International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.12, pp 873-883, 2016

The Potential Protective Impact of *Spirulina platensis* Against Thioacetamide-Induced Liver Fibrosis in Rats

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Abstract : The present study was carried out to investigate the possible protective role of *Spirulina platensis* in modulating hepatic fibrosis induced by thioacetamide. Forty eight male Sprague-Dawley rats were randomly allocated into four groups (Gps), twelve rats each; Gp1 received distilled water orally by gavage and served as control negative group, Rats of Gp2 were daily administered Spirulina in distilled water at a dose of 300 mg/kg body weight, Rats of Gp3 were intraperitoneally injected with thioacetamide (TAA) in distilled water twice weekly at a dose of 200mg/kg body weight. While, Rats of Gp4 were co-administered Spirulina and thioacetamide (TAA) as in Gp2 and Gp3. All treatments continued for eight weeks. Serum samples were separated and used for estimation of hepatic function tests, body and liver weights were estimated and tissue specimens were collected for histopathological and immunohistochemical studies. Our results revealed that Spirulina was able to improve body weight, liver function markers (AST, ALT, ALP and Bilirubin) and hinder the progress of fibroplasia induced by TAA to great extent. Spirulina co-administration induced marked decreased apoptotic figures among the hepatic parenchymal cells as well as decreased the immune positivity of α-SMA, which all suggesting its role as a hepatoprotective agent.
Key words: *Spirulina platensis*, Thioacetamide, Liver Fibrosis.

Introduction

Cirrhosis is a progressive disease and associated with severe clinical condition with considerable morbidity and high mortality. It leads to a wide spectrum of characteristic clinical manifestations, mainly attributable to hepatic insufficiency and portal hypertension[1]. Major complications include ascites, gastro-intestinal bleeding, hepatic encephalopathy (HE), renal failure, bacterial infections, and coagulopathy. Cirrhosis is also a risk factor for developing hepatocellular carcinoma (HCC) [2].

In contrast with the traditional view that cirrhosis is an irreversible disease, recent evidence indicates that even advanced fibrosis is reversible[3]. In experimentally induced fibrosis, cessation of liver injury results in fibrosis regression[4].

TAA is a classic hepatotoxic reagent used for induction of liver cirrhosis and has recently been used to induce fibrosis in rats. A decrease in the amount of sinusoidal endothelial fenestration and formation of a basement membrane-like structure in the Disse space were observed during the development of TAA-induced fibrosis in the rat [5].

TAA has been used extensively in the development of suitable animal models of acute and chronic liver injury[6]. More recently, the in vivo use of TAA in rodents as a model hepatotoxin produced highly selective liver damage including cirrhosis, fibrosis, hepatic necrosis and apoptosis[7].

The cirrhosis model induced by TAA in rats produces histopathological changes that are similar to those found in humans and animals and is considered as a valid model. Although histological and hemodynamics analysis show that the similarities between the TAA induced cirrhosis in experimental models and that in humans are greater than that observed in CC14-induced cirrhosis [8].

Spirulina is a microscopic blue-green filamentous alga that floats freely; it grows in fresh water, as well as in alkaline salt water. It is a cyanobacterium belonging to the class Cyanophyceae and the order Oscillatoriales[9].

Spirulina has been consumed for centuries in many parts of the world, ranking from the Aztec civilization in Latin America to the tribes that inhabit the Lake Chad region of central Africa. In 1996, the United Nations World Health Organization declared spirulina the best food for the future, and it has gained in popularity as a food supplement in recent years thanks to its high content of proteins and natural vitamins[10].

Currently, there is varying scientific evidence for its biological effects against health problems. In summary, the results of previous literature indicated that it was antioxidant, anti-inflammatory, hypolipemic, antihypertensive, antidiabetic, antimicrobial, neuroprotective, antianemic, immunostimulant, and anticarcinogenic and a hepatoprotector[9]. These activities were largely related to c-phycocyanin, an active protein of *Spirulina*, C-phycocyanin exhibits an anti-inflammatory, neuroprotective, hepatoprotective and anticancer activities[11].[12] indicated that the carotenoids derived from Spirulina had greater anti-hepatotoxic effects, compared to synthetic beta-carotene. The aqueous extracts of *Spirulina platensis* showed antiproliferative effects on Hepatic stellate cells (HSC) [13].

The aim of the study was to evaluate the potential hepatoprotective effect of *Spirulina platensis* (Sp) against thioacetamide (TAA) induced hepatic fibrosis.

Materials And Methods

Animals:

Forty eight male Sprague-Dawley rats (150-180 gm) were obtained from Helwan Animal Colony belonging to VACSERA. Rats were kept in plastic cages under standard hygienic conditions 6 rats/cage maximum, they were maintained in good ventilation, at a temperature of 25˚C ± 5˚C, 60% humidity, with suitable illumination conditions (light/dark cycle) and allowed free access to standard commercial rat pellets and water *ad libitum*. Animals were left one week for acclimatization to the laboratory conditions prior to use. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals (Intuitional Animal Care and Use Committee at Cairo University) approval number (CUIIS516).

Chemicals

Thioacetamide (TAA) was obtained from Sigma-Aldrich® Switzerland (≥99% purity, ASC Certified reagent) as white crystals.

Spirulina platensis was obtained as a fresh powder from Arabic Academy for Science, Technology and Maritime Transportation, in Alexandria Government, Egypt.

Experimental design

Rats were randomly allocated into four groups (Gps), twelve rats each; Gp1, received distilled water by gavage and served as control negative group, rats of Gp2 were daily administered *Spirulina platensis* in distilled water at a dose of 300 mg/kg body weight [14,15], rats of Gp3 were intraperitoneally injected thioacetamide (TAA) in distilled water twice weekly at a dose of 200mg/kg body weight[16]. While rats of Gp4 were daily co-administered Spirulina and intraperitoneally injected thioacetamide as in groups 2 and 3. All treatments were

continued for eight weeks**.** Rats of all groups were well monitored along the experimental period. Three animals from each group were euthanized under gentle diethyl ether anesthesia every two weeks prior to which blood samples were collected from the retro-orbital venous plexus for serum separation. Thorough PM examination was carried out for any abnormality in the liver.

Methods

Determination of body weight and Hepato/carcass index

Body weight was measured at the beginning of the experimental period as well as at the 2nd, 4th, 6th and 8th weeks post all administrations start. Liver was carefully dissected out, wiped of blood and weighted at each time of euthanization to calculate the hepato-carcass index.

Determination of liver function markers

Liver function markers AST, ALT [17] and ALP [18] as well as total bilirubin[19] were determined in serum samples of animals of all groups.

Histopathological examination

Representative liver specimens were collected from each animal and preserved in 10% neutral buffered formalin then routinely processed, dehydrated, cleared and finally embedded in paraffin**.** Paraffin blocks were serially sectioned at 4–5 µm thickness and stained with Hematoxyline and Eosin (H & E). Masson's trichrome stain was performed on need [20].

Immunohistochemical staining was performed on selected livers' sections of both control and all treated groups to evaluate the expression of alpha smooth muscle actin (α-SMA) [21] and caspase-3 as an apoptotic marker using avidin-biotin Peroxidase (DAB, Sigma Chemical Co.) according to method described by[22]**.** Briefly tissue sections were incubated with a monoclonal antibody to α-SMA and caspase-3, their expression was localized by the chromagen 3,3 -diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich®).

Statistical analysis Data were expressed as means ± SD and were analyzed using one way analysis of variance (ANOVA) within SPSS version 17.0 for windows. Duncan's multiple range test was used to differentiate between means at probability level of ($P<0.05$).

Results

Results of clinical follow up

No abnormal clinical signs or any deviation from the normal murine behavior were observed in rats of both control and Spirulina administered groups. However, rats of Gp3 showed severe depression with decreased appetite, loss of body condition and drowsy eyes with arched back and slight increase in the size of the abdomen. However, rats of Gp4 showed mild signs of depression and decreased appetite started at the 6th week of the experiment.

Body weight and Hepato/carcass index

The administration of TAA resulted in significant gradual decrease in body weight gain as well as significant increase in hepatocarcass index starting two weeks' post experimental onset. However, the co-administration of Spirulina with TAA in Gp4 resulted in significant restoration of both body weight gain and hepatocarcass index compared with control group **Table (1) and Fig. (1a and 1b).**

Table (1) Showing the mean values ± SD of rats' body weight (g) and hepatocarcass index

		Group (1)	Group(2)	Group (3)	Group (4)
Body weight (g)	2 weeks	172 ± 18.71	171±13.31	165±13.21	166±11.17
	4 weeks	176 ±14.9 [c]	182± 9.53 [c]	135± 8.34 [a]	150± 8.33 [b]
	6 weeks	184± 11.46 [c]	187± 9.81 [c]	127± 6.20 [a]	145± 15.7 [b]
	8 weeks	192±11.44 [c]	192± 6.77 [c]	127±1.70 [a]	143±4.5 [b]
Hepato-carcss index	2 weeks	2.3± 0.25 [a]	2.4± 0.05 [a]	3.5± 0.41[b]	2.6± 0.17 [a]
	4 weeks	2.6± 0.05 [a]	2.7± 0.05 [a]	4.2± 0.45 [b]	3.2± 0.45 [a]
	6 weeks	2.6± 0.11 [a]	2.6± 0.11 [a]	4.7± 0.11 [c]	3.1± 0.10 [b]
	8 weeks	2.6± 0.15 [a]	2.7± 0.25 [a]	4.5± 0.05 [c]	3.3± 0.15 [b]

Means with different letters (a, b, c) within the same row are significantly different at P value ≤ 0.05.

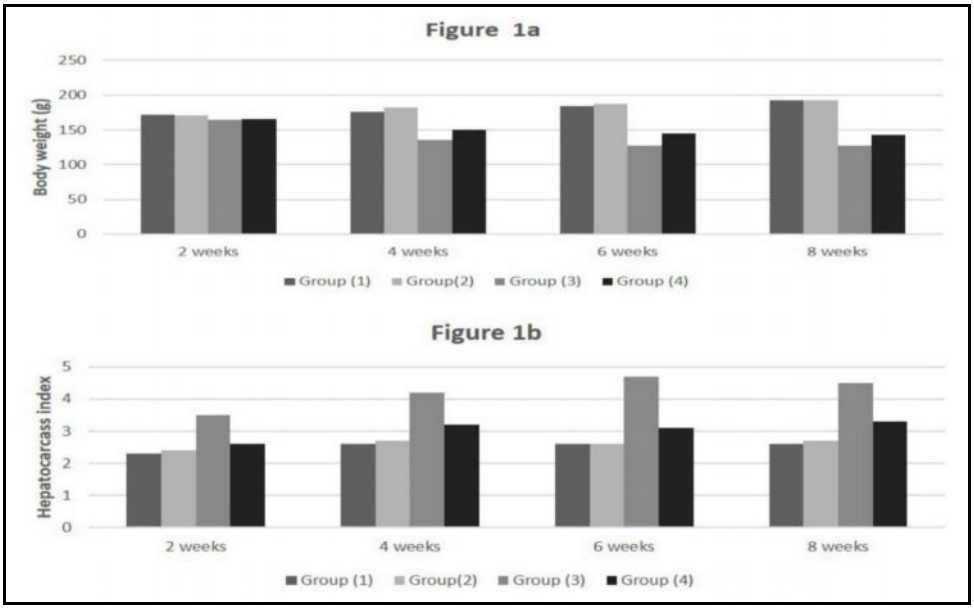

Figure (1) Showing the mean values ± SD of rats' body weight (a) and hepatocarcass index (b) in control and different treated groups.

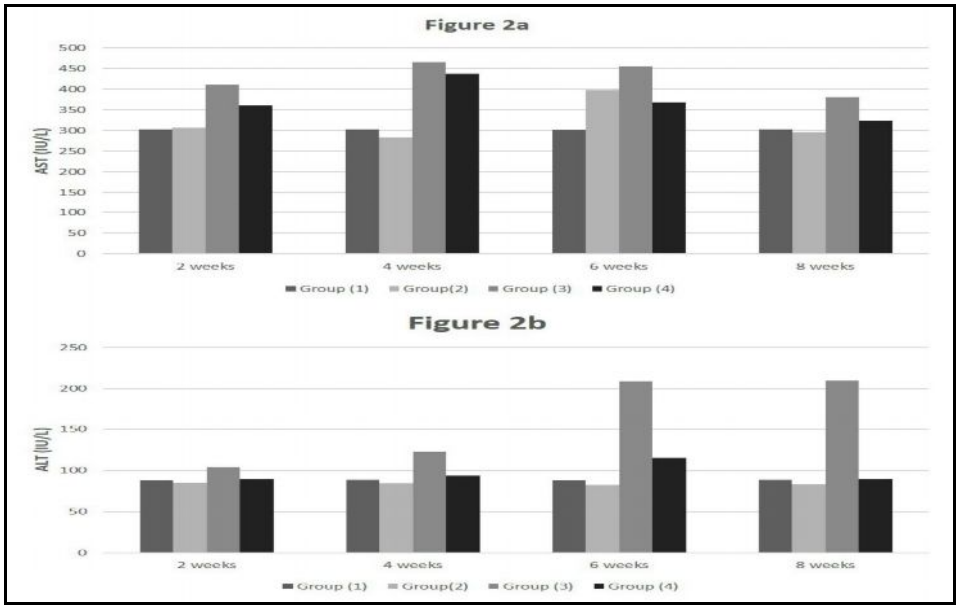

Figure (2) Showing the mean values of serum AST (a) and ALT (b) in control and different treated groups..

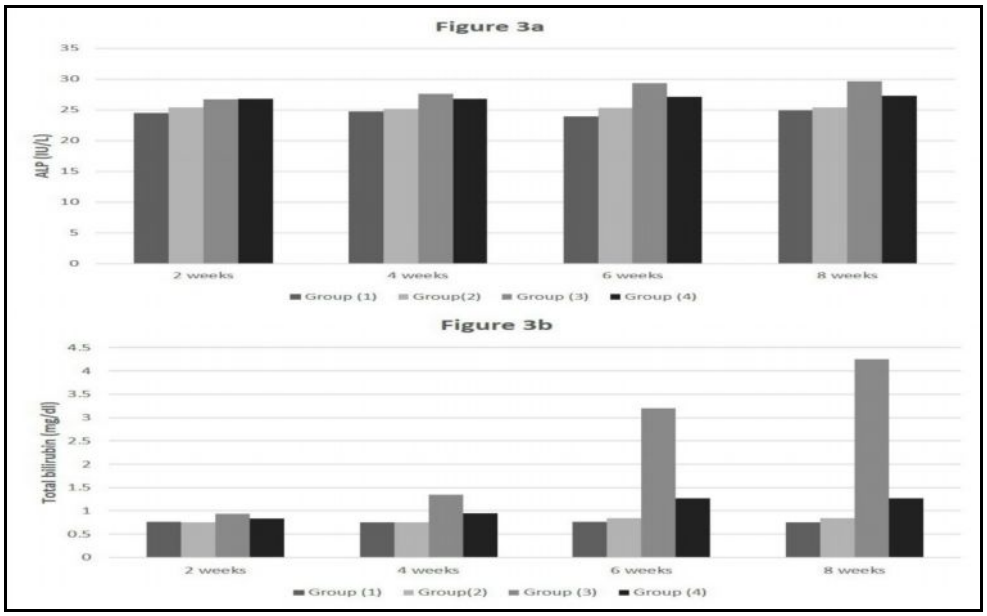

Figure (3) Showing the mean values of serum ALP (a) and Bilirubin (b) in control and different treated groups.

Liver function markers

In comparison to the control group significant increases were observed in the serum AST, ALT, ALP and total bilirubin in rats of Gp3, while those received Spirulina concurrently with TAA showed significant ameliorative decreases in serum levels of these parameters when compared to Gp3 **Table (2)** and **(Figs. 2a, 2b, 3a and 3b).**

Table (2) showing the mean values ± SD of serum AST, ALT, ALP and Bilirubin (mg/dl)

		Group (1)	Group(2)	Group (3)	Group (4)
AST (IU/L)	2 weeks	303± 25.1 ^{a}	306±1.1 ^{ab}	410± 51.6 ^{c}	360±2.3 ^{bc}
	4 weeks	303± 26.0 ^{a}	284± 13.8 ^{a}	465± 3.0 ^{b}	437± 41.0 ^{b}
	6 weeks	302± 12.28 ^{a}	398± 9.6 ^{a}	455± 41.0 ^{c}	368± 11.35 ^{b}
	8 weeks	303± 12.16 ^{a}	296± 16.8 ^{a}	380.6± 0.57 ^{b}	323± 27.2 ^{a}
ALT (IU/L)	2 weeks	88.33± 2.08 ^{a}	85.0± 4.3 ^{a}	104.33± 2.0 ^{b}	89.33± 2.08 ^{a}
	4 weeks	88.66± 8.08 ^{a}	84.33± 17.4 ^{a}	122.66± 2.5 ^{b}	93.66± 3.21 ^{a}
	6 weeks	88.33± 2.08 ^{a}	82.33± 17.4 ^{a}	209± 11.23 ^{c}	115± 1.5 ^{b}
	8 weeks	88.66± 8.08 ^{a}	83.33± 5.5 ^{a}	209.6± 11.2 ^{b}	89± 8.66 ^{a}
ALP (IU/L)	2 weeks	24.49± 0.23 ^{a}	25.37± 0.05 ^{b}	26.66± 0.57 c	26.77± 0.23 ^{c}
	4 weeks	24.7± 0.15 ^{a}	25.18± 0.45 ^{a}	27.6 ± 0.57 ^{c}	26.77± 0.12 ^{b}
	6 weeks	23.87± 0.70 ^{a}	25.27± 1 ^{b}	29.33± 0.07 ^{d}	27.1± 0.15 ^{c}
	8 weeks	24.9± 0.56 ^{a}	25.37± 0.05 ^{a}	29.66± 0.57 ^{c}	27.30± 0.05 ^{b}
Total bilirubin (mg/dl)	2 weeks	0.77± 0.03 ^{a}	0.75± 0.10 ^{a}	0.94± 0.05 ^{b}	0.83± 0.09 ^{a}
	4 weeks	0.75 ± 0.10 ^{a}	0.75± 0.15 ^{a}	1.35± 0.15 ^{b}	0.95± 0.02 ^{a}
	6 weeks	0.77 ± 0.30 ^{a}	0.84± 0.17 ^{a}	3.20± 1.3 ^{b}	1.27± 0.02 ^{a}
	8 weeks	0.75 ± 0.10 ^{a}	0.84± 0.18 ^{a}	4.25± 0.66 ^{b}	1.27± 0.10 ^{a}

Means with different letters (a, b, c, d) within the same row are significantly different at P value ≤ 0.05.

Gross pathology

No pathological abnormalities were detected in livers of both control and the sole Spirulina treated groups. Regarding livers of Gp3 rats, they were apparently normal up to 2 weeks post TAA administration, but by the 4th week livers of most animals appeared pale in color with distinct lobulation. While at the end of

experimental period livers of those rats were tough, rubbery, pale in color with clear multiple nodules giving its surface uneven pattern **(Fig. 4a)**. Ascites was a predominant finding at the end of the experimental period. Gross examination of livers of treated rats (Gp 4) revealed apparent normal color and texture at the last week **(Fig. 4b)**.

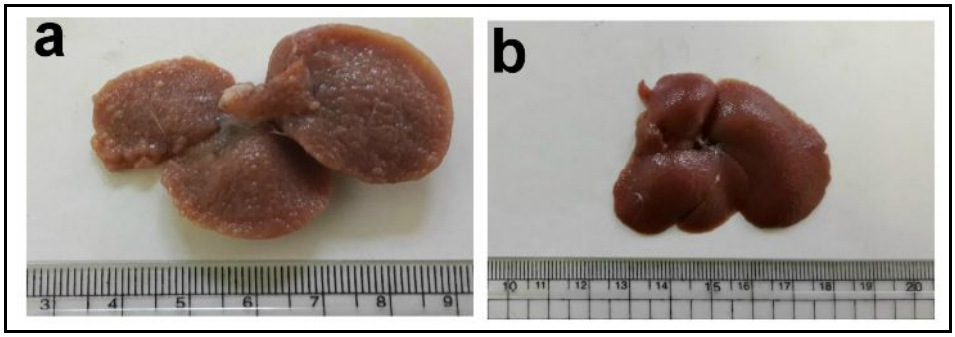

Figure 4: (a): Liver of rat of Gp3, 8 weeks post TAA administration showing paleness in color with multiple nodules and uneven surface. (b): Liver of Gp4 (8 weeks) showing apparently normal appearance.

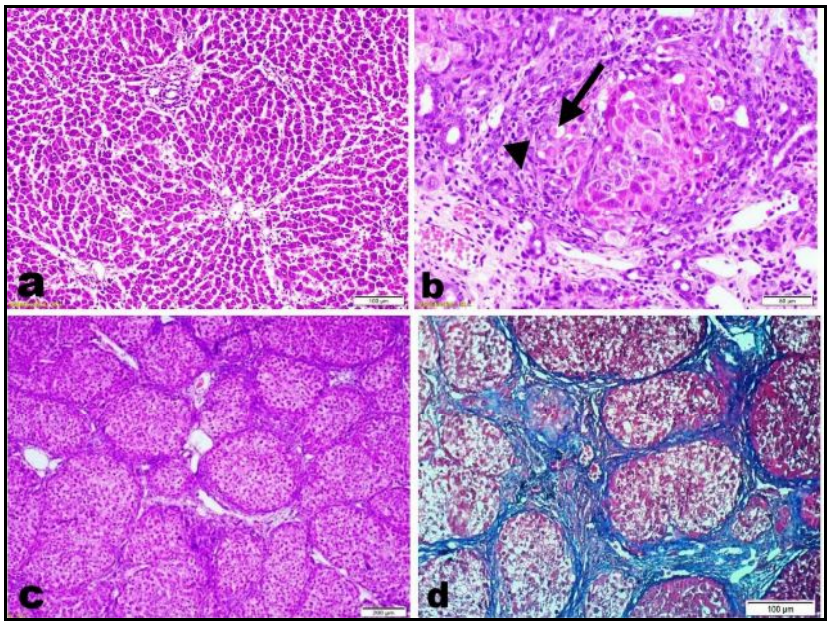

Figure 5: Liver of Gp3 rat showing; (a) Marked disorganization of the hepatic cords and mild portal fibroplasia was extending toward the hepatic lobules. (b) Hepatocellular degeneration, necrosis and apoptosis (arrow), notice the hyperplastic oval cells (dashed arrow). (c) Multilobular cirrhosis, notice the fibrous septa surrounding the atrophied hepatic lobules (a, b & c stained with H & E).. (e) Extensive fibrosis (Masson's Trichrome stain).

Histopathology

Livers of both Gp1 and Gp2 revealed normal histological structure without any pathological alterations. While, examination of livers of TAA administrated rats (Gp3) revealed marked tissue alterations, the intensity of which was time related. At two weeks period, the hepatocytes suffered from various degenerative and necrotic changes; karyomegally of some hepatocytes and few apoptotic bodies were also noticed. Multiple focal areas of hepatocellular necrosis with mononuclear cells infiltration were scattered here and there all over the examined liver sections**.** Some animals showed marked disorganization of the hepatic cords, widening of the portal areas by inflammatory cells infiltration and mild portal fibroplasia was observed which was extending toward the hepatic lobules **(Fig. 5a).** Four weeks post TAA administration, hepatic fibrosis was dramatically increased, and numerous thick fibrous septa were extending from the portal areas toward the parenchyma with

portal to portal bridging fibrosis. Hyperplastic oval cells were observed with formation of new tiny bile ductuoles along the proliferating fibrous septa. The hepatic architecture was completely missed, the central vein disappeared and hepatocytes suffered from marked degeneration, necrosis and apoptosis **(Fig. 5b)**. The most characteristic feature of liver sections 6weeks post TAA administration was extensive fibrosis; the fibrous septa were completely surrounding the atrophied hepatic lobules. Hepatocellular massive necrosis as well as numerous apoptotic bodies were observed. The conspicuous microscopical finding 8 weeks post TAA injection was the observation of the characteristic 'chicken-wire' appearance of multilobular cirrhosis, at which the fibrous septa were completely surrounding the atrophied hepatic lobules **(Figs. 5c and d)**. In addition, regenerative nodules of hyperplastic and vacuolated hepatocytes were apparent.

Regarding microscopic examination of livers of Gp4 rats, it revealed that Spirulina could protect the hepatic tissue against the action of TAA and could cause some sort of delay in the alterations induced by TAA. Two weeks post concurrent administration of Spirulina and TAA, only mild changes could be noticed, portal areas appeared slightly wider with or without mononuclear inflammatory cells infiltration. The hepatocytes showed mild necrobiotic changes. While after 4 weeks, the situation was not aggravated for the portal triads, fine strands of fibroblasts were scattered in the hepatic lobules **(Fig. 6a)**, mild mononuclear inflammatory cells infiltration and newly formed bile ductuoles were the most frequently encountered findings**.** Mild hepatocellular degenerative and necrotic changes with scattered apoptosis were frequently detected**.** The picture at the 6^{th} and 8^{th} weeks post concurrent administration of Spirulina and TAA was nearly the same, mild to moderate fibroplasia was noticed only in the vicinity of the portal triads but not extend peripherally except after eight weeks fine fibrous strands appeared extending toward the parenchyma cells. The hepatocytes showed vacuolar degeneration, scattered necrotic and apoptotic cells but the central vein was widely opened keeping the hepatic lobular architecture maintained **(Fig. 6b).** After 8 weeks, examined sections revealed mild portal fibroplasia with fine incomplete fibrous tissue strands **(Fig. 6c, 6d & 6e).**

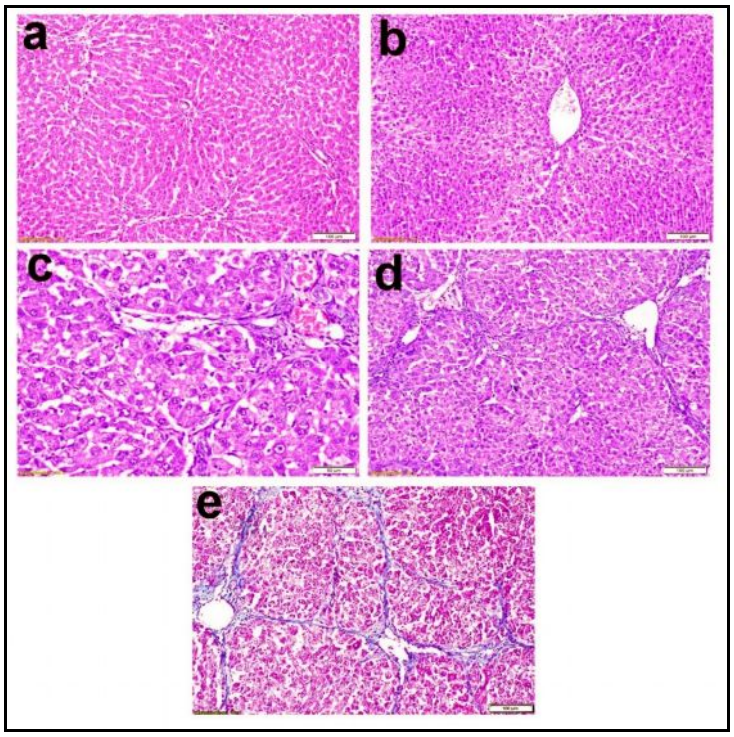

Figure 6: (a and b) Liver of Gp4 rat (after 4 weeks) showing (a) disorganized hepatic parenchyma and fine fibrous strands scattered in between the hepatic lobules. (b) vacuolar degeneration of hepatocytes, scattered necrotic and apoptotic cells with the central vein was widely opened keeping maintained hepatic lobular architecture. c, d and e (after 8 weeks) showing (c) mild portal fibroplasia with fine incomplete fibrous tissue strands.
(d) mild portal fibroplasia with fine fibrous tissue strands (a, b, c & d stained with H & E).
(e) fine fibrous septa (Masson's Trichrome stain).

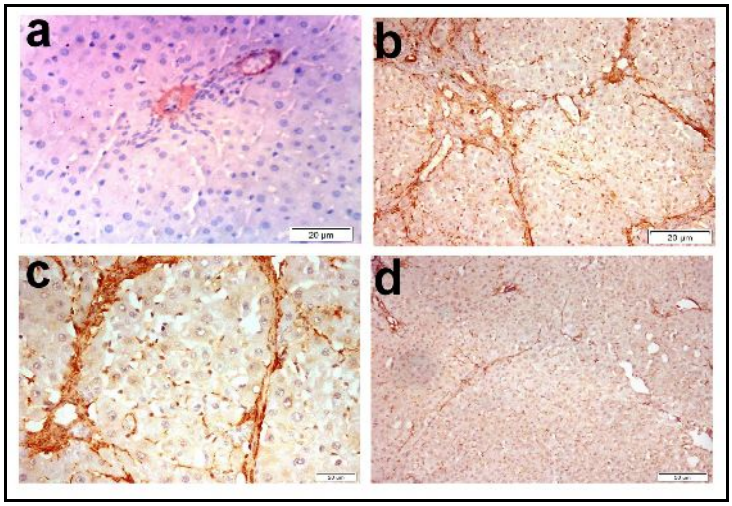

Figure (7): Immunostaining for α-Smooth Muscle Actin (α-SMA); (a) Normal positive expression of α-SMA in the portal area in liver of Spirulina treated rat. (b and c) Liver of Gp3 rat showing (b) strong expression of α-SMA in the portal areas, pericanalicular, along the fibrous septa and (c) around the atrophied lobules. (d) weak expression of α-SMA in the portal area and very rare extension in the parenchyma in live of Gp4 rat.

Immunohistochemistry

Immunostaining for α-SMA revealed normal weak positive expression around the central vein and in the portal areas of rats from Gps 1 and 2 **(Fig. 7a).** However, livers of Gp3 rats showed strong positive expression of α-SMA in the portal areas, pericanalicular, along the fibrous septa and around the atrophied lobules **(Fig. 7b and c).** However, weak to moderate expression of α-SMA was noticed in livers of Gp4 rats in the portal areas and sometimes along the fine incomplete septa **(Fig. 7d).**

Concerning Immunostaining for Caspase-3 revealed its negative expression in the livers of rats of groups 1 and 2 **(Figs. 8a and b***)*. In contrary, strong positive expression was noticed in the livers of rats from Gp**4 (Fig. 8c**). However, livers of rats of Gp4 showed scattered positive expression of caspase-3 in the hepatocytes **(Fig.8d).**

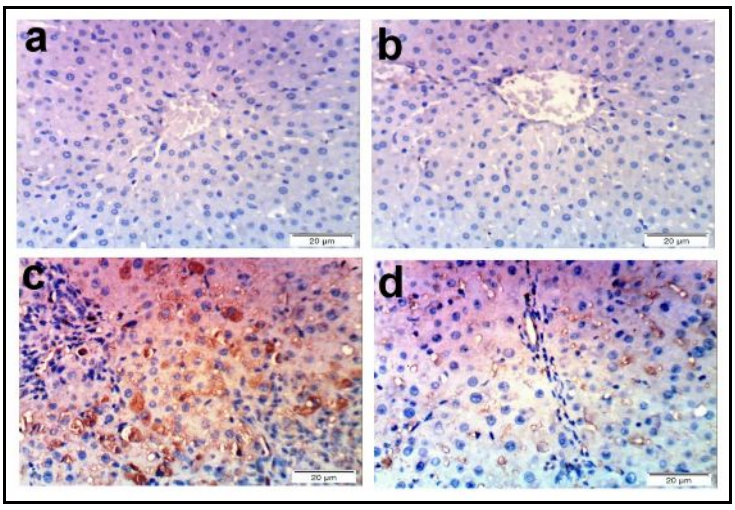

Figure (8): Immunostaining for Caspase 3, (a and b): Liver of rats Gp1 and Gp2 showing negative expression of caspase 3. (c): Liver of rat from Gp3 showing marked strong positive expression of caspase 3 (brown colour) in apoptotic cells and bodies. (d) Liver of rat Gp4 showing scattered weak expression of caspase-3.

Discussion

Results of the present study showed that the administration of TAA had deleterious effects on the experimental rats in terms of clinical follow up, hepatic macro and microscopic picture. The later effects included body weight loss, general weakness (completely loss of activity) and ascites**,** these findings came in accordance with that mentioned by [23].

Decline in the body condition was a remarkable sign at the end of cirrhosis induction, which was recorded also by [24,8] which might be a result of metabolic impairment which led to this phenomenon.

In our study, Spirulina succeeded to induce an improvement in body weight compared to TAA administrated group, which was also recorded by [25] that could be attributed to its higher content of some macro- and micronutrients including high quality protein.

Thioacetamide caused lower body weight and larger liver compared with untreated rats [26], which was consistent with the present study, at which liver weight of cirrhotic animals was higher than that of animals in the control group, that came in accordance with the results recorded by [8]**,** that increase in liver weight could be attributed to the increase in collagen content of the cirrhotic liver.

The relation between the hepatic tissue damage and elevation of the relevant serum enzymes is well documented [27]. In case of liver cirrhosis, liver function markers including AST,ALT and ALP were elevated[2]**.**

The recorded changes in serum liver biomarkers along the study proved that TAA model of cirrhosis is not limited to morphologic lesions but that functional changes also occurred [8]**.**

The administration of Spirulina with TAA reduced the liver serum biomarkers, the later could be attributed to the inhibition of reaction involved in the formation of reactive metabolites and its radical scavenging activity. The presence of β-carotene, enzyme superoxide dismutase, vitamins or selenium in Spirulina produced immunostimulant activities and protective effects. Furthermore, treatment with Spirulina resulted in the stabilization of plasma membrane as well as the repair of hepatic tissue damage [28]**.**

The gross appearance of livers of TAA injected rats was quite similar to that described by[8] in their experiment for development a chronic TAA induced cirrhosis model in rats, Cirrhotic animals showed a nodular surface and most animals showed macronodular cirrhosis.

Our histopathological findings in TAA administrated rats were similar to that mentioned by[8] including, loss of parenchymal architecture , bridging fibrosis between portal areas, centrolobular veins were of difficult identification, bile duct hyperplasia and proliferation of oval cells. As mentioned by[29] Livers of rats administrated TAA developed ballooning degeneration but fatty change was not prominent these findings were as that of our work.

Considering the severe undesirable side effects of synthetic antioxidant agents on liver, there is growing focus to evaluate scientific basis for natural compounds which are claimed to possess hepatoprotective activity through antioxidant action [30]**.** However**;** The true value of natural products in liver diseases prevention and/or their exact mechanisms of action remain largely unknown[31]**.**

Spirulina treated group showed better histological features than that receiving TAA only, The protective role of Spirulina may be attributed to the presence of β-carotene, enzyme superoxide dismutase and blue pigment phycocyanin. β-carotene of Spirulina was reported to reduce cell damage, especially the damage to DNA molecules, thus playing the role in the repair and regeneration process of damaged liver cells[27] and phycocyanin has been considered the predominant compound in the antioxidant activity of the Spirulina [25]**.**

In our results, the amount of fibroplasia was quietly restricted in the group receiving Spirulina which might be assigned to its antiproliferative effect on Hepatic stellate cells (HSC) [13] and its ability to block inflammatory infiltration through its anti-inflammatory activities.

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