Alleviation of Dimethylnitrosamine-Induced Liver Injury and Fibrosis by Supplementation of *Anabasis articulata* Extract in Rats

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Abstract *Anabasis articulata* (Forssk) Moq. (Chenopodiaceae) is an herb, grows in Egypt, and used in folk medicine to treat diabetes, fever, and kidney infections. The protective and therapeutic effects of the ethanol extract of *A. articulata* aerial parts were evaluated against dimethylnitrosamine (DMN)-induced liver fibrosis, compared with the standard drug, silymarin. Hepatic hydroxyproline content, serum transforming growth factor- β 1 (TGF- β 1), interleukin 10 (IL-10) and fructosamine were measured as liver fibrosis markers. Hepatic malondialdehyde (MDA), nitric oxide (NO), catalase (CAT), glutathione reductase (GR) and glutathione content (GSH) were measured as oxidant/antioxidant markers. Parallel histopathological investigations were also performed. Protective and therapeutic administration of *A. articulata* (100 mg/kg daily for 4 weeks), markedly prevented DMN-induced loss in body and liver weights. The extract significantly inhibited the elevation of hepatic hydroxyproline, NO and MDA ($P < 0.05$), as well as serum fructosamine, and TGF- β 1 ($P < 0.05$) induced by DMN while it restored IL-10 to normal level in both protective and therapeutic groups. Furthermore, *A. articulata*

prevented the depletion in CAT, GR, and GSH levels ($P \leq 0.05$). In addition, oral administration of *A. articulata* extract and silymarin to both protective and therapeutic groups reduced the increase in liver function enzyme activities; alanine and aspartate aminotransferases, gamma-glutamyl transferase in addition to alkaline phosphatase, and caused significant increase in serum albumin concentration as compared to DMN group. These data corresponded closely with those obtained for the drug silymarin. Histopathological studies confirmed the biochemical data and revealed remarkable improvement in liver architecture. Thus, it could be concluded that, *A. articulata* extract exhibited in vivo hepatoprotective and therapeutic effects against DMN-induced liver injury and may act as a useful agent in controlling the progression of hepatic fibrosis through reduction of oxidative stress and improving liver function.

Keywords Dimethylnitrosamine · Oxidative stress · Liver fibrosis · *Anabasis articulata*

Introduction

Hepatitis, or inflammation of the liver, is induced by a variety of causative agents, including viral infection, chronic alcohol consumption, constant cholestasis, nonalcoholic steatohepatitis, and drug use [1]. The net result of this process is liver damage and destruction, primarily through substantial hepatocellular necrosis. When the extent of the damage is low, the liver regenerates normal hepatic tissue. In contrast, when liver damage occurs repeatedly, the disease becomes chronic and is characterized by a different healing process. These results, accumulation of extracellular matrix proteins (ECM), including

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fibrillar collagen [2]. The harmful outcome of chronic hepatitis is fibrosis, which results from abnormal accumulation of collagen, the main source of which is hepatic stellate cells (HSCs), and can further develop into cirrhosis and ultimately to end-stage liver disease, liver failure, or hepatocellular carcinoma [2].

The direct role of abnormal glucose metabolism in the development of tissue fibrosis was previously reported [3]. Serum fructosamine, is one of the abnormal glucose metabolism markers, results from spontaneous non-enzymatic condensation of glucose and proteins [4], and has been shown to increase the expression of ECM and activation of protein kinase C, which has a central role in tissue fibrosis [5].

Injury of hepatocytes results in the recruitment and stimulation of inflammatory cells, as well as resident ones, including Kupffer cells. Factors released by these inflammatory cells lead to activation of HSCs and their transformation into a myofibroblast-like phenotype. Chronically activated HSCs produce large amounts of ECM and enhance fibrosis by secreting a broad spectrum of cytokines such as TGF- β 1. This exerts pro-fibrotic actions in other cells and in an autocrine manner perpetuates their own activation [6]. This is accompanied by a decrease in anti-inflammatory cytokines such as IL-10 [7]. Oxidative stress, including the decrease of antioxidant capacity, plays an important role in the pathogenesis of liver fibrosis via different pathways [8]. Increased ROS, reactive nitrogen species (RNS), and MDA, are commonly detected in most types of experimental liver fibrosis [9]. Oxidative stress induces activation of HSCs through the direct effects of ROS, reactive aldehyde products of lipid peroxidation and finally via the reaction of NO with ROS, yielding peroxynitrite (ONOO⁻). The latter is a very reactive, toxic and strongly oxidizing compound, able to induce DNA damage and lipid peroxidation. This process leads to membrane damage and correlates positively with fibrosis through inducing fibrogenic cytokines and increasing collagen synthesis in fibroblasts and HSCs [9].

DMN is a potent hepatotoxin, carcinogen and mutagen [10]. Repeated exposure to lower doses of DMN as small as 10 and 20 mg/kg causes sub-acute and chronic liver injury with varying degrees of necrosis, fibrosis, and nodular regeneration [11, 12]. Metabolism of DMN produces formaldehyde and methanol, coupled with hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), hydroxyl radicals (OH[•]), and reacting with nucleic acids and proteins to form methylated macromolecules, resulting in oxidative stress causing liver damage [13]. Conventional or synthetic drugs used in the treatment of liver fibrosis are sometimes inadequate and can have serious adverse effects [14]. Therefore, medicinal plants are of a great use in many countries [15]. In this concern, many natural products are in use for

the treatment of liver ailments including sho-saiko-to (TJ-9), or silymarin, which is the natural drug of choice [16]. Silymarin is a standardized extract from the seeds and fruits of the milk thistle *Silybum marianum* (L.) and contains, as its main constituents, the flavonolignans silybin, isosilybin, silydianin and silychristin. The major component of silymarin is silybin, also known as silibinin [17]. Silibinin was shown to stimulate hepatocyte RNA synthesis, act as a radical-scavenger and hepatoprotectant, suppress HSCs proliferation and collagen synthesis in vitro [18]. *Anabasis articulata* (Forssk) Moq. (Chenopodiaceae) locally named as ‘ajrem’, is a wild plant widely used in folk medicine to treat diabetes, kidney infections, fever, headache, and skin diseases such as eczema [19]. It is taken orally after boiling in water as a single herb or with other medicinal plants [20]. Previous phytochemical screening of *A. articulata* aerial part revealed the presence of saponin as a main component in addition to coumarins, flavonoids, alkaloids and others [21, 22]. It has been previously reported that, saponins of *A. articulata* possess antidiabetic activity [20, 23, 24]. In general, saponins are reported to have antifibrotic activity [25]. Therefore, the present study aims to evaluate the protective and therapeutic effects of the ethanol extract of *A. articulata* aerial part in comparison with the currently available antifibrotic drug (silymarin), against liver fibrosis induced by DMN. Histopathological investigations on fibrotic and treated rats liver were also conducted.

Materials and Methods

Chemicals

All chemicals used were of high analytical grade. Hydroxyproline was purchased from Merck (USA). DMN and all other chemicals were obtained from Sigma (Germany). Kits used for the quantitative determination of ALT and AST, ALP, GGT and albumin were purchased from Stanbio (USA), ELISA kits for TGF- β 1 and IL-10 were purchased from Invitrogen (USA) and fructosamine kit was purchased from Quimica Clinia Aplicada, (Spain).

Plant Material, Extraction and Isolation

The aerial parts of *A. articulata* were collected from Quatamia-Suez desert road in 2010 and were kept in the herbarium of National Research Center, Cairo, Egypt. Air dried powdered of the aerial parts of *A. articulata* (1 kg) were exhaustively extracted with petroleum ether (40–60 °C). Then the mark was dried at room temperature and re extracted with 70 % EtOH, and the extract was concentrated under vacuum yielded (40 g). The crude ethanol extract was

suspended in H₂O and partitioned with EtOAc, the EtOAc extract concentrated under vacuum afforded (10 g) of gummy residue. The EtOAc residue was loaded on silica gel column chromatography (80, 3 cm) eluted with n-hexane, n-hexane–EtOAc, EtOAc–CHCl₃, CHCl₃–MeOH and finally MeOH. Fractions (20 ml) were collected; similar fractions were combined and separated on TLC silica gel plates. The compounds were purified by HPTLC and on column Sephadex LH-20. The main pure compounds obtained were identified by different spectral analyses (1 H-NMR, MS) [26].

Previous work of Kambouche et al. [23] and Metwally et al. [26], determined the acute and chronic toxicity of *A. articulata* and confirmed the orally safety of *A. articulata* administration (LD₅₀ = 1.5 gm/kg body weight) .

Animals

Male Sprague–Dawley rats (120–150 g) were obtained from the animal house of the National Research Centre, Dokki, Cairo, Egypt. Rats were fed a standard diet (El-Kahira Co. for Oil and Soap) and free access to tap water. They were kept for two weeks to acclimatize to the environmental conditions.

Ethics

Ethical conditions for treatment of animals complied with the guidelines established by the Animal Care Committee and approved by the ethical committee of National Research Center (Approval No. 10031).

Induction of Liver Fibrosis *A. articulata* Extract Treatment

Hepatic fibrosis was induced by intra-peritoneal injections of DMN in saline (10 mg/kg body weight in 0.2 ml saline for each injection) [10], weekly on the first three consecutive days for 4 and 6 weeks. Control animals also received an equal volume of saline without DMN [24]. *Anabasis articulata* extract and silymarin were orally administered by gavages in similar doses for both protective and therapeutic groups (100 mg/kg body weight in 1 ml aqueous Tween 80 solution for each dose) [20, 26], daily for 4 weeks.

Experimental Design

Sixty rats were divided into three main classes:

Class I Four protective groups, six rats each. *Anabasis articulata* and silymarin were co-administered with DMN for 4 weeks. Group 1: control rats sacrificed post four weeks; Group 2: rats treated with DMN and sacrificed post

four weeks; Groups 3 and 4: rats treated with DMN co-administered with *A. articulata* extract and silymarin respectively and sacrificed post 4 weeks.

Class II Four therapeutic groups, six rats each. The plant extract and silymarin were given post 6 weeks of DMN injection, for 4 weeks and then sacrificed. Group 5: normal control rats sacrificed post 10 weeks; Group 6: rats treated with DMN for 6 weeks and sacrificed post 10 weeks; Groups 7 and 8: fibrotic animals (post 6 weeks of DMN injection), treated with the *A. articulata* and silymarin respectively for 4 weeks and then sacrificed (post 10 weeks).

Class III Two normal groups, six rats each. Group 9 and 10: normal animals treated with *A. articulata* extract and silymarin respectively, for 4 weeks and then sacrificed.

Sample Preparations

At the end of each experimental period (post 4 weeks in classes I, III and post 10 weeks in class II), blood samples were collected into sterilized tubes, and serum was separated by centrifugation at 3,000 rpm for 10 min, stored at –80 °C for subsequent biochemical analyses. Rats were then sacrificed under diethyl ether anesthesia, and the liver samples were rapidly removed and stored at –80 °C for biochemical determination of fibrotic, oxidative, and antioxidant markers. In addition, liver samples were removed and placed overnight in fixative containing 10 % formalin, liver samples from different groups were paraffin-embedded in stains and cut at 5 µm in the longitudinal plane.

Biochemical Analyses

Hepatic Hydroxyproline Determination

Liver collagen concentration was determined by measuring hydroxyproline content using the method of Jamall et al. [27]. Briefly, liver samples were homogenized and hydrolyzed in 6 N HCl at 110 °C for 18 h. After filtration of the hydrolysate, chloramine T was added to a final concentration of 2.5 mM. The mixture was then treated with 410 mM paradimethyl-amino-benzaldehyde and incubated at 60 °C for 30 min. After cooling to room temperature, the samples were read at 560 nm against a reagent blank which contained the complete system without added tissue. The concentration of hydroxyproline in each sample was determined from a standard curve plotted for known quantities of hydroxyproline.

Determination of Serum Fructosamine, TGF-β1 and IL-10 Levels

Fructosamine (glycated serum protein) was determined using diagnostic kit (Quimica Clinia Aplicada, Spain),

according to the method of Schleicher and Vogt [28]. Specific enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, USA) were used to measure serum TGF- β 1 and IL-10 according to the manufacturer's instructions.

Determination of Oxidative Stress and Antioxidant Markers

Nitric oxide was determined in tissue liver homogenate using a quantitative colorimetric assay based on the Griess reaction, according to Moshage et al. [29]. In brief, liver samples were homogenized in 10 % trichloroacetic acid (TCA), centrifuged, and the supernatant sample was added to sulfanilamide and mixed. After 5 min, *N*-1-naphthyl ethylene diaminedihydrochloride (0.1 %) was added and vortexed. The developed color was measured after 15 min at 540 nm against blank. Lipid peroxidation in the liver was determined by measurement of MDA [30]. In brief, liver tissue was homogenized in 10 % TCA. After centrifugation, the supernatant was added to 0.67 % thiobarbituric acid (TBA) and the solution heated in a boiling water bath for 15 min. The mixture was allowed to cool and the absorbance was read at 535 nm.

Determination of Antioxidant Markers

Catalase activity was assayed in tissue liver homogenate according to Lubinsky and Bewley [31]. The reaction was initiated by adding 20 μ l 5 % liver homogenate of concentration to 2.53 ml of the reaction mixture (sodium potassium phosphate buffer containing H₂O₂). The disappearance of hydrogen peroxide was monitored by following the decrease in absorbance at 230 nm by spectrometer using molar extinction coefficient for hydrogen peroxide of 62.4.

Glutathione reductase was assayed in tissue liver homogenate according to Erden and Bor [32]. To 0.01 ml of liver tissue homogenate, 1 ml of the reaction mixture containing the following solutions: 4.1 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 5.7 mM EDTA, 60 mM KCl, 2.6 IU GSSG and 0.2 mM of NADPH. The reaction was started by the addition of tissue homogenate. The decrease in absorbance was monitored at 340 nm.

Glutathione was estimated according to the method of Moron et al. [33]. Equal volumes of liver tissue homogenate and 20 % TCA acid were mixed. The precipitated fraction was centrifuged and to 0.25 ml of supernatant, 2 ml of 0.6 mM 5,5'-dithiobis(2-nitrobenzoic acid) reagent were added. The final volume was made up to 3 ml with phosphate buffer (0.2 M, pH 8.0). The color developed was read at 412 nm against reagent blank. Different concentrations of standard glutathione were prepared and processed as above for obtaining a standard graph.

Assessment of Liver Function

ALT, AST, GGT, ALP, and albumin were determined using diagnostic Kits (Stanbio, USA) according to the manufacturer's instructions.

Histological Examination

Tissue samples were taken from the right lobe of the liver of each rat and fixed with 10 % formaldehyde. Tissues were processed routinely and embedded in paraffin. Sections of 5–7 μ m in thickness were stained using masson-trichrome (MT) stain for collagen visualization and hematoxylin and eosin (H&E) for histopathological examination.

Statistical Evaluation

Data were analyzed by comparing values for different treatment groups with the values for individual control. All values were expressed as the mean $\pm$ SD. Significant differences between the groups were statistically analyzed using a one-way analysis of variances (ANOVA), followed by a non-parametric post hoc test, [least significance difference (LSD)], at *p* value of 0.05 or less was considered statistically significant.

Results

Effect of A. articulata on Body and Liver Weights

The protective and therapeutic effects of the used plant extract on body and liver weights of rats are shown in Table 1. Treatment with DMN (G2 and G6) caused a significant decrease in both body and liver weights as compared to control groups (G1 and G5, respectively). Oral administration of *A. articulata* extract and silymarin restored body and liver weights in protective and therapeutic groups as compared to DMN-treated groups.

Effect of A. articulata on Hepatic Hydroxyproline and Serum Fructosamine, TGF- β 1 and IL-10

As shown in Table 2, the hepatic collagen content, expressed as hydroxyproline, fructosamine and TGF- β 1 is significantly increased in DMN groups as compared to control groups. This elevation was diminished by the administration of *A. articulata* extract and silymarin in both protective and therapeutic groups. On the other hand, serum level of IL-10 showed a marked reduction in DMN-intoxicated groups as compared to the control groups. Treatments with *A. articulata* extract and silymarin significantly normalized the level of IL-10 in protective and therapeutic groups.

Table 1 Protective and therapeutic effects of *A. articulata* extract and silymarin on body and liver weights of DMN-treated rats

Index	Body weight (g)	Liver weight (g)
Prophylactic groups		
Control (G1)	269.33 ± 19.22	10.70 ± 1.11
DMN (G2)	220.00 ± 13.00 ^a	8.27 ± 0.21 ^a
DMN + <i>A. articulata</i> (G3)	251.00 ± 6.55 ^{n#}	9.27 ± 0.46 ^{n#}
DMN + Silymarin (G4)	259.00 ± 23.26 ^{n*}	10.24 ± 1.77 ^{n*}
Therapeutic groups		
Control (G5)	343.66 ± 23.71	12.51 ± 0.74
DMN (G6)	288.66 ± 11.67 ^a	10.84 ± 0.75 ⁿ
DMN + <i>A. articulata</i> (G7)	299.00 ± 10.82 ^{n#}	11.36 ± 0.77 ^{n#}
DMN + Silymarin (G8)	297.00 ± 14.73 ^{n#}	11.49 ± 0.73 ^{n#}

All data expressed as mean ± SD of ten rats

* Significant at $P \leq 0.05$; # not significance as compared with DMN group

^a Significant at $P \leq 0.05$ as compared to control group

ⁿ Not significance as compared to control group

Effect of *A. articulata* on Oxidative Stress and Antioxidant Markers

Table 3 shows the levels of hepatic oxidative stress and antioxidant markers. The results revealed that induction of fibrosis with DMN in rats showed marked increase in the level of hepatic NO and MDA while a significant reduction was observed in the levels of hepatic antioxidants, CAT, GR, and GSH as compared to control groups. The administration of *A. articulata* extract as well as silymarin significantly protected against DMN-induced elevation in NO

and MDA as evidenced by a significant inhibition in their levels. Also, the activities of antioxidant levels were significantly enhanced to near normal level.

Effect of *A. articulata* on Liver Function

As indicated in Table 4, DMN treatment resulted in an increase in serum ALT, AST, GGT, and ALP enzyme activities while serum albumin was significantly lower as compared to normal control groups. Oral administration of *A. articulata* extract and silymarin ameliorated enzyme activity levels and caused a significant increase in serum albumin concentration as compared to DMN groups.

Histopathological Examination

In control liver, normal hepatic architecture was noticed (Figs. 1a, 2a).

The dispensation of DMN to rats, caused histopathological changes in their livers as indicated by hepatic necrosis, collapse of the liver parenchyma, dilated and congested blood sinusoids, nuclear dysplasia and mixed acute and chronic cell eosinophilia (Fig. 1b). These changes were more severe after 6 weeks of DMN injection, as revealed by disorientation of lobular architecture associated with focal necrotic areas, degeneration in the cytoplasm and loss of cell boundaries and pyknotic nuclei (Fig. 2b). These alterations were remarkably reduced in the liver sections of the *A. articulata* and silymarin treated rats in both protective (Fig. 1c, d) as well as therapeutic groups (Fig. 2c, d).

Moreover, serial sections were stained with MT stain for collagen. In the liver sections of normal control groups,

Table 2 Protective and therapeutic effects of *A. articulata* extract and silymarin on hepatic hydroxyproline content, serum levels of fructosamine, TGF-β1 and IL-10 of DMN-treated rats

Index	Hydroxyproline (μg/g liver tissue)	Fructosamine (μmol/L)	TGF-β1 (ng/ml)	IL-10 (pg/ml)
Prophylactic groups				
Control (G1)	384 ± 41.00	211.66 ± 7.63	26.27 ± 1.74	59.77 ± 3.01
DMN (G2)	908 ± 47.00 ^a	801.66 ± 30.89 ^a	75.71 ± 7.65 ^a	30.96 ± 5.32 ^a
DMN + <i>A. articulata</i> (G3)	571 ± 58.00 ^{a*}	309.00 ± 30.00 ^{a*}	54.56 ± 4.77 ^{a*}	43.66 ± 4.04 ^{a*}
DMN + Silymarin (G4)	514 ± 24.00 ^{a*}	341.00 ± 7.54 ^{a*}	36.43 ± 3.80 ^{a*}	53.01 ± 7.92 ^{n*}
Therapeutic groups				
Control (G5)	339 ± 49.00	251.00 ± 7.54	26.45 ± 5.78	50.73 ± 2.55
DMN (G6)	944 ± 101.00 ^a	1045.0 ± 95.3 ^a	58.66 ± 4.36 ^a	29.16 ± 2.75 ^a
DMN + <i>A. articulata</i> (G7)	661 ± 27.00 ^{a*}	416.00 ± 37.26 ^{a*}	43.44 ± 7.48 ^{a*}	44.43 ± 4.17 ^{n*}
DMN + Silymarin (G8)	639 ± 65.00 ^{a*}	319.0 ± 27.87 ^{a*}	29.29 ± 1.87 ^{n*}	47.41 ± 7.41 ^{n*}

All data expressed as mean ± SD of ten rats

* Significant at $P \leq 0.05$ as compared with DMN group

^a Significant at $P \leq 0.05$ as compared with control group

ⁿ Not significance as compared to control group

Table 3 Protective and therapeutic effects of *A. articulata* extract and silymarin on hepatic oxidative markers, NO and MDA and hepatic antioxidant markers, CAT, GR and GSH, of DMN treated rats

Index	NO ($\mu\text{g/g}$ liver tissue)	MDA (nmol/g liver tissue)	Catalase ($\mu\text{mol/min/mg}$ protein)	GR (nmol/min/mg protein)	GSH ($\mu\text{mol/g}$ tissue)
Prophylactic groups					
Control (G1)	20.33 $\pm$ 2.88	26.33 $\pm$ 1.52	14.66 $\pm$ 0.90	4.36 $\pm$ 0.49	5.38 $\pm$ 0.74
DMN (G2)	113.66 $\pm$ 11.1 ^a	54.33 $\pm$ 5.48 ^a	6.77 $\pm$ 0.47 ^a	1.19 $\pm$ 0.23 ^a	0.65 $\pm$ 0.11 ^a
DMN + <i>A. articulata</i> (G3)	51.00 $\pm$ 5.19 ^{a*}	37.03 $\pm$ 1.07 ^{a*}	10.88 $\pm$ 0.24 ^{a*}	2.87 $\pm$ 0.62 ^{a*}	2.40 $\pm$ 0.50 ^{a*}
DMN + Silymarin (G4)	57.33 $\pm$ 2.0 ^{a*}	32.76 $\pm$ 2.44 ^{a*}	10.53 $\pm$ 0.30 ^{a*}	3.23 $\pm$ 0.40 ^{a*}	4.63 $\pm$ 0.73 ^{a*}
Therapeutic groups					
Control (G5)	20.33 $\pm$ 2.88	28.26 $\pm$ 0.46	16.86 $\pm$ 1.85	4.44 $\pm$ 0.38	6.02 $\pm$ 0.70
DMN (G6)	156.0 $\pm$ 14.52 ^a	66.50 $\pm$ 5.50 ^a	6.97 $\pm$ 0.66 ^a	1.64 $\pm$ 0.30 ^a	2.02 $\pm$ 0.30 ^a
DMN + <i>A. articulata</i> (G7)	95.00 $\pm$ 2.00 ^{a*}	33.56 $\pm$ 1.36 ^{a*}	14.23 $\pm$.49 ^{a*}	2.85 $\pm$ 0.48 ^{a*}	4.30 $\pm$ 0.45 ^{a*}
DMN + Silymarin (G8)	81.66 $\pm$ 2.51 ^{a*}	33.86 $\pm$ 2.70 ^{a*}	13.96 $\pm$ 0.30 ^{a*}	3.32 $\pm$ 0.40 ^{a*}	3.83 $\pm$ 0.39 ^{a*}

All data expressed as mean $\pm$ SD of ten rats

* Significant at $P \leq 0.05$ as compared to DMN group

^a Significant at $P \leq 0.05$ as compared to control group

ⁿ Not significance as compared to control group

Table 4 Protective and therapeutic effects of *A. articulata* extract and silymarin on the serum levels of ALT, AST, GGT, ALP and albumin of DMN treated rats

Index	ALT (U/L)	AST (U/L)	GGT (U/L)	ALP (U/L)	Albumin (g/dl)
Prophylactic groups					
Control (G1)	21.62 $\pm$ 1.25	16.47 $\pm$ 0.71	8.13 $\pm$ 2.70	38.66 $\pm$ 6.02	6.85 $\pm$ 0.18
DMN (G2)	64.50 $\pm$ 1.00 ^a	51.90 $\pm$ 2.65 ^a	59.16 $\pm$ 5.75 ^a	118.56 $\pm$ 5.00 ^a	3.14 $\pm$ 0.22 ^a
DMN + <i>A. articulata</i> (G3)	29.50 $\pm$ 1.80 ^{a*}	17.50 $\pm$ 0.00 ^{n*}	12.31 $\pm$ 0.33 ^{a*}	45.00 $\pm$ 1.73 ^{a*}	5.67 $\pm$ 0.37 ^{a*}
DMN + Silymarin (G4)	25.75 $\pm$ 1.70 ^{n*}	18.96 $\pm$ 0.49 ^{n*}	15.80 $\pm$ 1.35 ^{a*}	75.33 $\pm$ 2. 50 ^{a*}	6.27 $\pm$ 0.32 ^{n*}
Therapeutic groups					
Control (G5)	21.66 $\pm$ 2.08	13.60 $\pm$ 0.69	8.50 $\pm$ 1.47	42.66 $\pm$ 1.15	5.92 $\pm$ 0.31
DMN (G6)	95.87 $\pm$ 5.20 ^a	59.76 $\pm$ 2.21 ^a	66.60 $\pm$ 0.53 ^a	126.00 $\pm$ 7.21 ^a	3.68 $\pm$ 0.16 ^a
DMN + <i>A. articulata</i> (G7)	66.66 $\pm$ 4.16 ^{a*}	21.83 $\pm$ 2.26 ^{a*}	14.10 $\pm$ 2.49 ^{a*}	60.50 $\pm$ 2.18 ^{a*}	5.05 $\pm$ 0.07 ^{a*}
DMN + Silymarin (G8)	35.83 $\pm$ 13.07 ^{a*}	29.40 $\pm$ 0.52 ^{a*}	13.70 $\pm$ 1.37 ^{a*}	62.50 $\pm$ 2.78 ^{a*}	5.02 $\pm$ 0.92 ^{a*}

All data expressed as mean $\pm$ SD of ten rats

* Significant at $P \leq 0.05$ as compared with DMN group

^a Significant at $P \leq 0.05$ as compared to control group

ⁿ Not significance as compared to control group

almost no collagen was observed (Figs. 3a, 4a). Injection of DMN to rats revealed deposition of collagen fibers in the centrilobular and periportal regions in their livers after 4 weeks of DMN injection (Fig. 3b). The collagen depositions were more excessive displaying bundles of collagen surrounding the lobules after 6 weeks of DMN injection (Fig. 4b). The thickening of these collagen fiber bundles was markedly reduced in protective (Fig. 3c, d) as well as therapeutic groups (Fig. 4c, d).

All studied parameters were measured in control rats administered the *A. articulata* extract (G9-10) and the data

obtained showed, non-significant changes with respect to the control non-treated group (G1), confirmed its biosafety at the concentration used.

Discussion

The present results show hepatoprotective and therapeutic effects of *A. articulata* against DMN-induced liver injury through reduction of oxidative stress and improving liver function.

Fig. 1 Protective effect of *A.articulata* extract (100 mg/kg b.w) and silymarin (100 mg/kg b.w) on DMN-treated rats (10 mg/kg b.w, 3 days/week, for 4 weeks). **a** A section of control liver shows the normal architecture of a hepatic lobule. The central vein (CV) lies at the centre of the lobule surrounded by the hepatocytes (HC) with strongly eosinophilic granulated cytoplasm, and distinct nuclei (N). Between the strands of hepatocytes the hepatic sinusoids are shown (HS), **b** a section of liver of rat after 4 weeks of first DMN injection

shows mid and peripheral cloudy degeneration, and granular precipitation in the cytoplasm, **c** a section of liver of DMN intoxicated rat and co-administrated with *A.articulata* extract shows lobule architecture that appear like normal. Notice the activated Kupffer cells (arrow) and few vacuoles (arrowhead), **d** a section of liver of DMN intoxicated rat and co-administrated with silymarin shows few focal necroses (arrow), congested and dilated blood sinusoids (arrowhead) (H & E stain X 300)

It was found that, when the extent of liver damage is low, the liver regenerates normal hepatic tissue. In contrast, when liver damage occurs repeatedly, the disease becomes chronic and is characterized by a different healing process leading to fibrosis/cirrhosis or sometimes to hepatocellular carcinoma [1, 2]. Herbal medicines are becoming popular among patients with liver disease, and are attractive as putative antifibrotics or hepatotherapeutics [34].

The present study aims to investigate the antifibrotic effect of the ethanol extract of *A. articulata* aerial part against DMN induced liver fibrosis. The effect of the currently available antifibrotic drug, silymarin, was also investigated.

Hydroxyproline, is considered as a major component of the protein collagen, produced by hydroxylation of the amino acid proline, and helps provide stability to the triple-helical structure of collagen by forming hydrogen bonds [35]. Hydroxyproline content in the liver reflexes the biochemical and pathological status of liver fibrosis [36]. In parallel with the previous published data, the current study has demonstrated that, injection of repeated dose of DMN to rats induced significant elevation in the hydroxyproline

content in liver tissue post 4 and 6 weeks of DMN injection as compared to control groups. These results declare the initiation of hepatotoxicity in response to DMN [37, 38].

Histopathological examination of liver tissue stained with Masson's trichrome for collagen showed, bundles of collagen surrounding the lobules and confirmed the biochemical results demonstrated an increase in collagen content in liver of DMN-injected rats. These results are supported by the study of Lee et al. [39], who reported that DMN caused an abnormal deposition of collagen fibers.

Protective and therapeutic treatment with the ethanol extract of *A. articulata* improved the dramatic increase in liver hydroxyproline content proved by histomorphological pictures, which showed a successful reduction in the thickening of the collagen bundles as compared to DMN intoxicated rats. These results indicate the antifibrotic beneficial effects of this extract. The phytoconstituents of ethanol extract of *A. articulata* aerial part used in the present study revealed the presence of saponins [21, 22], which was documented to exert antifibrotic action by inhibiting HSCs proliferation and activation leading to suppression of collagen synthesis [25, 40]. Similar results

Fig. 2 Therapeutic effect of *A.articulata* extract (100 mg/kg b.w) and silymarin (100 mg/kg b.w) on DMN-treated rats (10 mg/kg b.w, 3 days/week, for 4 weeks). **a** A section of control liver showing the portal tract (PT), **b** a section of liver of rat after 6 weeks of first DMN injection showing disturbed lobular architecture, **c** a section of liver of DMN intoxicated rat and treated with *A.articulata* extract shows hepatic lobule that appears like normal except congested some blood sinusoids and few vacuoles in hepatocytes, and **d** a section of liver of DMN intoxicated rat and treated with silymarin shows normal structure of the hepatocytes with activated Kupffer cells (H & E stain X 300)

Fig. 3 Protective effect of *A.articulata* extract (100 mg/kg b.w) and silymarin (100 mg/kg b.w) on DMN-treated rats (10 mg/kg b.w, 3 days/week, for 4 weeks) and stained with MT **a** liver section of control rat showing the distribution of few of collagen fibers in the portal tract, **b** liver section of rat treated with DMN for 4 weeks shows increase in the distribution of collagen in the portal tract as compared to control, **c** liver section of rat treated with DMN co-administrated with *A.articulata* extract shows decrease in the thickening of collagen bundles as compared to DMN treated rat, and **d** liver section of DMN treated rat co-administrated with silymarin shows decrease in the thickening of collagen bundles as compared to DMN treated rat (MT X 300)

were obtained for silymarin in DMN-treated rats and confirmed by previous studies [38, 41], which attributed its antifibrotic effect to the main active constituent, silybin [18].

The current study also demonstrated that, DMN induced marked elevation in serum fructosamine as compared to control group. Fructosamine results from spontaneous non-enzymatic condensation of glucose and proteins [4]. It has

Fig. 4 Therapeutic effect of *A.articulata* extract (100 mg/kg b.w) and silymarin (100 mg/kg b.w) on DMN-treated rats (10 mg/kg b.w, 3 days/week, for 4 weeks) and stained with MT **a** liver section of normal rats shows the normal distribution of collagen, **b** liver section of rat after 6 weeks of first DMN injection shows excessive distribution of collagen in the portal tract as compared to control, **c** liver section of DMN treated rat and given *A.articulata* extract shows decrease in the thickening of collagen bundles as compared to DMN treated rats, and **d** liver section of DMN treated rats and given silymarin showing decrease in the thickening of collagen bundles as compared to DMN treated rats (MT X 300)

a main role in an increasing the expression of ECM and activating protein kinase C, thus plays a central role in tissue fibrosis [5]. Previously it was demonstrated that, protein glycation is considered as one of the causative factors in the etiology of tissue fibrosis [42].

Treatment with the current plant extract, effectively reduced serum fructosamine level as compared to DMN-treated rats, confirming their antiglycating beneficial effects, which may be considered as one of their antifibrotic mechanisms. A similar effect was also found in the protective and therapeutic effects of silymarin in DMN-intoxicated rats [42].

Hepatic fibrosis is usually initiated by hepatocyte damage, leading to the recruitment of inflammatory cells and platelets with the subsequent release of cytokines, chemokines, and growth factors [43]. These factors probably affect the inflammatory and repairing phase of liver fibrosis by activating HSCs [44]. This is accompanied by down-regulation of anti-inflammatory cytokines, which has an important role in attenuating the progression of fibrosis [7].

In parallel with the above information, the present study revealed that, the hepatotoxic agent, DMN, leads to pronounced increment in serum pro-inflammatory fibrogenic cytokine, TGF- β 1, accompanied with a decrease in serum anti-inflammatory cytokine, IL-10. These results are confirmed by previous studies indicating the alterations in these cytokines in response to liver fibrosis [26, 45].

Protective and therapeutic treatments of DMN-injected rats with the tested plant extract, effectively down-

regulated the level of serum TGF- β 1. While, therapeutic treatment, up modulated the level of serum IL-10 and ameliorated this anti-inflammatory cytokine to its normal level. These results may imply the anti-inflammatory and immunomodulatory effects of *A. articulata* extract as they may be related to the presence of saponins [46]. Similarly, silymarin, markedly ameliorated both cytokine levels, confirming its immunomodulatory effect [26, 47].

In this study, DMN induced oxidative stress in liver of rats as evidenced by the marked elevation in the NO and MDA levels as compared to the normal untreated ones ($P \leq 0.05$). The alterations in these oxidative stress markers were accompanied with a depletion of enzymatic antioxidants, CAT and GR, as well as in the non enzymatic antioxidant, GSH ($P \leq 0.05$). These findings are consistent with previous authors who reported the deterioration of these markers in DMN- induced hepatotoxicity of rat's liver [48, 49].

Nitric oxide is a biologically active radical synthesized by the enzyme nitric oxide synthase (NOS). It plays a dual role, mediating both protective effects and tissue damage by its overproduction. In addition, it able to react with ROS, yielding peroxynitrite (ONOO⁻), which is a very reactive, toxic and oxidizing compound, inducing DNA damage and lipid peroxidation [50]. This process leads to membrane damage and correlates positively with tissue fibrosis through eliciting fibrogenic cytokines and increasing collagen synthesis in fibroblasts and HSCs [9].

The decrease in the activities of CAT and GR in the present study could be attributed to their excessive

utilization in scavenging the free radicals overload generated during the metabolism of DMN. Enzymatic scavengers like CAT and GR, protect the system from the deleterious effects of ROS [51]. Catalase catalyses the detoxification of hydrogen peroxide into H₂O and O₂. Hence, its inhibition may damage the first line of defense represented in enzymatic antioxidants against hydrogen peroxide toxicity [52]. GR has an important accessory antioxidant function in regeneration of GSH (reduced) from GSSG (oxidized) in the presence of NADPH, therefore, preventing cellular loss of GSH [53]. Depletion of GSH and its dependent enzyme, GR, in liver of DMN treated rats indicated damage to the second line of antioxidant defense. GSH is the predominant low molecular weight thiol and the most important non-enzyme antioxidant in mammalian cells [54]. GSH and its precursors effectively protect cells against oxidative stress-induced damage by scavenging toxic free radicals, suppressing lipid peroxidation and also it has been shown to exert protective effects against HSCs activation [55].

In this study, protective and therapeutic effects of *A. articulata* extract (G3) and silymarin (G4) in DMN treated rats, greatly improved the levels of hepatic oxidative stress markers, NO and MDA ($P \leq 0.05$), as well as up-regulated the levels of the antioxidant markers CAT, GR and GSH as compared to DMN treated rats ($P \leq 0.05$). These results may indicate the antioxidant beneficial actions of the used plant extract, which may be attributed to its active constituents previously mentioned. Saponins were found to have hepatoprotective effect against both, oxidative stress of FeCl₂–ascorbate induced lipid peroxidation in liver, and on superoxide radical scavenging activity [56]. Furthermore, Kiruthiga et al. [57], showed, that administration of silymarin enhanced the activities of antioxidant enzymes, SOD, CAT, glutathione peroxidase (GPx), GR and glutathione-S-transferase (GST), together with a decrease in the levels of MDA, a marker for lipid peroxidation, in erythrocytes exposed to H₂O₂. Also, silymarin exhibited an excellent hepatoprotective and antioxidant properties by restoring the hepatic marker enzyme activities and reversing the oxidant-antioxidant imbalance during diethylnitrosamine-induced oxidative stress in rats [58].

The beneficial antifibrotic effect of the plant extract against DMN induced liver injury was also confirmed by its potent action in restoring liver function enzyme activities and albumin to their normal levels as well as improving histopathological architecture of liver. As previously reported injection of DMN to rats caused hepatic necrosis and collapse of the liver parenchyma, dilated and congested blood sinusoids, nuclear dysplasia and mixed acute and chronic cell eosinophilia. However, these changes were more severe after 6 weeks of DMN injection, as

evidenced by disorientation of lobular architecture associated with I hepatocellular degeneration [59, 60].

Conclusion

Anabasis articulata aerial parts exhibited in vivo hepatoprotective and therapeutic effects against DMN-induced liver injury via suppression of oxidative stress by its high level of saponins. However, further clinical studies are required for application of this extract (crude extract, fractions, sub-fractions or pure compounds), in order to be used as a drug remedy against human liver disorders.

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