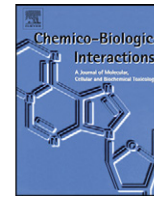
Chemico-Biological Interactions 181 (2009) 145–153

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint



Operculina turpethum attenuates *N*-nitrosodimethylamine induced toxic liver injury and clastogenicity in rats

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ARTICLE INFO

Article history:
Received 10 August 2008
Received in revised form 25 June 2009
Accepted 29 June 2009
Available online 7 July 2009

Keywords:
Clastogenicity
Hepatic fibrosis
LDH isoenzymes
NDMA
N-Nitrosodimethylamine
Operculina turpethum
Toxic liver injury

ABSTRACT

The root extract of *Operculina turpethum* (OTE) has been used as an anti-inflammatory, purgative, and hepato-protective agent. *N*-Nitrosodimethylamine (NDMA) is a potent hepatotoxin that induces fibrosis of the liver. In the present study, we examined the therapeutic effects of OTE root extract against NDMA-induced hepatotoxicity and clastogenicity in rats. Hepatic fibrosis was induced in adult male albino rats through serial intraperitoneal administrations of NDMA at a concentration of 10 mg/kg body weight on three consecutive days of each week over a period of three weeks. A group of rats received OTE orally in doses of 75, 150 and 200 mg/kg body weight at 5 h after the administration of NDMA. The controls and treated animals were sacrificed on days-7, 14 and 21 after the start of the administration of NDMA. The progression of hepatic fibrosis as well as the amelioration effect of OTE was evaluated through histopathologically as well as by immunohistochemical staining for the activation of hepatic stellate cells. Alterations in serum and liver biochemical parameters and LDH isoenzymes were also studied. Serial administration of NDMA resulted in well formed fibrosis in the liver and induction of micronuclei in the bone marrow cells. Staining of α-SMA demonstrated activated stellate cells from day-7 onwards which was dramatically increased on day-21. An elevation of micronuclei count, liver function enzymes, serum hydroxyproline levels and LDH isoenzymes 4 and 5 were also observed. All these changes were remarkably reduced in OTE administered animals and fibrogenesis was completely absent. Our results suggest that OTE has hepatoprotective and anti-clastogenic effects against NDMA-induced hepatic fibrosis. Therefore OTE may be used as a hepatoprotective agent against various liver diseases including toxic liver injury.

1. Introduction

N-Nitrosodimethylamine (NDMA) is a potent hepatotoxin, carcinogen and mutagen and it induces fibrosis and cirrhosis of the liver [1,2]. The toxicity produced by NDMA is mediated by its reactive metabolites and not by the parent compound. NDMA is used as a softener for copolymers' production in industries, in addition to synthesis as a chemical intermediate in the production of 1,1-dimethylhydrazine and nematocide [3]. As a component of tobacco smoke condensate and certain alcoholic beverages, NDMA can induce lung, liver or renal cancers [4,5].

It has been shown that NDMA induced hepatic fibrosis in rats is a suitable and appropriate animal model to study biochemical and pathophysiological alterations associated with the development of hepatic fibrosis and alcoholic cirrhosis of human [6–10]. Hepatic fibrosis is characterized by excessive accumulation of connective tissue components, especially matured collagen fibers in the extracellular matrix (ECM) of the liver. It is a complex dynamic process which reflects the balance between ECM synthesis and degradation [11]. The activation of hepatic stellate cells (HSCs) has been associated with the pathogenesis of liver fibrosis [12,13]. Activated HSCs are proliferative and fibrogenic and expresses α-smooth muscle actin (α-SMA) and various connective tissue proteins including collagen types I, III and IV [14,15]. Moreover, activated HSCs have been implicated in hepatic inflammation through their ability to secrete cytokines, including transforming growth factor-β1 (TGF-β1) during liver fibrogenesis [12]. Hepatic fibrosis may be induced by various chronic liver injuries including viral and autoimmune hepatitis, alcoholism or biliary obstruction [16]. In general, fibrosis requires years of exposure or sometimes decades to be visible clinically, but few notable exceptions are there in which cirrhosis develops in months [17].

Data are scarce on therapeutic potential of synthetic drugs to treat liver cirrhosis and the toxic side effects may remain a persistent risk [15,16,18]. Conversely, the use of a number of natural products and phytoextracts has been reported to show negligible or no side effects [19–23]. In case of botanicals also, the benign nature of the constituents or their anti-mutagenic potential has to be ensured, since several compounds produced within or derived from plants may possess mutagenic potential [24,25]. Once their

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0009-2797/$ – see front matter
doi:10.1016/j.cbi.2009.06.021

benign nature is ensured, anti-mutagenic/anti-clastogenic potential of phytoconstituents may also be exploited to treat genetic and biochemical ailments. The approach will be at par with that employed in cancer treatment, where the protective phytoconstituents were consumed as part of the diet or designed remedies [20]. Reports on anti-mutagenic or anti-clastogenic potential of a number of compounds extracted from different plant products are available [26,27].

In traditional medicine, natural or crude phytoextracts are considered as alternative medicines, because some natural constituents present in them counterbalance the side effects of synthetic medicines [28]. It is therefore obvious that the therapeutic potential and risk efficiency of traditional medicinal plants is based on the direct assessment of phytoextracts as well as effects of their purified compounds. *Operculina turpethum* (Family: Convolvulaceae), commonly known as *trivrit* or *nishot* in the western part of India and adjoining Pakistan, is a plant with immense ethnomedicinal value. *O. turpethum* is a perennial climber with slender, fleshy and branched roots, hard and twisted cord like stem with small ovate leaves [29]. Mainly, roots or stem bark of this plant are traditionally used for medicinal purpose. *O. turpethum* extract is used to treat wide range of ailments. For instance, it is used to relieve periodic fevers, constipation, flatulence and colic obesity, to treat anaemia, splenomegaly, raised lipid levels and obesity [19,30,31]. The present study was designed to evaluate whether *O. turpethum* (roots) aqueous extract (OTE) exerts hepatoprotective and anti-clastogenic effects in rats against NDMA induced liver toxicity.

2. Materials and methods

2.1. Chemicals

Acrylamide, 3,3′-diaminobenzidine tetrahydrochloride hydrate, *N*-nitrosodimethylamine, nicotinamide adenine dinucleotide (β-NAD), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS) and trizma base were purchased from Sigma. Biochemical kits were from Span Diagnostics Ltd., India, cyclophosphamide (500 mg Endoxan-N)™ of Baxter Oncology GmbH (Germany), fetal bovine serum (FBS) was of HiMedia, India, goat anti-mouse IgG-HRP conjugated was purchased from CALTAG Laboratories, Bangkok. α-Smooth muscle actin (α-SMA) antibodies were obtained from Trend Bio-products Pvt. Ltd., India. All other chemicals used were of analytical grade.

2.2. Animals

Adult male albino rats of Wistar strain were kept in well aerated polycarbonate cages at the departmental facility with light:dark exposure of 12:12 h. The rats were acclimatized for a week while being fed regularly with sterilized diet and water ad libitum. Healthy, 8–10-week-old rats weighing around 162 ± 10 g were selected for the experiments described here. Animals were handled complying with regulations of the University Ethical Committee.

2.3. Preparation of phytoextract

Authentic plant material, *O. turpethum*, was procured from Dawakhana (Pharmacy of Herbal Medicines), Ajmal Khan Tibbiya College, Aligarh Muslim University. Following verification of the plant material by an established plant taxonomist, some *O. turpethum* Linn. root specimens were stored in the Herbarium (Id. No. C1/94) of Department of Pharmacology (Ilm-ul Advia), Ajmal Khan Tibbiya College. Finely ground powder of the dried roots of *O. turpethum* L. (∼250 g) was extracted in distilled water under reflux and filtered on Whatman #1. The filtrate was dried in a rotary evaporator at a temperature of 40 ± 1 °C under reduced pressure [32]. The yield of *O. turpethum* powder from 250 g of dried roots was ∼10–12 g. The powder was stored at −2 °C in sterilized and labeled screw capped bottles.

2.4. Induction of hepatic fibrosis

The animals were divided into 4 groups of 5 each. One group served as the negative control and the second group received intraperitoneal injections of normal saline. The third group served as the positive control and received intraperitoneal injections of cyclophosphamide (150 mg/kg body weight). The fourth group was administered NDMA (10 μL diluted to 1 mL with 0.15 mol/L sterile NaCl) intraperitoneally in doses of 10 mg/kg body weight as described previously [33]. The injections were given on three consecutive days of each week for three successive weeks.

2.5. Administration of O. turpethum extract (OTE)

Here also, the animals were divided into 4 groups of 5 each. One group served as the OTE control and received OTE alone. The remaining three groups received NDMA injections as described above followed by OTE in concentrations of 75, 150 and 200 mg/kg body weight. OTE was administered after 5 h of NDMA administration. The doses of OTE were selected on the basis of a previous report [30]. The 5 h time interval of OTE administration was selected on the basis of pilot studies. A set of animals from each group was anesthetized with diethyl ether before sacrifice on days-7, 14 and 21 after the start of NDMA and OTE administrations. Blood was collected from a deep cut made on the right jugular vein with a scalpel. Urine was collected under a layer of toluene beginning from 24 h prior to sacrifice. The time course of the study was decided on the basis of pilot experiments and previous reports on NDMA induced toxic liver injury and hepatic fibrosis in rats [1,33].

2.6. Assessment of hepatic fibrosis

The progression of hepatic fibrosis was assessed histochemically following staining of serial sections of liver with hematoxylin and eosin (H&E). Stained slides were examined under Nikon microscope with an LCD attachment (Model: 80*i*) and photographed. The degree of hepatic fibrosis was monitored by following activation of hepatic stellate cells as indicated by immunohistochemical staining of α-smooth muscle actin (α-SMA) filaments.

2.7. Staining of α-smooth muscle actin (α-SMA)

For detection of α-smooth muscle actin (α-SMA), serial liver sections (5 μm) were stained immunohistochemically using α-SMA monoclonal antibodies. Endogenous peroxidase activity in the liver sections was quenched by incubating the sections with 3% hydrogen peroxide for 15 min. The sections were washed with PBS, layered with prediluted monoclonal α-SMA antibody and incubated overnight at 2–8 °C in the moist chamber. After washing off unbound antibody with PBS for 5 min, the sections were reacted with HRP conjugated goat anti-mouse IgG immunoglobulins (secondary antibody) and incubated for 30–45 min at room temperature. The slides were washed with cold PBS and developed using 3% 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB) solution for 5–15 min. Developed slides were rinsed with PBS, counter stained with Mayer's hematoxylin and mounted with DPX.

2.8. Micronucleus test

Micronucleus test was performed in controls and treated groups of rats which received NDMA alone as well as in mixes with 200 mg kg^{-1} body weight OTE as described previously [34]. Slides

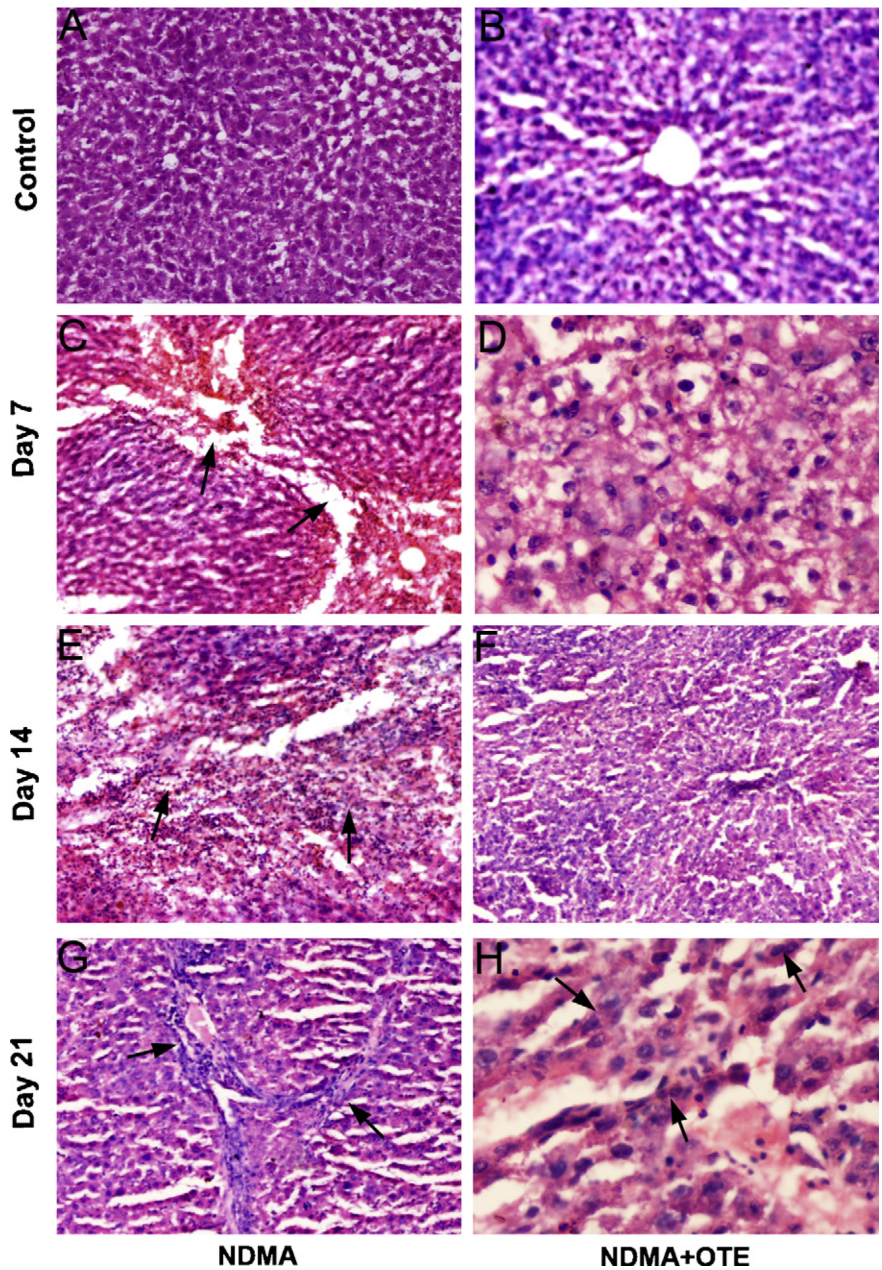

Fig. 1. Hematoxylin and Eosin (H&E) staining of rat liver sections during the pathogenesis of NDMA induced hepatic fibrosis and concurrent administration of OTE. (A) Control liver (×125). (B) OTE control (×125). OTE was administered 200 mg/kg body weight (C) NDMA, day 7 (×125). Severe centrilobular congestion (arrow) and hemorrhagic necrosis. (D) NDMA + OTE, day 7 (×250). Kupffer cells hyperplasia and fatty changes. (E). NDMA, day 14 (×125). Massive hepatic necrosis (arrow), severe neutrophilic infiltration and multifocal collapse of liver parenchyma. (F) NDMA + OTE, day 14 (×125). Moderate infiltration of mononuclear cells and regeneration of hepatocytes. (G) NDMA, day 21 (×125). Marked hepatic fibrosis (arrow) and deposition of collagen fibers. (H) NDMA + OTE, day 21 (×250). Restoration of normal liver architecture with increased number of regenerating hepatocytes.

of bone marrow smears were stained in Giemsa. A total of 7000 cells, at a magnification of 10 × 100×, were examined. Best stained slides were selected and scored by a pathologist to avoid overlapping during the counting process.

2.9. Biochemical parameters

Commercial kits of Span Diagnostics Ltd., India were used for determination of alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and bilirubin levels in rat sera. Hydroxyproline (HP) was estimated in sera and urine samples of control and treated group of rats according to Woessner [35].

Lactate dehydrogenase (LDH) isoenzymes were analyzed in sera and liver homogenates of all group of rats. Livers were homogenized in ice cold Tris–HCl buffer (pH, 7.5), centrifuged at 10,000 × *g* and ∼10 μL of clear supernatants were resolved on native 7.5% vertical slab polyacrylamide gel electrophoresis [36]. LDH isoenzymes were visualized by histochemical staining using L-lactate-βNAD and phenazine methosulphate (PMS)-tetrazolium (NBT) protocol [37]. LDH isoenzyme bands developed at 37 °C within 25–30 min.

2.10. Densitometry

Quantitative estimates of LDH isoenzyme were made by densitometry of LDH gel-scans using Scion Imaging (Scion Corporation:

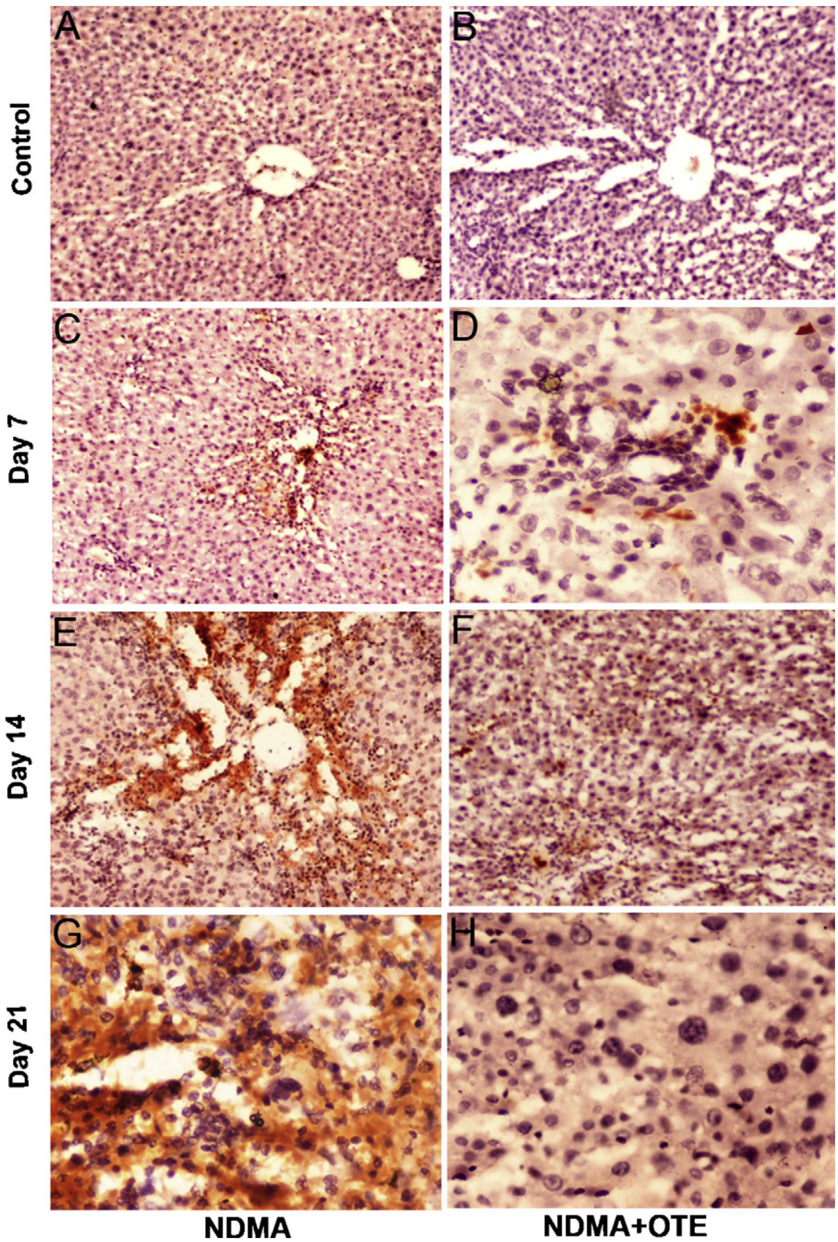

Fig. 2. Immunohistochemical staining of α-smooth muscle actin (α-SMA) demonstrating activated hepatic stellate cells during the pathogenesis of NDMA induced hepatic fibrosis and ameliorating effects of OTE (A) control liver (×100). Absence of α-SMA staining. (B) OTE control (×100). OTE was administered 200 mg/kg body weight. α-SMA staining is absent. (C) NDMA day 7 (×100). Staining of α-SMA demonstrates activated hepatic stellate cells in the necrotic zone. (D) NDMA + OTE, day 7 (×250). Focal staining of α-SMA around central vein indicating ameliorative effects of OTE. (E). NDMA, Day 14 (×100). Intense staining of α-SMA indicating extensive activation of hepatic stellate cells in the fibrotic zone. (F) NDMA + OTE, day 14 (×100). Mild staining of α-SMA (G) NDMA, day 21 (×200). Remarkable staining of α-SMA demonstrating enormous number of activated stellate cells in fibrotic zone. (H) NDMA + OTE, day 21 (×250). Absence of α-SMA staining and restoration of normal liver parenchyma with regeneration of hepatocytes.

Beta release-4.0) and GelPro (Media Cybernetics, USA) software programs and presented as fractions in arbitrary units (AU) per gram wet weight of the tissue.

2.11. Statistical analysis

The micronuclei count values of selected biochemical markers, hydroxyproline and LDH isoenzymes were recorded in their corresponding units and presented as mean ± SD (n = 5). Analysis of variance (ANOVA) was applied to demonstrate significant differences among the frequencies of micronuclei counts and the variations among liver function test parameters.

3. Results

3.1. Animal body weight and liver weight

It was evaluated in the groups of rats treated with NDMA and those treated with NDMA + OTE. During NDMA administration, the mean body weight of the animals decreased significantly on days-14 and 21. As compared with controls, a similar decline of the magnitude of 25% and 45% was noted in the liver weight on days-14 and 21, respectively. The animals which received NDMA + OTE showed significant increase in the mean body weight as well as liver weight during the course of treatment, as compared to NDMA injected animals. In OTE receiving animals, an increase of 55% and

Table 1
Micronuclei induction in the bone marrow cells of rats treated with NDMA and *O. turpethum* extract (200 mg kg^{-1} body weight for 21 days).

Exposure (in days)	Chemical/extract	Concentration (mg/kg body weight)	Number of cells scored	No. of micronuclei/cell			Total number of micronuclei	Micronucleated cells/1000 cells scored ± SD	Per cent anti-clastogenic effect [100–(Y/X × 100)]
				1	2	3			
7	C-1	–	7000	6	×	×	6	0.85 ± 0.12	31
	C-2	8.76	7000	7	×	×	7	1.00 ± 0.17	
	C-3	150	7000	116	4	×	124	17.71 ± 2.41	
	NDMA	10	7000	127	5	×	137	19.57 ± 2.2 (X)	
	NDMA + OTE	10 + 200	7000	93	1	×	95	13.57** ± 1.2 (Y)	
14	C-1	–	7000	6	×	×	6	0.85 ± 0.11	48
	C-2	8.76	7000	8	×	×	8	1.14 ± 0.13	
	C-3	150	7000	159	8	×	175	25.00 ± 2.53	
	NDMA	10	7000	173	11	2	201	28.71 ± 3.01(X)	
	NDMA + OTE	10 + 200	7000	99	3	×	105	15.00* ± 1.27 (Y)	
21	C-1	–	7000	7	×	×	7	1.00 ± 0.09	65
	C-2	8.76	7000	7	×	×	7	1.00 ± 0.16	
	C-3	150	7000	294	27	1	351	50.14 ± 4.32	
	NDMA	10	7000	346	38	5	437	62.42 ± 5.11 (X)	
	NDMA + OTE	10 + 200	7000	125	7	×	139	19.85* ± 1.07 (Y)	

Values are significant at $^{*}P < 0.05$ and $^{**}P < 0.001$.
Note—C-1: solvent control (distilled water); C-2: solvent control (*NaCl = mg/mL); C-3: positive control (cyclophosphamide); NDMA: *N*-nitrosodimethylamine; OTE: *Operculina turpethum* extract.

81% was observed in the body weight on days-14 and 21, respectively. Similarly, about 63% and 87% increase in liver weight was noted on days-14 and 21 in animals given OTE.

3.2. Hematoxylin and eosin staining

3.2.1. Histological changes during NDMA treatment and progression of hepatic fibrosis

The hematoxylin and eosin stained slide of liver specimens during NDMA administration and progression of hepatic fibrosis is demonstrated in Fig. 1. The control liver sections showed normal lobular architecture (Fig. 1A). On day-7 disintegration of liver parenchyma, hepatic necrosis and severe centrilobular congestion were observed (Fig. 1C). Massive hepatic necrosis along with neutrophilic infiltration and multifocal collapse of parenchyma were the prominent features in liver specimens (sections) of day-14 (Fig. 1E). On day-21, NDMA treated liver specimens demonstrated disruption of normal liver architecture, inflammation, hemorrhage and intensive fibrosis with deposition of thick collagen fibers (Fig. 1G). At certain places sign of early cirrhosis was also visible.

3.2.2. Effect of OTE treatment

The hematoxylin and eosin staining of liver sections during amelioration by OTE is also displayed in Fig. 1. The control liver specimens showed normal cellular and lobular architecture with radiating hepatic cords (Fig. 1B). A sort of fatty change and a low level of Kupffer cells hyperplasia were the peculiar features of OTE treated rats on the day-7 (Fig. 1D). On day-14, moderate infiltration of mononuclear cells and regeneration of hepatocytes was observed in liver sections of OTE treated rats (Fig. 1F). Remarkable decrease in the hepatic fibrosis was visualized on day-21 of OTE treatment (Fig. 1H). The liver architecture restored to normalize with more number of regenerating hepatocytes.

3.3. Staining of α-smooth muscle actin (α-SMA)

3.3.1. Histological changes during NDMA treatment and progression of hepatic fibrosis

The staining of α-SMA for the visualization of activated hepatic stellate cells is demonstrated in Fig. 2. In control liver specimens, the α-SMA staining was absent (Fig. 2A). A large number of α-SMA positive stained stellate cells were observed in the necrotic zone on day-7 of treatment with NDMA (Fig. 2C). From day-7 onwards, the feature of staining of α-SMA was quite persistent. The staining was quite deep (intense) and confined to the fibrotic area in liver specimens on day-14 (Fig. 2E). The count of positively stained hepatic stellate cells further increased in the fibrotic zone. On day-21, the α-SMA staining was almost restricted to the fibrotic zone (Fig. 2G). The number of positive stained cells in the fibrotic area was much higher when compared with normal areas of the liver sections.

3.3.2. Effect of OTE treatment

The α-SMA staining in liver specimens of rats treated with NDMA + OTE is also shown in Fig. 2. Similar to the saline control, the α-SMA staining was also absent in OTE control liver samples (Fig. 2B). On day-7, focal staining of α-SMA around the central vein indicated the ameliorative action of OTE (Fig. 2D). In liver specimens of day-14, the α-SMA staining was of very low intensity with a few numbers of activated stellate cells distributed sporadically (Fig. 2F). On day-21, the α-SMA staining was almost absent in liver sections of OTE treated rats. This indicated restoration of liver parenchyma and regeneration of hepatocytes (Fig. 2H).

3.4. Evaluation of clastogenicity

Clastogenicity was evaluated by the micronuclei count in bone marrow stem cells of experimental rats. *N*-Nitrosodimethylamine (NDMA) produced clastogenic effects in experimental rats after 21 days of its treatment (Table 1). NDMA induced toxicity in rats increased with the exposure time and caused higher micronuclei formation than cyclophosphamide, the positive control. Maximum micronuclei induction was observed in rats (62.42 ± 5.11) due to NDMA treatment on day-21. To assess the maximum amelioration produced by OTE, we have compared the following groups: (1) rats injected only with NDMA and (2) received both NDMA and OTE (200 mg kg^{-1} body weight of rat day^{-1}). This much dose of OTE is considered safe in rats [19,30] and therefore chosen as the highest concentration in order to demonstrate the anti-clastogenic potential of the OTE in this study. Administration of 200 mg of the OTE/kg^{-1} body weight of rat day^{-1} three times a week significantly reduced ($P < 0.05$) the micronuclei counts to the extent of ∼31%, ∼48% and ∼65% on days-7, 14 and 21 respectively (Table 1).

Table 2
Biochemical markers in the sera of rats treated with NDMA and different doses of *O. turpethum* extract for 21 days.

Biochemical parameters	Control group (A)	Treated groups					
		NDMA		OTE			
		10 mg kg^{-1} body weight (B)	Per cent increase ($B - A/B \times 100$)	75 mg kg^{-1} body weight	150 mg kg^{-1} body weight	200 mg kg^{-1} body weight (C)	Per cent reduced ($B - C/B \times 100$)
Hydroxyproline (μg/mL serum)	11.63 ± 1.3	14.59** ± 1.57	20.28	14.12 ± 1.02	13.71 ± 1.14	12.16** ± 0.24	16.65
Hydroxyproline (μg/mL urine)	28.17 ± 1.49	66.82* ± 3.11	57.84	64.9 ± 2.87	52.5 ± 2.31	35.22** ± 2.08	47.29
Serum ALP (U/L)	317 ± 42.06	716* ± 40.64	55.72	707 ± 37.89	581 ± 41.76	392* ± 45.85	45.25
SGOT (U/L)	126 ± 21.19	190* ± 22.9	33.68	186 ± 19.58	159 ± 16.55	135* ± 18.89	28.94
SGPT (U/L)	56 ± 8.95	65** ± 9.53	13.84	63.8 ± 7.88	61.5 ± 9.65	58.11 * ± 10.02	10.6
LDH (AU/g wet weight of liver)							
LDH-1	0.596 ± 0.2	0.92* ± 0.23	35.21	0.9 ± 0.17	0.83 ± 0.23	0.655* ± 0.23	28.80
LDH-2	0.596 ± 0.17	0.976* ± 0.20	38.93	0.912 ± 0.22	0.85 ± 0.19	0.681** ± 0.2	30.22
LDH-3	0.617 ± 0.17	0.742** ± 0.14	16.84	0.737 ± 0.15	0.701 ± 0.11	0.646* ± 0.089	12.93
LDH-4	0.627 ± 0.14	1.05* ± 0.22	40.28	1.0 ± 0.18	0.81 ± 0.14	0.652* ± 0.1	37.90
LDH-5	0.755 ± 0.2	0.86** ± 0.17	12.2	0.85 ± 0.12	0.82 ± 0.1	0.77* ± 0.13	10.46
Total bilirubin (mg/dL)	0.316 ± 0.05	1.49* ± 0.18	78.79	1.3 ± 0.16	1.1 ± 0.12	0.51** ± 0.047	65.77
Direct bilirubin (mg/dL)	0.13 ± 0.02	0.15** ± 0.04	13.33	0.15 ± 0.05	0.15 ± 0.05	0.14** ± 0.016	6.66

Values are significant at $^{*}P < 0.05$ and $^{**}P < 0.001$.
Note—ALP: alkaline phosphatase; AU: arbitrary units; LDH-1–5: lactate dehydrogenase isoenzyme-1–5; OTE: *Operculina turpethum* extract; SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase.

3.5. Sera ALP, GOT, GPT and bilirubin levels

Hepatotoxicity due to NDMA in rats was assessed by the levels of liver function test enzymes (ALP, GOT, GPT) and bilirubin in the sera. As compared to their respective control group, significant increase in the activities of SALP, SGOT, SGPT ($P < 0.05$) and bilirubin ($P < 0.001$) was recorded in rats treated with the evaluated doses of NDMA (Table 2). Time and dose dependent amelioration was shown by OTE against NDMA induced hepatotoxicity in rats. Hepatoprotective effect of OTE that was evaluated at three different concentrations: 75, 150 and 200 mg kg^{-1} body weight day^{-1} showed outstanding recovery in LFT values of rats receiving 200 mg kg^{-1} body weight day^{-1} of OTE along with NDMA regimen. In comparison with NDMA infused group at this dosage of OTE, significant reduction in the levels of SALP, SGOT, SGPT (45.25%, 28.94%, 10.6%) and bilirubin (65.77%) was observed (Table 2).

3.6. Hydroxyproline levels in the sera and urine

Hydroxyproline levels were estimated in the sera and urine of NDMA treated rats on day-21 of NDMA treatment. The increase of about 20.28% ($P < 0.001$) and 57.84% ($P < 0.05$) was noted in sera and urine hydroxyproline levels, respectively (Table 2). Remarkable decline in the activity of sera and urine hydroxyproline was observed in rats which received OTE regimen (200 mg kg^{-1} body weight day^{-1}) for 21 days. In sera and urine samples of OTE receiving rats, the decrease in hydroxyproline levels was of the magnitude of 16.65% and 47.29%, respectively.

3.7. Total and fractional activity of LDH isoenzymes

Lactate dehydrogenase (LDH) isoenzymes activity was estimated in the sera and liver samples of treated animals and compared with their control values. The increase in total LDH (TLDH) activity was noticed on day-7 onwards in sera samples of NDMA treated rats. Particularly, the rise in the activity of LDH-4 and LDH-5 was prominent in the sera (Fig. 3A). OTE treated groups showed significantly decreased levels of sera LDH-4 and LDH-5 within 21 days and the ranking of the LDH isoenzymes was comparable to the control values (Table 3).

In liver samples of NDMA administered animals, an increase in the levels of LDH-4 was observed on day-7, while LDH-5 was detected in significantly higher levels at all the selected durations (Fig. 3B). On day-21, the ranking of LDH isoenzymes was LDH-5 > -4 > -3 > -1 > -2 as compared with the control (LDH-5 > -4 > -3 > -2 > -1). Treatment with OTE remarkably reduced the levels of LDH-4 and -5 in liver samples of rats at all the durations. On day-21 of OTE treatment, LDH isoenzymes ranking in liver samples was observed LDH-5 > -4 > -3 > -2 > -1 which was similar to control values (Table 4).

4. Discussion

Our data lends support to hepatic fibrosis and cirrhosis-inducing potential of *N*-nitrosodimethylamine (NDMA) in liver of rats [1,2,9,10,17,33]. The consequent histological and biochemical changes include increased synthesis and deposition of connective tissue proteins, changes in hepatic lobular architecture and concomitant impairment of several biochemical processes [38,39] leading to shrunken liver. For the first time in this study, we report that aqueous extract of *O. turpethum* root (OTE) can ameliorate NDMA-induced toxic effects on liver. OTE treatment restored normal lobular architecture of the liver through extensive regeneration of hepatocytes.

During NDMA-induced hepatic fibrosis in rats, decrease in liver weight and liver to body weight ratio has been reported previ-

Table 3
Apparent ranking and per cent distribution of lactate dehydrogenase (LDH) isoenzymes in the sera samples of rats during NDMA induced hepatic fibrosis and treatment with OTE (200 mg kg^{-1} body weight of rats).

LDH isoenzymes	NDMA				NDMA + OTE			
	Control	Day-7	Day-14	Day-21	Control[a]	Day-7	Day-14	Day-21
LDH-1	18.32 ± 2.01	12.63 ± 1.11	9.14 ± 1.2	4.13* ± 0.35	19.02 ± 1.39	21.43 ± 2.01	32.59± 2.56	24.39* ± 1.91
LDH-2	13.66 ± 1.5	7.45 ± 0.74	5.66 ± 0.87	3.81* ± 0.07	18.42 ± 1.89	19.11 ± 1.1	19.77 ± 1.37	21.75* ± 2.2
LDH-3	8.09 ± 0.78	7.23 ± 0.69	3.31 ± 0.06	2.55* ± 0.03	7.07 ± 0.7	7.54 ± 0.91	7.94 ± 0.66	11.49* ± 1.17
LDH-4	11.53 ± 1.03	8.45 ± 0.98	14.67 ± 1.5	20.81** ± 1.89	16.73 ± 1.33	18.86 ± 1.44	11.8 ± 1.42	12.39* ± 1.3
LDH-5	48.34 ± 2.33	64.17 ± 3.08	67.15 ± 4.3	67.59** ± 3.2	38.76 ± 2.81	33.06 ± 3.08	27.9 ± 2.09	29.98* ± 2.39
Rank of LDH isoenzymes	5 > 1 > 2 > 4 > 3	5 > 1 > 4 > 2 > 3	5 > 4 > 1 > 2 > 3	5 > 4 > 1 > 2 > 3	5 > 1 > 2 > 4 > 3	5 > 1 > 2 > 4 > 3	1 > 5 > 2 > 4 > 3	5 > 1 > 2 > 4 > 3

Values are mean ± SD of 5 samples.
Values are significant at $^{*}P < 0.05$ and $^{**}P < 0.001$.
[a] Treated with OTE (200 mg kg^{-1} body weight).

Table 4
Apparent ranking and per cent distribution of LDH isoenzymes in liver homogenates of rats during NDMA induced hepatic fibrosis and treatment with OTE (200 mg kg^{-1} body weight of rats).

LDH isoenzymes	NDMA				NDMA + OTE			
	Control	Day-7	Day-14	Day-21	Control[a]	Day-7	Day-14	Day-21
LDH-1	11.72 ± 1.1	6.65 ± 0.88	7.31 ± 1.06	6.7** ± 0.81	9.17 ± 0.66	8.94 ± 0.49	9.91 ± 0.69	6.11** ± 0.55
LDH-2	13.05 ± 1.4	8.98 ± 0.79	7.72 ± 0.97	5.36** ± 0.47	12.99 ± 0.83	12.95 ± 1.1	14.25 ± 1.22	13.55 ± 1.23
LDH-3	17.94 ± 1.2	15.26 ± 1.05	12.25 ± 1.56	7.76** ± 0.53	15.98 ± 2.01	13.1 ± 1.12	14.66 ± 1.08	18.27* ± 0.98
LDH-4	25 ± 2.3	30.41* ± 2.9	25.07 ± 1.99	25.91 ± 0.93	25.17 ± 1.66	27.3 ± 2.41	25.17 ± 1.83	25.6*3 ± 2.61
LDH-5	32.3 ± 2.57	38.16 ± 3.1	47.65 ± 2.88	54.24* ± 4.1	36.66 ± 2.09	37.68 ± 1.05	35.98 ± 1.31	36.41 ± 2.59
Rank of LDH isoenzymes	5 > 4 > 3 > 2 > 1	5 > 4 > 3 > 2 > 1	5 > 4 > 3 > 2 > 1	5 > 4 > 3 > 1 > 2	5 > 4 > 3 > 2 > 1	5 > 4 > 3 > 2 > 1	5 > 4 > 3 > 2 > 1	5 > 4 > 3 > 2 > 1

Values are mean ± SD of 5 samples.
Values are significant at $^{*}P < 0.05$ and $^{**}P < 0.001$.
[a] Treated with OTE (200 mg kg^{-1} body weight).

ously [38,40,41]. Under the conditions applied in this study, NDMA treated rats showed the decrease in body weight and liver weight from day-14 onwards. Our histopathological data supports the suggestions that the decreased protein synthesis, cell necrosis and deterioration of liver parenchyma cause weight losses [33]. The treatment with OTE caused significant increase in body and liver weight of rats on days-14 and 21. It may therefore be inferred that OTE has the potential to induce the activation of cellular enzymes responsible for increased metabolic activity in liver and the regeneration of hepatic tissues.

We have taken up two aspects of histopathological changes in liver tissue: (i) the changes which can be observed after hematoxylin and eosin (H&E) staining and, (ii) those detectable by immunohistochemical staining for the expression of α-smooth

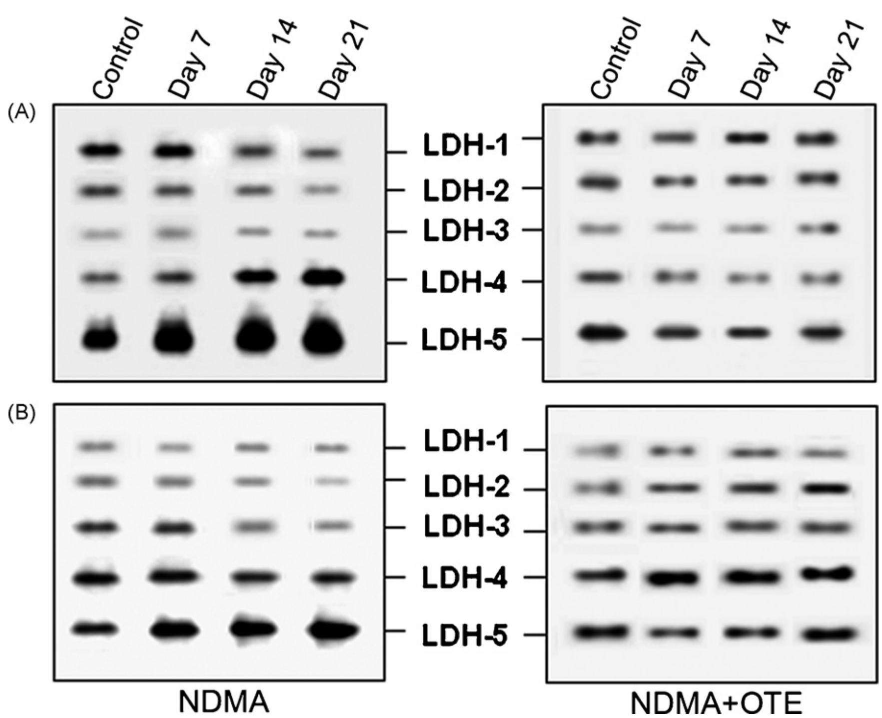

Fig. 3. Polyacrylamide gel electrophoretic (PAGE) patterns of lactate dehydrogenase (LDH) isoenzymes in the serum (A) and liver homogenate (B) during the pathogenesis of NDMA induced hepatic fibrosis and concurrent administration of OTE in rats.

muscle actin (α-SMA). H&E stained slides show synthesis as well as continued accumulation of collagen in liver from the day-7 onwards. In fact, by day-21 post-treatment with NDMA, complete architecture of the liver deteriorated. During hepatic fibrosis, the role of activated stellate cells in excessive collagen synthesis is well established [17,42,43]. OTE administration in rats significantly reduced the inflammation, necrosis and fibrotic area produced during NDMA-induced hepatic fibrosis. The reversal in necrosis and hepatic fibrosis due to the treatment with OTE was most remarkable on days-14 and 21.

The expression of α-smooth muscle actin (α-SMA) by the stellate cells is considered as the most reliable marker for activated hepatic stellate cells which add to collagenous connective tissue [44]. The presence of high number of activated stellate cells on day-7 of NDMA administration indicates that the fibrosis had started much before day-7 [1,9,33]. The late stage of hepatic fibrosis and cirrhosis was characterized by more enhanced expression of α-SMA in the activated stellate cells in the fibrotic zone on days-14 and 21, respectively. OTE treatment caused constant decline in the density of activated stellate cells on days-7 and 14. Antifibrotic and hepatoprotective potential of OTE is evident by a decrease in the α-SMA staining and increase hepatocytes regeneration on day-21 indicating the elimination of activated hepatic stellate cells.

Scanty literature is available on anti-clastogenic/anti-mutagenic potential of the OTE. A recent study has reported that administration of methanolic extract of *O. turpethum* (stems) brings some relief to the rats suffering from 7,12-dimethyl benz(*a*)anthracene-induced breast cancer [45]. Anti-clastogenic action of OTE against NDMA induced clastogenicity in rats is demonstrated by our data on micronuclei counts (Table 1). When compared with NDMA administered group, a significant ($P < 0.05$) decline of respectively 31%, 48% and 65% in micronuclei counts occurred on days-7, 14 and 21 of OTE treatment. It is likely that the presence of glycoside turpethins [19,30,46] or similar active constituents in OTE modulates destabilization of intermediate metabolites which cause chromosomal breakage [34].

Similar to previously published reports [47,48], we also recorded significant increases in sera total LDH activity with major contribution by LDH-4 and LDH-5 isoenzymes. These changes begin on day-7 and become more intense on days-14 and 21. The initial increase has been attributed to an increase in the synthesis of LDH due to the presence of higher number of functional hepatocytes during early fibrosis [47]. However, the increase in LDH levels at advanced stage of fibrosis appears to be the consequence of tissue injury and necrosis leading to leakage of the enzymes into blood stream [37,47,49]. That the enzymatic changes may actually be the result of cellular injury is supported by a concordance between our histopathological and micronuclei data with the changes in enzyme and isoenzyme levels. While the induction of fibrosis by NDMA disrupts cellular architecture of hepatic tissue, which is also collateral to micronuclei induction and elevated enzyme levels, OTE significantly reverses all of these changes.

No report is available on the hepatoprotective effect of OTE against NDMA induced liver injury in rats, though its use as hepatoprotectant against paracetamol-induced toxicity [30] and in other clinical and pathological conditions has been recognized [19,29]. According to our data, the protection rendered by OTE against NDMA-induced hepatotoxicity was time and dose dependent. OTE at 200 mg kg^{-1} body weight of rat for 21 days reduced the levels of hydroxyproline (sera/urine), SALP, SGPT, SGOT and total bilirubin up to 16.65%/47.29% ($P < 0.001$), 45.25%, 10.6%, 28.94% ($P < 0.05$) and 65.77% ($P < 0.001$), respectively. The values in NDMA treated rats on day-21 showed an increase of 20.28%/57.84%, 55.72%, 13.84%, 33.68%, 78.79% in sera/urine hydroxyproline, SALP, SGPT, SGOT levels and bilirubin, respectively. This trend of increase is in agreement with the observations published by other workers who administered 10 mg kg^{-1} body weight day^{-1} NMDA thrice a week [1,33].

Our data shows that liver detoxification and the prevention of collagen accumulation are simultaneous processes. This is evident from the correspondence between histopathology of OTE treated rat livers (which show decreased collagen contents) and the shift in sera ALP, GPT, GOT, LDH isoenzymes and bilirubin levels towards control values. That there occurs lesser hydrolysis of collagen in OTE injected rats on day-21 is evident by lower hydroxyproline levels, which indicates reduced spill of this non-essential amino acid in the sera/urine. Obviously, OTE restores hydroxyproline levels by blocking the pathways to collagenesis and also decreases the levels of collagenolytic enzymes in rat liver. An increase in hepatic collagenolytic enzymes (or increased hydroxyproline levels) and liver function test enzymes has been recorded in cases of carbon tetrachloride-induced early fibrosis [50,51] and the patients with chronic liver diseases [52].

In summary, the present study suggests that *O. turpethum* (roots) aqueous extract (OTE) exhibits hepatoprotective, antifibrotic and anticlastogenic effects by decreasing collagen contents, restoring α-SMA skeleton, overcoming chromosomal breakage and sera enzyme markers of the liver injury towards normal levels. Thus, it is suggested that OTE in its maximum selected dose may be potentially useful in preventing hepatic fibrosis and clastogenicity in rats. Therefore, it may be desirable to include OTE in various pharmaceutical preparations so as to prevent liver damage caused by NDMA.

Conflict of interest

There are no conflicts of interest among the authors of the manuscript.

Acknowledgements

The authors sincerely thank to the Chairman, Department of Zoology for providing necessary laboratory facilities. Thanks are also due to Prof. S.H. Afaq, Pharmacognosy Section, A.K. Tibbiya College for generously providing us authentic material. Authors extend their gratitude to Prof. Veena Maheshwari and Dr. Kiran Alam, Department of Pathology and Dr. Mumtaz Alam, Department of Plastic Surgery, JN Medical College and Hospital for their timely help. Photographic facility extended by Prof. Waseem Ahmad and support of Dr. Banyamuddin, Department of Zoology, is sincerely acknowledged. The authors are grateful to Dr. Christopher W. Brey, Rutgers University, USA for reading the manuscript. Assistance of Mr. Ramesh Chander in handling/caring of laboratory animals is thankfully appreciated. The authors are also thankful to the anonymous reviewers for their kind suggestions that helped us to improve the manuscript. Lastly, part of the work was supported by the grants sanctioned by DST to RA and University Fellowship to SA.

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