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Original Article

Alternative therapeutic approaches: hepatoprotective effect of neriumoleander extract in thioacetamide induced hepatotoxicity

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Abstract: In the folk medicine system different parts of Nerium oleander are utilized for the treatment of a variety of human disorders. In this study, the therapeutic potential of aqueous extract of N. oleander leaves extract was assessed in thioacetamide (TAA) directed chronic hepatotoxicity in Wistar rats. TAA administration caused severe hepatic damage as evidence from significant decrease in level of serum total protein, albumin, ALT, total lipids, HDL (30%, 32%, 37%, 37% & 48% respectively) and concurrent rise in ALP and bilirubin total (129% & 153% respectively). The results illustrated that the treatment of N. oleander effectively increased the TAA induced low levels of serum total protein, albumin, ALT, total lipids, HDL (12%, 24%, 64%, 46% & 53% respectively) and reduced the TAA induced serum level of ALP and bilirubin total (66% & 35% respectively). Histopathology of hepatic tissues displayed that N. oleander extract has lessened the occurrence of liver lesions, along with infiltration, cloudy swelling of hepatic cells and low degree of hepatic necrosis induced by TAA in rats. While histochemical findings revealed blue stained hemosiderin granules in the peripheral area of hepatic lobules and mainly in Kupffer cells in TAA treated group while lesser degree of iron deposition was observed after the use of N. oleander leaves extract. Findings suggest that N. oleander leaves extract has significant hepatoprotective activity, as evident by biochemical parameters and histopathological studies.

Keywords: Hepatotoxicant, hepatoprotective, cytotoxicity, necrosis, infiltration, thioacetamide

Introduction

Nerium oleander belongs to the family Apocynaceae. It is indigenous to Indo-Pak subcontinent, broadly scattered in subtropical Asia, southern United States, Mediterranean region and many other warms areas [1] where it grows outdoors in parks, gardens and along roadsides by people who may not consider its toxic potential [2].

In the folk medicine system, different parts of N. oleander are used for the cure of various human disorders. The leaves are utilized as a diuretic, cardiotonic, anti-bacterial in cutaneous eruptions and are also effectual against snake-bites [3, 4]. Roots are found to be effective for treatment of various types of cancers, leprosy and ulcers [5]. The root-bark is specially used for ring worm and other parasitic infections [6, 7]. On the contrary, the aqueous extracts of flowers, branches, leaves and roots are reported hazardous to certain insects and other species of living creatures. It is considered that cardiac glycosides of N. oleander cause poisoning by inhibiting plasmalemmal Na^+, K^+-ATPase [8].

TAA is a model hepatotoxicant and prolonged oral intake of this chemical directs to hyperplastic liver nodules, hepatocarcinomas, cholangiomas and liver cell adenomas [9]. TAA induced inflammation leads to cirrhotic conditions in rat liver that resembles the human alcoholic liver fibrosis [10]. TAA is classified as indirect intrinsic [11]. The direct hepato-toxins subclass was found intrinsic and employed its toxicity by the formation of free radicals which leads to membrane peroxidation of hepatocyte, in contrast the indirect hepatotoxins are concerned

with disruption of cell integrity due to their highly reactive metabolites which interact with cellular membrane or intracellular molecules [12-14].

Metabolic activation is required to provoke TAA toxicity [15, 16]. Hepatic cytochrome P450 mediates the two-step bio activation of TAA by the formation of FAD-containing monooxygenase (FMO) as well as sufenes (TAASO) and sulfines (TAASO2). The latter one exists in two tautomeric forms, interacts and alter the lysine residues of proteins. This most likely leads to impairment of function and cytotoxicity [17, 18]. The relative contributions of hepatic cytochrome P450 and FMO in TAA bioactivation differ under changed circumstances [19]. It has been reported that TAA can also injure different organ systems besides liver, including lungs [20], intestine [21], kidneys [22], spleen [23], thymus [22] and pancreas [24].

The current study aims to examine the potential of N. oleander leaves extract as a curative agent against TAA tempted hepatotoxicity in Wistar rats.

Materials and methods

Experiment was conducted on adult male Wister rats (175±25g) and two experimental groups (n=5) were established against a control group. Dose of TAA purchased from Sigma-Aldrich Switzerland was prepared by dissolving 200mg of TAA in 1L of distilled water and stirred well until all crystals were dissolved. N. oleander leaves extract was prepared by boiling air-dried leaves in 0.9% NaCl solution (1:1, w/v) for 3h by steam distillation. The extract was then filtered and used to the experimental animals [25]. Animals weighing 175±25g were randomly divided into three groups (n=5) Con, I & II. Normal drinking water was given to Con group. Among the treated groups, group I was given TAA (200mg/L) orally in drinking water for a period of 18 weeks and group II was provided with TAA (200mg/L) for 18 weeks and further 7 days oral ingestion of N. oleander leaves extract.

The rats were anesthetized by intraperitonial administration of Ketamine distilled water mixture (1:1). All the animals were dissected in aseptic maintained environment to collect the blood and excise the liver out. Blood was shifted to clotting factor free vacutainers to separate the serum. Liver of each animal, obtained after dissection was placed in Petri dish containing 0.9% saline, cut into 1×1cm pieces and stored with 10% formalin in labeled glass bottles.

Evaluation of serum biochemical variations

Serum was obtained after centrifugation of sample for 20minutes at 4000rpm. Afterwards collecting serum in new labeled eppendorf cups, serum was kept at -20°C, till further use. The analysis of serological parameters was performed by using ready to use kits (Chemhouse). Control and experimental samples were processed according to manufacturer's instructions.

Evaluation of histological variations

Tissue samples were fixed in 10% formaldehyde and then dehydrated in ethanol (40%-100%). After dehydration tissues were cleared in xylene and embedded in paraffin wax followed by sectioning. Sections (5μm thick) were stained with heamatoxylin and eosin for histopathological findings and Prussian blue iron staining for histochemical findings. After staining sections were observed under 100X & 400X magnifications using light microscope (Olympus VANOX).

Statistical analysis

Prism Graph pad 5 software (San Diego, CA) was used to analyze the data. Results were presented as mean ± SEM. Statistical significance was assessed by means of one-way ANOVA test followed by Tukey's post hoc analysis and *P*-values less than 0.05 were considered to indicate a significant difference among different groups.

Results

Effect on serological parameters

Hepatotoxin thioacetamide is recognized to induce noticeable liver damage in exposed animals. This is evident by the changes, which comprise 30%, 32% and 37% reduction in total proteins, albumin and ALT level respectively in serum analysis of group I as compared to control animals while *N. oleander* extract exhibited novel hepatoprotective potential against thioacetamide tempted liver injury by lowering the

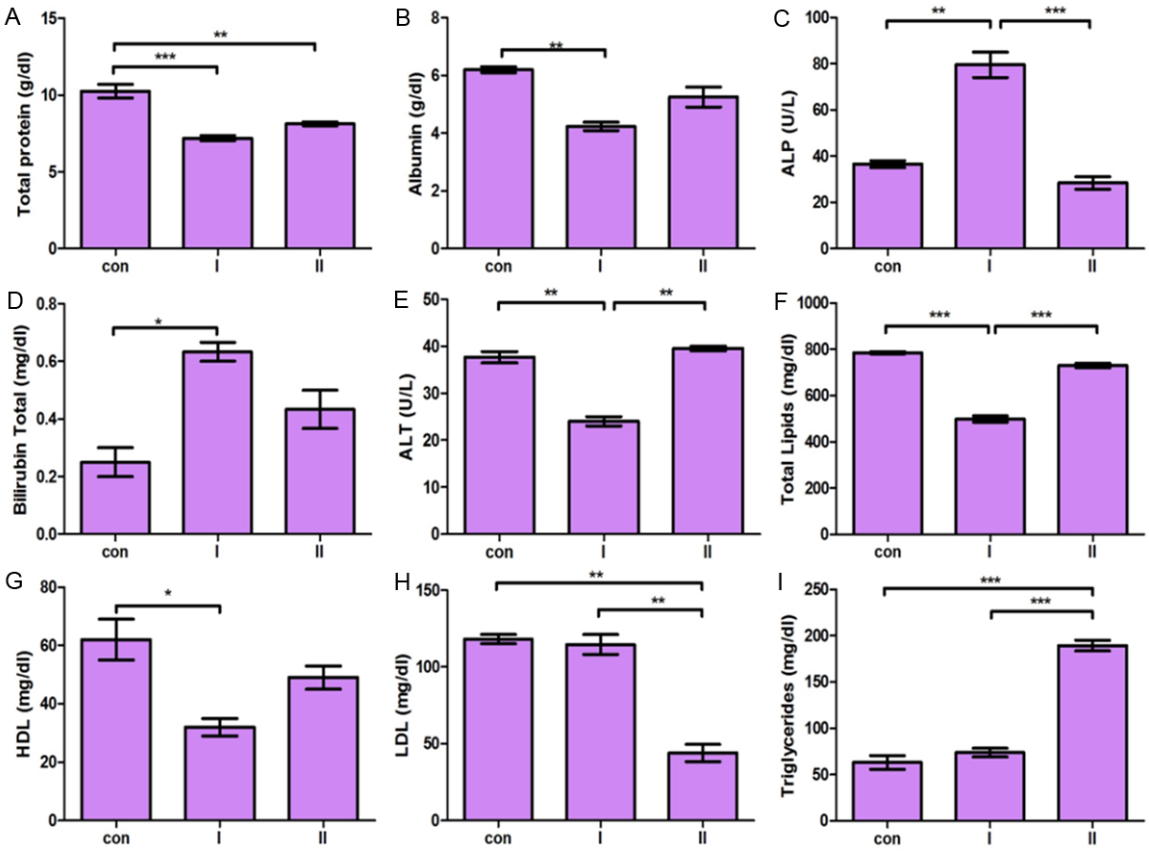

Figure 1. Changes in serum (A) total proteins, (B) albumin, (C) ALP, (D) bilirubin total, (E) ALT, (F) total lipids, (G) HDL, (H) LDL and (I) triglycerides level in control group, TAA administration group (I) and TAA plus N. oleander leaves extract administration group (II). Values are mean ± SEM, error bar indicating the standard error of mean. $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$.

Table 1. Effect of TAA & TAA+*N. oleander* leaves extract on serum biochemical parameters (Data are Mean ± S.E.M.)

	Control	Group I	Group II
Total proteins	10.25±0.45	7.20±0.17***	8.13±0.12**
Albumin	6.20±0.10	4.23±0.14**	5.25±0.35
ALP	36.50±1.50	79.50±5.50**	28.33±2.72
Bilirubin total	0.30±0.05	0.63±0.03*	0.43±0.06
ALT	37.67±1.20	24.00±1.00**	39.50±0.500
Total lipids	785.0±5.00	498.3±13.6**	730.0±10.0
HDL	62.00±7.00	32.00±3.00*	49.00±4.00
LDL	118.0±3.00	114.5±6.50	44.00±5.50**
Triglycerides	63.00±7.23	73.67±4.80	189.3±5.69***

Con, control; group I, TAA oral intake for 18 weeks; group II, TAA oral intake for 18 weeks plus additional 7 days oral intake of *N. oleander* leaves extract. Data were processed by one-way ANOVA followed by Tukey's *post hoc*: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

reduction level of total proteins, albumin and ALT to 12%, 24% & 64% respectively in group II (*P*=0.0007; **Figure 1A**, *P*=0.005; **Figure 1B** & *P*=0.001; **Figure 1E** respectively). ALP and bilirubin total level show a marked increment of 129% and 153% respectively in group I concerning control group at the same time these values were brought down to near normal range in group II (*P*=0.001; **Figure 1C** & *P*=0.012; **Figure 1D** respectively). A decreasing trend of total lipids and HDL (37% & 48% respectively) in group I was successfully countered by *N. oleander* leaves extract in group II (*P*=0.0001; **Figure 1F** & *P*=0.05; **Figure 1G** respectively). Group II shows a significant decrease (63% & 200%) in LDL and triglycerides level with reference to control group (*P*=0.0009; **Figure 1H** & *P*≤0.0001; **Figure 1I** respectively) (**Table 1**).

Histopathological findings

The liver sections of control animals were devoid of any kind of histopathological alterations, and H & E stained sections having regular

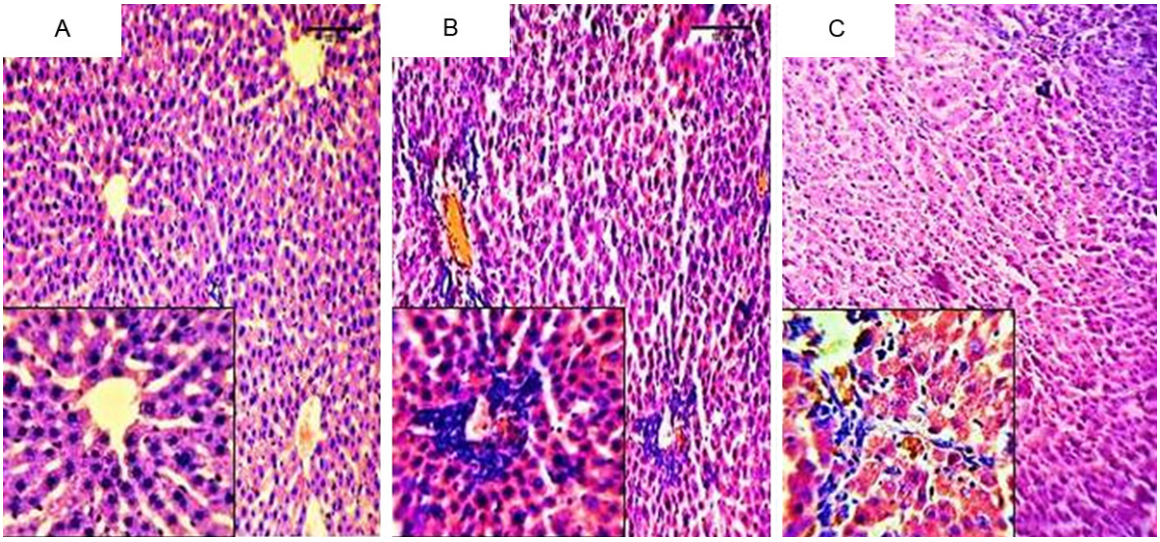

Figure 2. H & E staining on liver tissue of (A) Control group, provided with normal drinking water, (B) Group I, provided with 18 weeks oral intake of TAA (200g/L) and (C) Group II, provided with 18 weeks oral intake of TAA (200g/L) plus additional 7 days oral intake of N. oleander leaves extract. Microphotographs were taken at 100X & 400X magnifications.

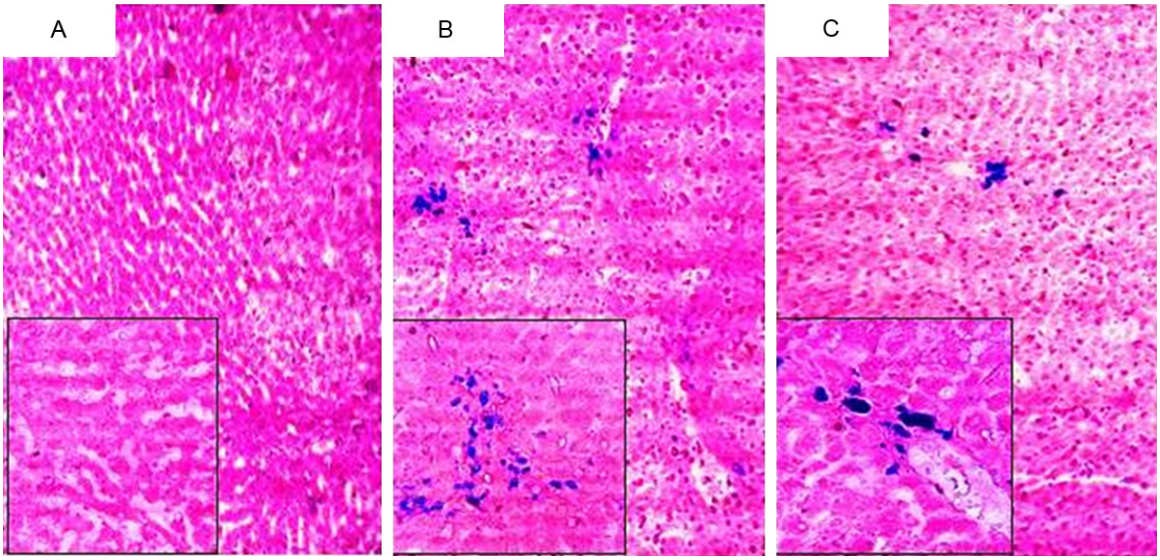

Figure 3. Staining on liver tissue of (A) Control group, provided with normal drinking water, (B) Group I, provided with 18 weeks oral intake of TAA (200g/L) and (C) Group II, provided with 18 weeks oral intake of TAA (200g/L) plus additional 7 days oral intake of N. oleander leaves extract. Microphotographs were taken at 100X & 400X magnifications.

cellular architecture appeared normal. The hepatic cells had undamaged sinusoidal spaces, cytoplasm, prominent nucleus and nucleolus (**Figure 2A**). The stained sections of group I exposed hepatic cells with intense toxicity represented by aggregation of lymphocytes in fibrotic area, giant hepatocytes, degenerated hepatic lobular architecture, increased rate of hepatopoises, low degree cirrhosis, high level of necrotic cells infiltration, occurrence of plasma cells in sinusoidal spaces particularly in vicinity of necrotic areas, increased sinusoidal Kupffer cells density and mild level of hemorrhage (**Figure 2B**). Treatment with N. oleander appeared to minimize the TAA-induced toxicity as conformed by lower degree of hepatic lobu-

lar architecture deformation, intra lobular occurrence of inflammatory cells, site containing-macrophages and low degree of hepatocytic necrosis (**Figure 2C**).

Histochemical findings

There was no noticeable hemosiderin granule were observed within hepatocytes, sinusoidal spaces and Kupffer cells, therefore no iron deposition in liver sections of control samples. In group II extensive blue stained hemosiderin granules were observed in hepatic sections of rats, distinctly marked bluish granules in the liver section were directed attention towards the iron storage in the liver. These hemosiderin granules were predominantly visible within the peripheral area of hepatic lobules and Kupffer cells. Meanwhile, lesser degree of iron deposition was observed in group III (**Figure 3**).

Discussion

In the present study, level of serum total protein, albumin, ALT, total lipids, HDL, ALP & bilirubin level was significantly reduced due to TAA toxicity. The difference in level of these biochemical parameters was contributed by many factors. Hypoproteinemia condition was usually concurrent with reduction in albumin concentration. The total protein level is low in hepatotoxic conditions owing to instability in the protein metabolisms or anxious biosynthesis of protein in the cirrhotic liver, carbohydrate and lipid metabolism [26]. Hypoalbuminaemia occurs as a consequence of dietary deficiency, liver disease, increased catabolism and nephritic syndrome. Hypoalbuminemia during the inflammatory reaction can be due to decrease in the rate of albumin mRNA transcription and thus translation [27]. Under inflammatory conditions, albumin gene transcription and translation is reduced due to different cytokines including IL-1, IL-6 and tumor necrosis factor-α [28-30].

Raised ALP activity is often observed in bone disease or liver disease involving the biliary tract. Rise is most likely a sign of enzyme activity on cell membrane [31]. Pathology that mainly blocks the flow of bile, either intrahepatic or extrahepatic, is originally linked with a rise in serum ALP activity chased by an increase in serum total bile acid concentration and finally hyperbilirubinemia [32]. The cause for the hyperbilirubinaemia can be obstruction of the bile duct, cirrhosis, hepatitis and several enzyme deficiencies. Bilirubin is also increased by pre-hepatic causes such as haemolytic disorders or periportal liver injury involving degenerative cell swelling [33, 34]. TAA disrupts the plasma membrane of erythrocytes stability by converting it to a labile membrane which was easily to go through lyses. The liver excretes bilirubin (hemoglobin breakdown product). During chemically induced hepatotoxicity, elevated bilirubin level reflects the necrotic conditions of hepatocytes [35]. An impaired lipid metabolism is commonly associated with infection and inflammation. Progressive decrease in serum HDL level may reflect reduced hepatic apoprotein A-1 synthesis and lecithin-cholesterol aceyltransferase deficit [36, 37].

The aqueous extract of N. oleander try to retain the structural integrity of the membranes of hepatocytes was visible from the protection provided in comparison to the enzyme level in the TAA treated rats. Its protective activity might be due to its effect against cellular leakage and loss of functional integrity of the cell membrane in liver. Along side its hepatoprotective activity, a few side effects of extract were also observed. An increase in triglycerides level in animals having N. oleander treatment was an indication that extract has some components that show adverse effect on LDL and triglycerides production. An increase in triglycerides level provides the roots for cardiovascular disorders [38].

Histopathological studies also displayed the potential of the extract as a hepatoprotective. Extract ingestion after TAA shows a low level of damage to hepatocytes in comparison to the TAA treated animals. In the hepatocytes of the experimental animals treated with N. oleander leaves extract after intoxication with TAA, the nuclei are not very clear as in normal hepatocytes, but compared to the TAA damaged nuclei, the number of hepatocytes with normal nuclei are greater. Only some cells shows higher number of vacuoles in the cytoplasm and necrosis. Lobular architecture was well preserved as compared to architecture in toxic cells. Histochemical assessment of hepatic tissues revealed massive iron deposition in TAA treated rats. Iron is an essential element for virtually every form of life due to its key role in

most biological systems and metabolic pathways [39]. During inflammatory situation, a distraction of iron transfer happens that is iron stores in reticuloendothelial system instead of circulation so as to reduce the accessibility of this vital element [40]. Liver production of hepcidin promotes iron sequestration by macrophages, while limiting its recycling [41] N. oleander leaves extract possibly hold back the liver Hepcidin production to avoid annoyance in iron accessibility.

Conclusion

Taken together these findings, we can conclude that TAA provides the foundation of chronic liver injury whereas N. oleander extract has the ability to protect liver by reversing the action of damaging events associated with TAA or at least lessen the liver damage owing to its possible hepatoprotective nature. However, an extensive research regarding N. oleander extract is required which includes the purification of different chemical components with further research on their anti carcinogenic ability with no inflammation is necessary before the extract can be utilized for any type of protective therapy.

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Disclosure of conflict of interest

None.

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