA. 2002. Liver triacylglycerols and free fatty acids in streptozotocin-induced diabetic rats have atypical n-6 and n-3 pattern. **Comp Biochem Physiol C Toxicol Pharmacol** 132: 349 - 354.

Gonzalez de Rivera C, Miranda-Zamora R, Diaz-Zagoya JC, Juarez-Oropeza MA. 1993. Preventive effect of Spirulina maxima on the fatty liver induced by a fructose-rich diet in the rat, a preliminary report. **Life Sci** 53: 57 - 61.

Iwata K, Inayama T, Kato T. 1990. Effects of Spirulina platensis on plasma lipoprotein lipase activity in fructose-induced hyperlipidemic rats. **J Nutr Sci Vitaminol (Tokyo)** 36: 165 - 171.

Jassey Y, Berlot JP, Baron C. 1971. [Comparative study of the nucleic acids of 2 species of Spirulina: Spirulina platensis Geitler and Spirulina maxima Geitler]. **CR Acad Sci Hebd Seances Acad Sci D** 273: 2365 - 2368.

Kay RA. 1991. Microalgae as food and supplement. **Crit Rev Food Sci Nutr** 30: 555 - 573.

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. 2012. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. **Osteoarthritis Cartilage** 20: 256 - 260.

Kishino T, Tanno M, Yamada H, Saito S, Matsumoto S. 2000. Changes in liver fatty acid unsaturation after partial hepatectomy in the rat. **Lipids** 35: 445 - 452.

Kravchenko LV, Gladkikh OL, Gmoshinskii IV, Mazo VK. 2008. Selenium enriched spirulina and phycocyanin are sources of bioavailable selenium. **Vopr Pitan** 77: 63 - 65.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. **J Biol Chem** 193: 265 - 275.

Lu J, Ren DF, Wang JZ, Sanada H, Egashira Y. 2010. Protection by dietary Spirulina platensis against D-galactosamine--and acetaminophen-induced liver injuries. **Br J Nutr** 103: 1573 - 1576.

Ludwig J, McGill DB, Lindor KD. 1997. Review: nonalcoholic steatohepatitis. **J Gastroenterol Hepatol** 12: 398 - 403.

Marles RJ, Barrett ML, Barnes J, Chavez ML, Gardiner P, Ko R, Mahady GB, Dog TL, Sarma ND, Giancaspro GI, Sharaf M, Griffiths J. 2011. United States Pharmacopeia Safety Evaluation of Spirulina. **Crit Rev Food Sci Nut** 51: 593 - 604.

Mirza MS. 2011. Obesity, Visceral Fat, and NAFLD: Querying the Role of Adipokines in the Progression of Nonalcoholic Fatty Liver Disease. **ISRN Gastroenterol** 2011: 592404.

Molloy JW, Calcagno CJ, Williams CD, Jones FJ, Torres DM, Harrison SA. 2012. Association of coffee and caffeine consumption with fatty liver disease, nonalcoholic steatohepatitis, and degree of hepatic fibrosis. **Hepatology** 55: 429 - 436.

Morio LA, Chiu H, Sprowles KA, Zhou P, Heck DE, Gordon MK, Laskin DL. 2001. Distinct roles of tumor necrosis factor-alpha and nitric oxide in acute liver injury induced by carbon tetrachloride in mice. **Toxicol Appl Pharmacol** 172: 44 - 51.

Mukherjee S. 2011. Betaine and nonalcoholic steatohepatitis: back to the future? **World J Gastroenterol** 17: 3663 - 3664.

Nakaya N, Homma, Y, Goto, Y. 1988. Cholesterol lowering effect of spirulina. **Nutr Rep Int** 37: 1329 - 1337.

Obika M, Noguchi H. 2012. Diagnosis and evaluation of nonalcoholic fatty liver disease. **Exp Diabetes Res** 2012: 145754.

Otogawa K, Kinoshita K, Fujii H, Sakabe M, Shiga R, Nakatani K, Ikeda K, Nakajima Y, Ikura Y,

Ueda M, Arakawa T, Hato F, Kawada N. 2007. Erythrophagocytosis by liver macrophages (Kupffer cells) promotes oxidative stress, inflammation, and fibrosis in a rabbit model of steatohepatitis: implications for the pathogenesis of human nonalcoholic steatohepatitis. **Am J Pathol** 170: 967 - 980.

Paredes-Carbajal C, Torres-Durán PV, Rivas-Arancibia S, Zamora-González J, Mascher D, Juárez-Oropeza MA. 1998. Effects of dietary Spirulina maxima on vasomotor responses of aorta rings from rats fed a fructose-rich diet. **Nutr Res** 10: 1769 - 1782.

Paredes-Carbajal MC, Torres-Duran PV, Diaz-Zagoya JC, Mascher D, Juarez-Oropeza MA. 1997. Effects of dietary Spirulina maxima on endothelium dependent vasomotor responses of rat aortic rings. **Life Sci** 61: 211 - 219.

Paredes-Carbajal MC, Torres-Duran PV, Diaz-Zagoya JC, Mascher D, Juarez-Oropeza MA. 2001. Effects of the ethanolic extract of Spirulina maxima on endothelium dependent vasomotor responses of rat aortic rings. **J Ethnopharmacol** 75: 37 - 44.

Park HJ, Lee JY, Chung MY, Park YK, Bower AM, Koo SI, Giardina C, Bruno RS. 2012. Green tea extract suppresses NFkappaB activation and inflammatory responses in diet-induced obese rats with nonalcoholic steatohepatitis. **J Nut** 142: 57 - 63.

Ponce-Canchihuaman JC, Perez-Mendez O, Hernandez-Munoz R, Torres-Duran PV, Juarez-Oropeza MA. 2010. Protective effects of Spirulina maxima on hyperlipidemia and oxidative-stress induced by lead acetate in the liver and kidney. **Lip Health Dis** 9: 35.

Ratziu V, de Ledinghen V, Oberti F, Mathurin P, Wartelle-Bladou C, Renou C, Sogni P, Maynard M, Larrey D, Serfaty L, Bonnefont-Rousselot D, Bastard JP, Riviere M, Spenard J. 2011. A randomized controlled trial of high-dose ursodesoxycholic acid for nonalcoholic steatohepatitis. **J Hepatol** 54: 1011 - 1019.

Rolo AP, Teodoro JS, Palmeira CM. 2012. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. **Free Radic Biol Med** 52: 59 - 69.

Saha SK, Ohno T, Ohinata H, Kuroshima A. 1997. Effects of nitric oxide synthase inhibition on phospholipid fatty acid composition of brown adipose tissue. **Jpn J Physiol** 47: 477 - 480.

Sakane N. 2011. Pharmacology in health foods:merits and demerits of food with health claims for the prevention of metabolic syndrome. **J Pharmacol Sci** 115: 476 - 480.

Sato H, Mohamed T, Goto A, Oikawa S, Kurosawa T. 2004. Fatty acid profiles in relation to triglyceride level in the liver of dairy cows. **J Vet Med Sci** 66: 85 - 87.

Shargorodsky M, Omelchenko E, Matas Z, Boaz M, Gavish D. 2012. Relation between augmentation index and adiponectin during one-year metformin treatment for nonalcoholic steatohepatosis: effects beyond glucose lowering? **Cardiovasc Diabetol** 11: 61.

Torres-Duran PV, Ferreira-Hermosillo A, Juarez-Oropeza MA. 2007. Antihyperlipemic and antihypertensive effects of Spirulina maxima in an open sample of Mexican population: a preliminary report. **Lip Health Dis** 6: 33.

Torres-Duran PV, Miranda-Zamora R, Paredes-Carbajal MC, Mascher D, Diaz-Zagoya JC, Juarez-Oropeza MA. 1998. Spirulina maxima prevents induction of fatty liver by carbon tetrachloride in the rat. **Biochem Mol Biol Int** 44: 787 - 793.

Torres-Duran PV, Paredes-Carbajal MC, Mascher D, Zamora-Gonzalez J, Diaz-Zagoya JC, Juarez-Oropeza MA. 2006. Protective effect of Arthrospira maxima on fatty acid composition in fatty liver. **Arch Med Res** 37: 479 - 483.

Zheng JF, Wang HD, Liang LJ. 2002. Protective effects of nitric oxide on hepatic steatosis induced by total parenteral nutrition in rats. **Acta Pharmacol Sin** 23: 824 - 828.