Protective effects of the carotenoids-rich alga *Dunaliella salina* against thioacetamide-induced liver fibrosis in rats

Dalia R. Hassan* and Salem A. Salem*

Home Economic Department, Faculty of Specific Education, Fayoum – University

*dr.dal.refaat@gmail.com

**ABSTRACT**

*Dunaliella salina* is naturally occurring source of beta-carotene which acts as antioxidant and also, it is beneficial in the treatment of liver disorders. The goal of this research is to investigate the impact of *D. salina* algae as an antioxidant on liver fibrosis in rats. In the current investigation, male Albino rats (*N* = 30 rats) weighing (180-200g) were used. They were separated into five groups: (1) negative control; (2) Positive control which was treated with Thioacetamide (TAA); groups (3), (4), and (5) TAA induced liver fibroses rats fed 100, 200, and 300mg *D. salina* powder/kg diet. The experiment extended to 6 weeks.

The following were tested in the serum samples of each group: aspartate aminotransferase "AST", alanine aminotransferase "ALT", alkaline phosphatase, total bilirubin, albumin, Malondialdehyde "MDA", Superoxide dismutase "SOD", and glutathione "GSH". The results showed that rats in group (2) with liver fibrosis had considerably higher levels of their serum AST, ALT, total bilirubin and MDA and were significantly lower in serum albumin, SOD, and GSH. On the other hand, treatment of the induced liver fibrosis with different doses of *D. salina* powder had a significant decline in the rat serum levels of AST, ALT, ALP, bilirubin, and MDA, as well as a significant rise in serum antioxidant SOD, GSH, and serum albumin. However, liver histological examination of rats with generated hepatic fibroses in groups (3, 4 & 5) revealed using *D. salina* at different doses can decrease liver injury, necrosis, and inflammatory cell infiltration. This was attributed to the presence of high levels of carotenoids (especially β–carotene) in *D. salina* which has protective activity opposed to TAA-induced hepatic fibrosis in rats.

**Keywords**: *D. salina*, liver, antioxidants, β-carotene, MDA, AST, ALT, SOD, GSH, histopathology.

**INTRODUCTION**

Algae have a high nutritional value with a wide range of bioactive compounds (Singh and Jialal, 2006). *Dunaliella salina* is a member of the Chlorophyaceae family, which includes unicellular biflagellate green algae. It is safe to use as a food additive or as a protective and curative agent in a variety of ailments (El-Baz *et al.*, 2019). It is a naturally occurring source of β-carotene (Borowitzka, 2013; Borovkov*et al.*, 2020). The oral acute toxicity assessment for *D. salina* powder or extract revealed no fatalities or toxicity indications a maximum dosage of 5g/kg, suggesting that it was safe (Farouk *et al.*, 2020). Under appropriate conditions, *D. salina* can accumulate the maximum amount of β-carotene, making this aquatic algae far more effective as an antioxidant because. It is regarded as a critical biomolecule in the treatment of atherosclerosis, and retinal degeneration (Bansal and Sapna, 2009; Xu and Harvey, 2019). The most common colors in nature are carotenoids, which include carotene, lycopene, lutein and zeaxanthin and these are powerful antioxidants in healthy human diets (Martin, 2007).
Liver fibrosis is a long-term condition which acts on the global population, and considered one of the leading morbidity and mortality causes (Schuppan and Kim, 2013). Also, it being the most common cause of death for 1.3 million around the world (Wong and Huang, 2018). Fibrosis develops at different speeds depending on the source of the liver disease. Cirrhosis is an advanced stage of liver fibrosis, it induces direct diverting of portal and arterial blood circulation into the hepatic (central veins), altering transfer between the liver subcarriers and the surrounding liver epithelium, i.e., hepatocytes (Detlef and Nezam, 2008).

Thioacetamide "TAA" is commonly used in the food, and many industries, like leather processing, laboratory, beverages, textile and motor fuel industries (Akhtar and Sheikh, 2013). It is recognized as a human carcinogen and as a liver toxic that necessitates the oxidative biosynthetic route in order to deactivate its hepatotoxic effect (Low et al., 2004; Ghosh et al., 2016). TAA causes glomerular necrosis in liver cells as well as elevations in plasma liver enzymes and bilirubin, resulting in acute liver injury, whereas prolonged exposure causes hepatic fibrosis, liver tumor development, and cytomegaly (Zarger et al., 2017; Bashandy et al., 2018).

TAA treatment also causes structural alterations in renal corpuscles, including as glomerular's capsule and tubule deterioration (Omar, 2018). It damages DNA, induces oxidative stress, produces cytokines, and induces renal failure in rats (Zarger et al., 2019). TAA bioactivation, results in the formation of thioacetamide S-oxide, which generates superoxide radicals and ROS (reactive oxygen species). These oxidative damage are subsequently distributed throughout the body's various organs (Ghosh et al., 2019). TAA may be absorbed by skin or inhaled/ingested by humans (Zarger et al., 2019).

The present study aims to evaluate the role of using *Dunaliella salina* powder as a protectant agent in rats against TAA-induced liver fibrosis.

**MATERIALS AND METHODS:**

**Materials:**

**Algae:**

Fresh *Dunaliella salina* in freshwater was received from the National Research Center's plant, biochemistry division. BG11 media was used to extract and filter algae from water, which was then grown for two weeks in 2 L of medium. Following growth, the algal biomass was extracted and cultivated for an additional two weeks, then grown in a 17 L canning jar with 15 L of culture media under oxygenation, and the culture temperature was 20±3°C. For cultivation, a 2500 lx continuous light was used.

**Animals:**

Thirty male adult rats weighed 180-200g were procured from Helwan Cairo's animal house and fed a regular laboratory diet while also having unrestricted access to tap water. The animals were kept in a climate-controlled environment with 12-hrs light/dark cycle facility and temperatures ranging from 22-25°C. All animals had been treated with kindness.

**Kits:**

Thioacetamide (TAA) kit was purchased from Sigma-Aldrich Co. Transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and albumin kits were supplied by Biodiagnostic Co., Egypt.

**β-carotene of D. salina:**

Using an Agilent 5 prep-C18 Scalar column and an analytical HPLC system (5m, 150mm, 4.6mm). At a flow rate of 1.25ml/min, the following solvents were used: (A) acetone and (B)
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methanol/water (9:1, v/v) containing 0.05% butylated hydroxytoluene (BHT). A gradient of solvents (A and B) was used to separate β-carotene for 40 minutes, including 25 minutes at 80 to 20%, 10 minutes at 20%, and 5 minutes at 20-80%. A colorimetric detection was used to measure the segregated β-carotene.

**Experimental design:**
After a week on a baseline diet, all 30 rats were separated into five groups (each with six rats).
- **Group (1):** fed a baseline diet and served as the negative control.
- **Group (2):** TAA was administered to rats intraperitoneally (dose 200 mg/kg of rat weight) two days weekly for four weeks and fed a baseline diet for six weeks.
- **Group (3):** Rats with induced liver fibrosis were daily given *D. salina* powder (100 mg/kg diet).
- **Group (4):** Rats with induced liver fibroses were daily given *D. salina* powder (200 mg/kg diet).
- **Group (5):** Rats with induced liver fibroses were daily given *D. salina* powder (300 mg/kg diet).

All rats were given light anaesthesia at the end of the trial, and blood samples were collected from the retro-orbital vessels. Blood samples were left to coagulate before being separated for 15 minutes at 3000 rpm to extract the serum.

**Histopathological examination:**
Pieces of liver from each group were preserved in formalin solution (10%), dehydrated using ascending grades of alcohol, imbedded in paraffin wax cutting 5 μ thick. Sections were hydrated using descending grades of alcohol, then dist. water and stained with hematoxylin and eosin (H&E).

**Statistical Analysis**
The statistical significance of standard deviation across groups was determined using analysis of variation in one direction (ANOVA). At (P<0.05), the significance of mean differences is determined and the Least Significant Difference (LSD) test was used. For all analysis of data, SPSS software was employed (Version 16; SPSS Inc., Chicago, USA).

**RESULTS AND DISCUSSION**
The chemical composition of *Dunaliella salina* powder indicated that it contains proteins (22.84%), carbohydrates (32.5%), lipids (5.8%), ash (4.87%) and total carotenoid (14.19%) of its dry weight and that of β-carotene was 12.98%. Moisture represents 4.87% of dry weight of this alga. Abd El-Baky *et al.* (2007) found that *D. salina* accumulates carotenoids (15.2% of its dry weight) and β-carotene (12.6%), making it more beneficial in the prevention of liver fibrosis. Furthermore, Farouk *et al.*, (2020) stated that phytochemical analysis of *D. salina* bioactive extract revealed a considerable quantity of carotenoids, notably β-carotene, which has numerous benefits in liver illnesses. *D. salina*, which has a high concentration of carotenoids and xanthophylls, was demonstrated to be an effective source of antioxidants versus a wide range of oxidative stresses (HU *et al.*, 2008; Hsu *et al.*, 2008).

**Table (1): Chemical composition of Dunaliella salina powder.**

<table>
<thead>
<tr>
<th>Moisture</th>
<th>Protein %</th>
<th>Fat %</th>
<th>Carbohydrates</th>
<th>Ash %</th>
<th>β-carotene %</th>
<th>Total carotenoid %</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.87</td>
<td>22.84</td>
<td>5.8</td>
<td>32.5</td>
<td>19.8</td>
<td>12.98</td>
<td>14.19</td>
</tr>
</tbody>
</table>
Carotenoids are made up of molecules such as β-carotene and xanthophylls like lutein and zeaxanthin, which are needed to protect cells from the damaging consequences of pollutants, light, air, and irritant pigments (Noeman, 1989). Carotenoids’ ability to diminish excited sensitizer particles and superoxides is the fundamental system of action for this phenomenon. Furthermore, β-carotene prevents liposomes from superoxide, hydroxyl radical-induced lipid auto-oxidation, peroxidation and hydrolysis of lipids caused by Fe2+-generated radicals (LO• and LOO•) (Shinmoto, 1992.). Furthermore, degradation of the lipid hydroperoxide produces alkoxy (LO) and peroxy radicals (LOO•). They ultimately produce a large number of carbonyls, which cause DNA deterioration and the development of cancer and ageing process disorders (Shinmoto,1992.; Yavuz et al., 2014).

Table (2) Mean blood ALT, AST, ALP, bilirubin, and albumin levels in TAA-rats fed meals containing varying doses of *D. salina* (Mean ± S.E.).

<table>
<thead>
<tr>
<th>Group (1): Negative control</th>
<th>ALT U/L ± S.E.</th>
<th>AST U/L ± S.E.</th>
<th>ALP U/L ± S.E.</th>
<th>Bilirubin mg/dl ± S.E.</th>
<th>Albumin g/dl ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (2): Positive control</td>
<td>76.48 ± 1.47</td>
<td>103.17 ± 2.50</td>
<td>127.92 ± 1.79</td>
<td>3.05 ± 0.20</td>
<td>3.39 ± 0.14</td>
</tr>
<tr>
<td>TAA+D. salina 100mg/kg diet</td>
<td>54.87 ± 1.73**</td>
<td>87.37 ± 1.57**</td>
<td>109.08 ± 3.37**</td>
<td>2.33 ± 0.10**</td>
<td>3.76 ± 0.07</td>
</tr>
<tr>
<td>TAA+D. salina 200mg/kg diet</td>
<td>45.95 ± 2.16**</td>
<td>83.17 ± 1.10**</td>
<td>84.92 ± 1.59**</td>
<td>1.62 ± 0.10**</td>
<td>4.22 ± 0.09**</td>
</tr>
<tr>
<td>TAA+D. salina 300mg/kg diet</td>
<td>40.05 ± 1.47**</td>
<td>78.57 ± 0.95**</td>
<td>62.28 ± 1.31**</td>
<td>1.46 ± 0.12**</td>
<td>4.60 ± 0.14**</td>
</tr>
</tbody>
</table>

*Significant differences from Positive group (2) at P< 0.05.
**Significant differences from Positive group (2) at P< 0.01.

In comparison to the negative group, TAA (200 mg/kg body weight) i.p. injection induced severe hepatic fibrosis as well as significant rises in serum levels of AST, ALT, ALP, and total bilirubin. This finding is consistent with the findings of Kuriakose and Kurup (2010), who discovered that animals administered paracetamol had substantial liver damage 24 hours later, as seen by considerably (P<0.05) greater levels of hepatotoxic AST, ALT, ALP, and bilirubin. However, after treatment with *D. salina*, all of the previous measures were considerably (P<0.05) decreased, suggesting that consuming 1000 mg/kg of this algae may be capable of causing liver cell regeneration and decreasing the leakage of these enzymes into the blood.

Albumin levels are low in chronic liver damage due to the liver cells’ diminished ability to produce proteins, which were according to Zhao et al., (2014). Furthermore, serum ALP and
biltrubin levels are linked to liver cell activity, whereas ALT is linked to liver disease and AST is linked to liver damage, myocardial infarction, and severe muscle injury.

Treatment with different doses of *D. salina* powder acted as a hepatoprotective in the current investigation, as evidenced by lowering all liver enzymes, particularly at high doses, which led in the equilibrium of the enzyme levels in the treated rats. These results are comparable to those of Madkour and Abdel-Daim (2013), who discovered that a 1000 mg/kg pretreatment with *D. salina* microalga restored hepatic enzyme levels in paracetamol-intoxicated rats.

Data in Table (3) show that, TAA induced highly significant decrease in SOD, GSH and TAC by percentage of 59.40%, 59.12% and 67.89%, respectively, and resulted in a highly considerable rise in MDA level by 145.11% as compared to the negative group. Also, it was noticed that there was a highly significant (P < 0.01) reduction in (MDA), and highly considerable rise in (SOD, GSH and TAC) in TAA rats administered 300mg/kg diet *D. salina* at group (5). But on the other hand, there was no substantial differences in both MDA and TAC levels in TAA rats given 100mg/kg diet *D. salina* at group (3) in comparison to the positive control group.

According to El-Baz et al. (2020), oxidative stress (OS) is an imbalance in the equilibrium between free radical generation and antioxidant defences that has been associated to the etiology of numerous liver disorders (Li et al., 2015). OS also induces structural and functional changes in physiologically macro-molecules like proteins, carbohydrates, and lipids, according to Sutti et al. (2014). RO are produced by the electron transport chain in mitochondria and peroxidases, largely in hepatocytes during metabolic or detoxifying activities (Murphy, 2009). To counteract ROS and mitigate their harm, living organisms have evolved complex antioxidant systems comprised of endogenous and food-derived antioxidants. Antioxidant collaboration provides more resistance to oxygen radicals or nitrogen species. As a result, TAC might give more valuable biological information and must take into consideration the whole impact of all antioxidants found in plasma and bodily fluids (Kuriakose and Kurup, 2010).

The primary line of defence against free radicals caused by oxygen has been recognized as SODs (Jung et al., 2011; Blackney et al., 2014). Superoxide is a free radical with a negative charge was generated when oxygen receives a free electron (Hayyan et al., 2016). SOD also plays an important role in the cellular antioxidant defence system. It eliminates superoxide (O2) by converting it to H2O2, 

**Table (3): The mean serum MDA, TAC, SOD, and serum GSH levels in TAA-rats fed meals containing varying doses of *D. salina* (mean ± S.E.).**

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>MDA nmol</th>
<th>TAC mmol/L</th>
<th>SOD U/L</th>
<th>GSH mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (1): Negative control</td>
<td>8.09 ± 0.67**</td>
<td>1.90 ± 0.11**</td>
<td>84.87 ± 1.88**</td>
<td>20.06 ± 0.75**</td>
</tr>
<tr>
<td>Group (2): Positive control</td>
<td>19.83 ± 0.93</td>
<td>0.61 ± 0.02</td>
<td>34.45 ± 2.11</td>
<td>8.20 ± 0.49</td>
</tr>
<tr>
<td>Group (3): TAA+D. salina 100mg/kg diet</td>
<td>18.56 ± 0.55</td>
<td>0.76 ± 0.03</td>
<td>46.36 ± 1.50**</td>
<td>10.44 ± 0.61*</td>
</tr>
<tr>
<td>Group (4): TAA+D. salina 200mg/kg diet</td>
<td>17.53 ± 0.55*</td>
<td>0.91 ± 0.04*</td>
<td>54.17 ± 1.70**</td>
<td>12.89 ± 0.78**</td>
</tr>
<tr>
<td>Group (5): TAA+D. salina 300mg/kg diet</td>
<td>14.75 ± 0.63**</td>
<td>1.63 ± 0.15**</td>
<td>68.76 ± 2.36**</td>
<td>19.24 ± 1.02**</td>
</tr>
</tbody>
</table>

* Significant differences from Positive G (2) P ≤ 0.05.
** Significant differences from Positive G(2) P ≤ 0.01.
which quickly convert to water by both (catalase and glutathione peroxide) (GPx).

MDA is a byproduct of the lipid peroxidation process (Barriuso, 2013) and when the number of free radicals created surpasses the cell's ability to eliminate them, oxidative stress occurs. The increase in MDA and decrease in TAC and SOD levels in TAA-induced liver fibrosis rats implies to a rise in lipid peroxidation, which causes tissue necrosis and failure of antioxidant defense systems to prevent uncontrolled free radical generation. The *D. salina* therapy adequately reversed these changes, in addition, *D. alina* demonstrated antioxidant effects against TAA in the current study, which were mediated by an increase in GSH levels, which is an antioxidant and anticarcinogenic tripeptide. Furthermore, this was linked to a reduction in MDA. The current result was consistent with Sukalingam and Ganesan (2018).

**Histopathological results:**

The microscopic anatomy of a normal rat liver (group 1) reveals the existence of normal hepatocytes, as well as central and portal veins (Fig. 1A). However, as seen in group (2), rats with TAA-induced hepatic fibroses had hepatocyte degradation, fibrosis, and mononuclear cell infiltration (Fig. 1B). In TAA-induced liver fibrosis rats fed 100mg/kg diet of *D. salina* (group 3); there was fine fatty alteration of hepatocytes and portal infiltration with inflammatory cells (Fig. 1C). In TAA-induced liver fibrosis rats fed a 200mg/kg diet of *D. salina*, considerable multifocal macrovascular and microvascular steatosis in hepatocytes surrounding the central vein and minor hydropic degeneration of hepatocytes were detected (Fig. 1D) (group 3). TAA-induced liver fibrosis in rats given 300mg/kg *D. salina* diet (group 5) resulted in normal histological pattern of hepatocytes, modest perivascular fibrosis in the portal, and isolated regions of coagulative necrosis (Fig. 1E).

The current findings showed that *D. salina* provided antioxidant protection against TAA-induced fibrosis in the livers of the rats studied. TAA insult caused glomerulus deformation and congestion. This was demonstrated in rats from groups (3, 4, and 5) that were given 100, 200, and 300mg/kg diets of *D. salina* powder. As revealed by the biochemical studies, this can be explained by an increase in GSH levels and an inhibition of MDA levels. GSH includes a tripeptide that is both antioxidant and anticarcinogenic, which increases protection against oxidant-induced cell damage (Tsai *et al.*, 2012). Sukalingam and Ganesan's (2018) findings are compatible with the present findings. *D. salina* inhibited thioacetamide-induced inflammatory cell infiltration, indicating that it may have antihepatotoxic effects (Farouk *et al.*, 2020). Furthermore, the presence of β-carotene in *D. salina* can be related to the improvement in TAA-induced liver fibrosis group of rats (group 6). El-Baz *et al.*, (2020) found that β-carotene in *D. salina* reduced liver fibrosis in rats by lowering inflammatory mediators.
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Figure (1A-E): Show microphotographs of sections of liver of rats from different groups stained with HE, with Magnification 400X.

(Fig 1-A): Negative G1, rats with normal histological pattern of liver hepatocytes, central and portal veins.

(Fig 1-B): Positive G2, rats with TAA induced liver fibrosis showing degeneration of hepatocytes, presence of fibrosis and mononuclear cells infiltration (arrows).

(Fig 1-C): Group (3), rats with TAA induced liver fibrosis and fed 100mg/kg diet of *D. salina* showing hepatocyte fatty changes and portal congestion with granulocytes (arrows).

(Fig 1-D): rats with TAA induced liver fibrosis and fed 200mg/kg diet of *D. salina* showing moderate multifocal macrovesicular and microvesicular steatosis in hepatocytes surrounding the central vein and slight hydropic degeneration of hepatocytes (arrow).

(Fig 1-E): rats with TAA induced liver fibrosis and fed 300mg/kg diet of *D. salina* showing normal histological pattern of hepatocytes, mild perivascular fibrosis in the portal area and focal areas of coagulative necrosis (arrows).
Conclusion:
The inclusion of β-carotene in *D. salina* powder significantly reduced hepatic steatosis. These findings suggest that *D. salina* can be used as an effective and valuable medication in treatment of liver cirrhosis. Additional research and medical investigations are needed to assess the medicinal value of *D. salina* in anti-fibrotic rats and individuals with liver cirrhosis.

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