Protective Effect of *Nanjunda* Extract and/or Silymarin Against Thioacetamide-induced Hepatic Fibrosis in Rats

B.E. Bayoumy¹, Akaber T. Keshta²,³, Fatma El-Tomy³.

¹ Professor of organic chemistry, Chemistry department, Faculty of Science, Zagazig University, Egypt.
² Lecture of biochemistry, Chemistry department, Faculty of Science, Zagazig University, Egypt.
³ 3Biochemistry Division, Chemistry department, Faculty of Science, Zagazig University, Egypt.

**ABSTRACT**

**Background:** Liver fibrosis is a hallmark histologic event of chronic liver diseases and is characterized by the excessive accumulation and reorganization of the extracellular matrix (ECM). Thioacetamide (TAA) exerts hepatotoxic effects induced liver fibrosis. Herbal plants play a role in the management of various liver disorders most of which speed up the natural healing processes of the liver. One such plant is *Nanjunda* (*Balanites aegyptiaca*). **Aim:** The main purpose of the present study is to evaluate the hepato-protective and antioxidant effects of *Nanjunda* (*Balanites aegyptiaca*) extract on liver fibrosis induced by TAA in rats. **Materials & Methods:** Swiss male adult albino rats were divided into 5 groups: Group 1, kept as control group; groups 2-5 were injected intraperitoneally (i.p.) with TAA (200 mg/Kg) twice weekly for 8 weeks to induce hepatic fibrosis. Groups 3-5 were administered daily doses of *Balanites aegyptiaca* extract (100 mg/kg), Silymarin (100 mg/kg) and combined treatments, respectively. **Results:** Our results indicated that TAA caused significant alterations in biochemical and histological manner of liver [increase in serum levels of aminotransferases, metalloproteinase, and galactin-3, elevation in oxidative stress (increase malondialdehyde (MDA), nitric oxide (NO), and decrease antioxidants enzyme activities)]. While, administration of *Balanites aegyptiaca* extract and/or Silymarin attenuated TAA-induced hepatic fibrosis, improved liver enzymes and reduced the oxidative stress. *Balanites aegyptiaca* extract exhibited a significant effect showing increasing levels of superoxide dismutase (SOD), catalase (CAT), by reducing MDA and NO levels. **Conclusion:** In conclusion, the combined effect of *Nanjunda* (*Balanites aegyptiaca*) extract with Silymarin had powerful hepato-protective and antioxidant effects.

**Introduction**

Liver fibrosis remains one of the serious health problems. Liver fibrosis is part of the structural and functional alterations in most chronic liver diseases (¹). During the development of liver fibrosis, activated hepatic stellate cells (HSCs) are able to synthesize and deposit excessive extracellular matrix components into the liver parenchyma (²). Advanced liver fibrosis leads eventually to cirrhosis and liver failure, for which no effective medical treatments are available.

Liver injury induced by various hepatotoxins has been recognized as a major toxicological problem for years (³). Thioacetamide (TAA) is a thiono sulfur containing compound. It has been used as a fungicide, organic solvent, accelerator in the vulcanization of rubber, and as a stabilizer of motor oil (⁴). The toxicity of TAA results from its bio-activation by a mixed-function oxidase system (⁵). Therefore, prevention of liver fibrosis is a critical step for protecting the liver against...
the occurrence of cirrhosis and failure. Although interferon has been widely used to treat chronic viral hepatitis, the effect of the therapy for liver fibrosis has not always been satisfactory. Herbal medicines have been reported to show protective effects from liver fibrosis and injury (6). _Balanites aegyptiaca_ (L.) Del belongs to the family Balanitaceae. It is commonly known as nanjunda. It has been used in a variety of folk medicine in India and Asia. Various parts of the plant are used in Ayurvedic and other folk medicine for the treatment of various ailments such as syphilis, jaundice, liver and spleen problem, epilepsy and yellow fever and the plant also has insecticidal, antihelminthic, antifeedant, molluscicidal and contraceptive activities (7).

The fruit mesocarp contains a large variety of chemicals amongst which are the pregnane glycosides, coumarins, flavonoids, 6-methylidiosgenin and saponins (8). The saponins are a structurally and biologically diverse class of glycosides of both steroids and triterpenes that are widely distributed in terrestrial plants and in some marine organisms (9). Galectin-3 is a member of a family of proteins comprising soluble β-galactoside–binding lectin that appears to be a direct mediator of pro-fibrotic pathways and is a potential marker of adverse cardiac remodeling (10).

The present study is to investigate the anti-fibrotic, Hepato-protective and antioxidant effects of ethanolic extract of _Balanites on thioacetamide induced liver fibrosis in male rats._

**Materials and Methods**

**Plant extraction:**
The ethanolic extract of _Balanites aegyptiaca_ was introduced from Prof. Dr. Faten Zahran.

**Chemicals**
Thioacetamide was purchased from Sigma-Aldrich Chemical Co., (St Louis, MO, USA), Galectin 3 (Gal-3) and metalloproteinase-8 (MMP-8) a sandwich ELISA Rat Kit method from (BG Medicine, Waltham, MA).

**Animals**
Adult male Swiss albino rats weighing (150-200g) were housed in experimental animal house of the Faculty of Science, Zagazig University. The animals were maintained in controlled environment of temperature, humidity, light, and fed on a commercial standard diet and tap water ad libitum.

**Experimental design**
Swiss male adult albino rats were divided into 5 groups: Group 1: Rats received normal saline was served as a normal control; Group 2: fibrotic group: Rats were injected intraperitoneally (i.p.) with TAA (200 mg/Kg) (11) twice weekly for 8 consecutive weeks. Groups 3 "Balanites treated group": rats were injected intraperitoneally (i.p.) with TAA (200 mg/Kg) twice weekly for 8 weeks, then administered daily doses of _Balanites aegyptiaca_ extract (100 mg/kg) (12); Group 4 "Silymarin treated group": rats were injected intraperitoneally (i.p.) with TAA (200 mg/Kg) twice weekly for 8 weeks, then administered Silymarin daily doses of (100 mg/kg) orally; and Group 5 "combined treatments": rats were injected intraperitoneally (i.p.) with TAA (200 mg/Kg) twice weekly for 8 weeks, then administered daily doses of _Balanites aegyptiaca_ extract (100 mg/kg) and Silymarin daily (100 mg/kg) doses for 28 days. At the end of the experiment animals, animals were weighed then anaesthetized under light diethyl-ether and dissected. Blood samples and liver tissues were collected for biochemical and histopathological analysis.

**(A) Biochemical analysis**

**Anti-oxidant assays:** The plasma was prepared for different antioxidant assays. Malondialdehyde (MDA), nitric oxide (NO) levels, Superoxide dismutase (SOD), and Catalase (CAT) activities, were determined by using Bio-diagnostic kit method according to Satoh (13), Montgomery and Dymock (14), Nishikimi et al., (15), and Aebi (16), methods; respectively.

**Liver Biomarkers measurements:**
Galectin-3 (GAL-3) and metalloproteinase-8 (MMP-8) levels were determined by using a sandwich ELISA Rat Kit method in serum according to the method of Christenson et al., (17) and Zhang et al., (18), respectively. Alanine transamines (ALT), asparatate transamines (AST), total protein and albumin were determined by using Bio-diagnostic kit method according to Schumann and Klauke, (19) Karmenet al., (20) Doumas, (21) and Doumas et al., (22) methods; respectively.

**(B) Histopathological studies**
Histological evaluation was performed on other portion of the liver tissues. Specimen were fixed in 10% formalin and embedded in paraffin wax. Liver sections were cut at 5μm in thickness, stained with hematoxylin and eosin (H&E) and viewed under light microscopy and examined the histological changes according to Lillie, (23).

Statistical analysis
Data were evaluated by one-way analysis of variance (ANOVA) by "SPSS" 14.0 for Microsoft Windows, SPSS Inc. Chicago USA (24) and considered statistically significant at a two-sided P<0.05. Numerical data were expressed as mean ± SD.

Results
Effect of Balanites aegyptiaca extract on antioxidants in all studied groups:
Table (1) summarized the mean values of MDA, NO, SOD, and CAT in all groups. On a hand, the mean value of MDA and NO levels were found to be 18.7±1.3 (nmol/ml), and 25.0±2.1 (μmol/ l) in negative control group; respectively. Fibrotic group showed a significant increase in both MDA levels to 67.3 ±6.0 (nmol/ml) by 259.8%, and NO levels to be 76.0 ±9.4 (μmol/l) by 204.0%, (p<0.001) compared to negative control group. While, administration of Banalities extract, Silymarin alone or in combination resulted in a significant decrease in MDA levels to 36.6 ±1.0, 39.9±4.2, and 22.4±0.9 (nmol/ml) by 45.6%, 40.7%, and 66.7%, (p<0.001) respectively; compared to the fibrotic group. Also, NO levels were decreased significantly in Banalities extract, Silymarin and combination groups to 32.4 ±1.8, 37.9±1.6, and 28.4±0.8 (μmol/l) by 57.4%, 50.1%, and 62.6%, respectively, (p<0.001) compared to fibrotic group.

On the other hand; CAT and SOD activities were decreased from 236.1±10.0 (U/l), 141.8±9.0 (U/ml) in negative control group to 97.8 ±4.2, 75.1±5.4 in fibrotic group by 58.6%, and 47.0%; respectively, (p<0.001). While, their activities were significantly increased to 213.7±10.9, and 157.3±12.1 in Banalities group, to 199.1 ±6.7 and, 114.5±9.8 in Silymarin group, and to 354.1±30.3, and 226.6±20.4 by 262.1%, 201.7% in combination group; respectively, (p<0.001) compared to fibrotic group.

Effect of Balanites aegyptiaca extract on liver functions in all studied groups:
Table (2) summarized the effect of extract on liver function tests in sera of studied groups. Measurement of liver enzyme activities demonstrated significant increase in ALT, and AST activities in fibrotic group to 96.8 ±4.6, and 217.6 ±3.6 (U/L) by 185.7%, 290.5%, and 109.4%; respectively compared to negative control group, (p<0.001). These high activities of liver enzymes were significantly reduced to 42.8 ±1.4, and 154.2 ±1.1by -55.8%, and 29.1%; respectively in Banalities group, to 61.3±3.3 and, 148.1±6.1 by 36.7%, and 31.9% in Silymarin group, and to 55.3±4.0, and 124.8±2.8 by 42.9%, and 42.6% in combination group; respectively, (p<0.001) compared to fibrotic group. Also, enzyme ratio decreased in fibrotic group and then this ratio was increased as a result of administration of treatments.

Total proteins and albumin concentrations were significantly decreased in fibrotic group from 9.7±0.3 to 7.7 ±0.4 (g/dl) by 20.6%, and from 4.1±4.50.21 to2.7±0.2 (g/dl) by 34.1%; respectively, (p<0.01) compared to negative control group. These concentrations were significantly increased to 9.3±0.1, and 4.1±0.07 by 20.8%, and 51.9%; respectively in Banalities group, to 8.0 ±0.09 and, 3.6±0.4 by 3.8%, and 33.3% in Silymarin group, and to 9.0±0.07, and 4.1±0.1 by 16.9% and 51.8% in combination group; respectively, (p<0.001) compared to fibrotic group.

Effect of Balanites aegyptiaca extract on Gal-3 concentrations and MMP-8 activity in all studied groups:
Gal-3 concentrations and MMP-8 activities were demonstrated in table (3) in all studied groups. Gal-3 and MMP-8 were significantly elevated in the fibrotic group to 20.7 ±2.1 (ng/ml) & 451.7 ±19.6 (pg/ml); respectively compared to negative control group 3.7±0.08 & 83.5±7.6 by 459.5% & 440.9%; respectively (p<0.001). Meanwhile, Gal-3 and MMP-8 were significantly decreased to 5.6 ±0.3, and 197.1 ±6.4 by 72.9%, and 56.4%; respectively in Banalities group, to 8.3 ±0.3 and, 278.4±22.7 by 59.9%, and 38.4% in Silymarin group, and to 4.3±0.4, and 116.4±8.6 by 79.2% and 74.2% in combination group; respectively, (p<0.001) compared to fibrotic group.
**Histological studies in all studied groups:**
The histological examinations of liver tissues with Hematoxylin and Eosin stain in the different studied groups confirm the biochemical study in all different groups, as shown in Fig. (1 A, B, C, D, E).
The histological examination of control rats (Negative control Group) revealed that, the normal histological appearance of liver with clearly outlined figs of hepatocytes along with adjacent sinusoids radiating from the central veins towards periphery of liver lobule. However, Fibrotic Group showed disturbance of the normal architecture of liver tissue. Histological abnormality in the fibrotic model rat was characterized by depositing bundles of collagens in the pericentral and mid-zonal areas of liver tissues. Nevertheless, Banalities group showed showing congestion of the hepatoporal blood vessel. Silymarin group showed mild liver affection, as liver showing congested blood vessel. Combination group demonstrated marked regression of the degree of fibrosis with a large amount of alleviation in abnormal area compared with model group and almost similar to normal group.

**Discussion**
Liver fibrosis is part of the structural and functional alterations in most chronic liver diseases. It is one of the main prognostic factors as the amount of fibrosis is correlated with the risk of developing cirrhosis and liver-related complications in viral and non-viral chronic liver diseases. Establishing an easy and reproducible model for hepatic fibrosis is absolutely necessary for research on liver reperfusion injury. The animal model of hepatic fibrosis is important for experimental research to apply to clinical uses for reperfusion injury or anti-fibrosis. Among various hepatotoxins, TAA is known to be the most potent because of its rapid elimination and cumulative injury. In the present study, TAA induced elevation of MDA and NO levels, reductions of endogenous antioxidant enzymes (SOD and CAT). It was suggested that TAA induced liver fibrosis by forming free radicals, which then react with cellular lipids to promote lipid peroxidation. The higher MDA level in TAA control rats observed in the present study also supports this suggestion. The hepatotoxicity of TAA results from its metabolic conversion to free radical products: thiaacetamide sulfoxide and thiaacetamide-S, S-dioxide which attacks microsomal lipids leading to their peroxidation and production of reactive oxygen species (ROS), such as the H$_2$O$_2$, super oxide anion O$_2^−$, and the hydroxyl radical. ROS affects the antioxidant defense mechanisms, decreases the activity of SOD that causes liver injury, cirrhosis development, and hepatocarcinoma. While the treatments with ethanol extract of *Balanites aegyptiaca* or Silymarin significantly reversed these changes and showing a significant improvement of the oxidative stress in these animals. It may be possible that the mechanism of hepato-protection by ethanol extraction of *Balanites aegyptiaca* is due to its antioxidant effect. Mohan et al., stated that the saponins of ethanol extract of *Balanites aegyptiaca* was able to stabilize reactive oxygen species by reacting with them and oxidizes subsequently to more stable and less reactive radicals. Also, balanitoside has significant inhibition on NO formation that might be due to its anti-inflammatory effect. Lawrence et al., reported that flavonoids of *Silybum marianum* (Silymarin) had potent antioxidant effect, indicated by significant increase of superoxide anions, and lipid oxygen radicals due to lipid peroxidation as proven in this study. Several investigators previously reported the potent in vivo antioxidant activity of *Silybium marianum*, they referred it’s in vivo antioxidant activity via increasing the levels of glutathione, which is an important antioxidant that detoxifies an array of hormones, drugs and chemicals. These findings are consistent with previous investigations which indicated that TAA caused a significant decrease in the levels of liver SOD and CAT. Our results are in agreement with Mayba et al., who reported that the increase in malondialdehyde (MDA) levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with ethanol extract of *Balanites aegyptiaca* significantly reversed these changes, and it may be possible that the mechanism of hepato-protection by ethanol extraction of *Balanites aegyptiaca* is due to its antioxidant effect.
Serum liver biomarkers (ALT, AST) are important criteria for the evaluation of liver toxicity. The amounts of enzymes that leak into the bloodstream indicate the severity of hepatic damage. In the present study, the rats intoxicated with TAA experienced hepatic injury evidenced by significant increase in serum liver biomarkers (AST and ALT) when compared to normal control rats. However, ethanol extract of Balanites aegyptiaca or/and Silymarin significantly exhibited hepato-protective effects to attenuate the elevated serum liver parameters, but the effectiveness of ethanol extract of Balanites aegyptiaca in the attenuation of the elevated liver enzymes was observed more in combined treated group than single treated one. The increased serum levels of AST and ALT are due to the damage to the structural integrity of the liver, since these enzymes are normally located in the cytoplasm and released into the circulation after cellular injury. Also Hajovsky et al. reported that thioacetamide produces free radicals, which affect the cellular permeability of hepatocytes leading to elevated levels of serum biochemical parameters like ALT, and AST. Administration of TAA for a period of 10 weeks increased the levels of serum ALT, AST, while the levels of total protein, and albumin were statistically decreased in experimental male rats. As Balanties and Silymarin have both hepato-protective and regenerative actions. Our results are in agreement with Abdel-Salam et al., reported that the Silymarin decreased leakage of hepatocellular enzymes ALT and AST into the plasma, and lessened the development of liver necrosis and fibrosis caused by carbon tetrachloride (CCl4). Our results revealed that, galectin-3 and MMP-8 were increased in induced hepatic fibrosis as a result of activation of hepatic stellate cells (HSCs), while Balanites and/or Silymarin reduce these alterations. Central to fibrogenesis and the scarring of organs is the activation of fibroblasts into matrix-secreting myofibroblasts. Galectin-3 expression is up-regulated in established human fibrotic liver disease and is temporally and spatially related to the induction and resolution of experimental hepatic fibrosis. Disruption of the Galectin-3 gene blocks myofibroblast activation and procollagen (I) expression in vitro and in vivo, markedly attenuating liver fibrosis. These data suggest that Galectin-3 is required for TGF-β mediated myofibroblast activation and matrix production. It has been suggested recently that the therapeutic strategy for protecting against oxidative stress will be to target simultaneously the free radicals in both the lipid and the aqueous phases, in extracellular and intracellular spaces. To confirm our biochemical data, Histopathological study was done. Histologically, in our study, TAA administration produced depositing bundles of collagens in the pericentral and mid-zonal areas of liver tissues. The post-treatment of animals with Balanites aegyptiaca or/and Silymarin, reversed significantly TAA-intoxicated pathogenic changes in liver. These findings were line with Saad et al., acute TAA toxicity leads to the prominent changes in the liver tissue architecture including the appearance of necrotic cells and inflammatory cells, mostly macrophages around the central vein. TAA produces centrilobular necrosis.

Conclusion:
In conclusion Balanites aegyptiaca extract may be has a significant anti-fibrotic and antioxidant effect on the liver fibrosis induced by thioacetamide. As Balanites aegyptiaca extract and Silymarin reverse the hepatic biochemical and histological alterations induced by thioacetamide.

References


Table (1): Non-enzymatic and Enzymatic anti-oxidants in all studied groups:

<table>
<thead>
<tr>
<th>Variables</th>
<th>Negative control Group (1)</th>
<th>Fibrotic Group** (2)</th>
<th>Banalities Group (3)</th>
<th>Silymarin Group (4)</th>
<th>Combination Group (Banalities + Silymarin) (5)</th>
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<tbody>
<tr>
<td></td>
<td>Mean±SD.</td>
<td>% Change</td>
<td>Mean±SD.</td>
<td>% Change</td>
<td>Mean±SD.</td>
</tr>
<tr>
<td>MDA (nmol/ml)</td>
<td>18.7±1.3</td>
<td>-</td>
<td>67.3***±6.0</td>
<td>259.8%</td>
<td>36.6±1.0**</td>
</tr>
<tr>
<td>NO (μmol/l)</td>
<td>25.0±2.1</td>
<td>-</td>
<td>76.0±9.4***</td>
<td>204.0%</td>
<td>32.4±1.8**</td>
</tr>
<tr>
<td>CAT (U/L)</td>
<td>236.1±10.0</td>
<td>-</td>
<td>97.8***±4.2</td>
<td>-58.6%</td>
<td>213.7±10.9***</td>
</tr>
<tr>
<td>SOD (U/ml)</td>
<td>141.8±9.0</td>
<td>-</td>
<td>75.1±5.4***</td>
<td>-47.0%</td>
<td>157.3±12.1***</td>
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Significant difference at (*P < 0.05, **P < 0.01, ***P < 0.001)

Table (2): Liver functions in all studied groups:

<table>
<thead>
<tr>
<th>Variables</th>
<th>Negative control Group (1)</th>
<th>Fibrotic Group (2)</th>
<th>Banalities Group (3)</th>
<th>Silymarin Group (4)</th>
<th>Combination Group (Banalities + Silymarin) (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD.</td>
<td>% Change</td>
<td>Mean±SD.</td>
<td>% Change</td>
<td>Mean±SD.</td>
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<tr>
<td>ALT (U/L)</td>
<td>63.9±6.1</td>
<td>-</td>
<td>96.8***±4.6</td>
<td>51.5%</td>
<td>42.8±1.4**</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>97.8±5.3</td>
<td>-</td>
<td>217.6**±3.6</td>
<td>122.5%</td>
<td>154.2**±1.1</td>
</tr>
<tr>
<td>TP (g/dl)</td>
<td>9.7±0.3</td>
<td>-</td>
<td>7.7±0.4**</td>
<td>-20.6%</td>
<td>9.3±0.1*</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.1±4.50 .21</td>
<td>-</td>
<td>2.7±0.2*</td>
<td>-34.1%</td>
<td>4.1±0.07**</td>
</tr>
</tbody>
</table>

Significant difference at (*P < 0.05, **P < 0.01, ***P < 0.001)
Table (3): Gal-3 concentration and MMP-8 activity in all studied groups:

<table>
<thead>
<tr>
<th>Variables</th>
<th>Negative control Group (1)</th>
<th>Fibrotic Group* (2)</th>
<th>Banalities Group (3)</th>
<th>Silymarin Group (4)</th>
<th>Combination Group (Banalities + Silymarin) (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD.</td>
<td>% Change</td>
<td>Mean ± SD.</td>
<td>% Change</td>
<td>Mean ± SD.</td>
</tr>
<tr>
<td>Gal-3 (ng/ml)</td>
<td>3.7±0.08</td>
<td>--------</td>
<td>20.7 ±2.1***</td>
<td>459.5%</td>
<td>5.6 ±0.3***</td>
</tr>
<tr>
<td>MMP-8 (pg/ml)</td>
<td>83.5±7.6</td>
<td>--------</td>
<td>451.7*** ±19.6</td>
<td>440.9%</td>
<td>197.1 ±6.4***</td>
</tr>
</tbody>
</table>

Significant difference at (*P < 0.05, **P < 0.01, ***P < 0.001)

Fig. (1) The histological examinations of liver tissues in all studied groups, (H&E X 400).
(A): A photomicrograph of Negative Control Group showing normal showed normal lobular architecture. (B): A photomicrograph of fibrotic group Liver showing portal fibrosis which encircle multiple hepatic lobules. (Arrows) (C): A photomicrograph of Banalities group Liver showing congestion of the hepatoporal blood vessel (arrow). (D): A photomicrograph of Silymarin group Liver showing hepatoporal blood vessel congestion with fibrotic nodules. (E): A photomicrograph of Combination group Liver showing congestion of the hepatoporal blood vessel (arrow) with marked regression of the degree of fibrosis