Chapter-3
Antimicrobial and hepatoprotective effects of *operculina turpethum*
3. Introduction

*Operculina turpethum* (Family: Convolvulaceae), commonly known as *trivrit* or *nishot* in the Western part of India and adjoining Pakistan, is a plant with immense ethno-medicinal value. This plant is widely grown throughout India and it is occasionally cultivated in gardens as an ornament. This plant appears to be rarely cultivated in non-Indian regions of Asia [Ooststroom, 1953; Verdcourt, 1963; Fosberg and Sachet, 1977; Chang 1978] however, it is expected that the medicinal use of this plant continued by Indian immigrants.

*O. turpethum* is a perennial climber with slender, fleshy and branched roots, hard and twisted cord like stem with small ovate leaves [The Wealth of India, 2001]. Mainly, roots or stem bark of this plant are traditionally used for medicinal purpose. The plant produces two types of roots: *sufed* or *sveta* or white roots that are mild, and *kala* or *krishna* or black roots that give drastic, often poisonous effects [Watt, 1889]. The root, bark and seeds are reported to contain cardio-active glycosides, formerly designated as neriodoprin, neriodorein and karabin which are anti-inflammatory, stimulant and good pain relievers. Active constituents like β-sitosterol, α- and β-turpethins, coumarin, scopoletin, lupeol and betulin [Husain et al., 1992; Yoganarasimhan, 2000] glycosides, saponins flavonoids, steroids and carbohydrates [Ashok Kumar et al., 2009] have been reported to constitute bulk in the plant. *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 [Austin, 1982; Vasudevan, 1995; Suresh Kumar et al., 2006]. The oil extracted from the root bark is used in skin diseases of a scaly nature. The fresh juice of leaves is dropped into the eyes for
inducing lachrymation in ophthalmia. It is also reported to use in the treatment of piles, tumors and jaundice [Kirtikar and Basu, 1987].

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 [Ahmad et al., 2008 a]. 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. 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 [Ames, 1983; Agner et al., 2001]. Once their 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 [Newman et al., 2003]. Reports on anti-mutagenic or anti-clastogenic potential of a number of compounds extracted from different plant products are available [Bruni et al., 2006; Barcelos et al., 2007]. Data are scarce on therapeutic potential of synthetic drugs to treat liver cirrhosis and the toxic side effects may remain a persistent risk [Kang et al., 2002; Lotersztajn et al., 2005; Kisseleva and Brenner, 2006]. Conversely, the use of a number of natural products and phytoextracts has been reported to show negligible or no side effects [Austin, 1982; Newman et al., 2003; Colvard et al., 2006; George et al., 2006; Arif et al., 2007] against the ill effects produced by many synthetic compounds. Therefore, in our opinion, it will be an interesting aspect to evaluate the therapeutic potential of *O. turpethum* (roots) against those chemical compounds which are of
routine use by industrial workers and cause serious damage. One such compound selected during the present investigations is \textit{N}-Nitrosodimethylamine (NDMA). The present study was designed to evaluate whether \textit{O. turpethum} (roots) aqueous extract (OTE) exerts hepatoprotective effects in rats against NDMA induced liver toxicity.

\textit{N}-Nitrosodimethylamine (NDMA) is a potent hepatotoxin, carcinogen and mutagen and it induces fibrosis and cirrhosis of the liver [George and Chandrakasan, 1996 a; George et al., 2001]. 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 [IARC, 1971]. As a component of tobacco smoke condensate and certain alcoholic beverages, NDMA can induce lung, liver or renal cancers [Magee and Barnes, 1967; Lijinsky and Epstein, 1970].

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 [Jenkins et al., 1985; Jezequel et al., 1987; Jezequel et al., 1990; George and Chandrakasan, 1996 b; George, 2003]. 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 [Friedman and Bansal, 2006]. The activation of hepatic stellate cells (HSCs) has been associated with the pathogenesis of liver fibrosis [Friedman, 2003; Bataller and Brenner, 2005]. Activated HSCs are proliferative and fibrogenic and expresses \textit{\alpha}-smooth muscle actin (\textit{\alpha}-SMA) and various connective tissue proteins including
collagen type I, III and IV [Pinzani and Marra, 2001; Lotersztajn et al., 2005]. 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 [Friedman, 2003]. Hepatic fibrosis may be induced by various chronic liver injuries including viral and autoimmune hepatitis, alcoholism or biliary obstruction [Kisseleva and Brenner, 2006]. 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 [Das and Vasudevan, 2007].

In the present study *Operculina turpethum* (roots) aqueous extract (OTE) was used for hepatoprotective, antibacterial and antifungal activity.

**3.1. Materials and Methods**

**3.1.1. Microbial studies**

**3.1.2. Preparation of phytoextract**

Authentic plant material, *Operculina 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. (~250g) 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 [Harbone, 1973]. The yield of *O. turpethum* powder from 250g of dried roots was ~10-12g. The powder was stored at -2 °C in sterilized and labeled screw capped bottles.
3.1.3. Microorganisms used

The test microorganisms included one gram –ve bacterium, *Salmonella typhimurium* (Clinical isolate) and one gram +ve bacterium, *Listeria monocytogenes* (Standard strain) and two fungi, *Candida albicans* and *Cryptococcus neoformans* (Clinical isolates).

3.1.4. Culture media

The media used to grow *Candida albicans* and *Cryptococcus neoformans* was YPD (Yeast extract, peptone, dextrose) and to culture *Salmonella typhimurium* nutrient broth (NB) was used. *Listeria monocytogenes* was cultured using brain heart infusion agar. All culture media were prepared and treated according to the manufacturer’s guidelines (Hi-media Pvt. Ltd. India).

3.1.5. Inoculum

The microorganisms were inoculated into their respective medium as given above. Inoculated broths were kept at 37°C for 12 hours. After 12 hours of incubation absorbance was read against sterilized medium (used as Blank) at 580 nm. Culture was diluted to obtain $1 \times 10^6$ CFU/ml.

3.1.6. Antimicrobial susceptibility testing

The MICs of the *Operculina turpethum* extract (OTE) for different organisms were determined by broth micro dilution method described by National committee for clinical laboratory standards [NCCLS, 1995]. The antimicrobial agents were tested over the final concentration range of 50 to 0.048 µg/ml. Tests were performed in 96 well round bottom microtitre plates. Cell suspensions of organisms were prepared to give final inoculums concentration of $1 \times 10^6$ cells/ml. The wells containing microbial inoculum with different concentrations of
compounds were incubated for 48 hours. The MIC was defined, as the lowest concentration of compound at which there was complete inhibition of growth.

3.2. Biochemical Toxicology Studies

3.2.1. Chemicals

Acrylamide, 3, 3′-Diaminobenzidine tetrahydrochloride hydrate, N′-DimethylNitrosamine, Nicotinamide adenine dinucleotide (β-NAD), Nitro blue tetrazolium (NBT), Phenazine methosulphate (PMS) and Trizma base were purchased from Sigma-Aldrich, India. Biochemical kits were from Span diagnostics Ltd (India), Cyclophosphamid (500 mg Endoxan-N)™ of Baxter Oncology GmbH (Germany), Fetal Bovine Serum (FBS) was of Hi-Media, 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.

3.2.2. Animals

Adult male albino rats of Wistar strain were kept in well-aerated cages at the departmental facility of Zoology department with equal interval of light-dark exposure. The rats were acclimatized for a week and 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.

3.2.3. 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 in their recommended doses. The fourth group was administered NDMA intraperitoneally in doses of 10 mg/kg body weight as
described previously [George and Chandrakasan, 2000]. The injections were given on three consecutive days of each week for over 21 days.

3.2.4. Administration of *Operculina turpethum* extract (OTE)

Here also, the experimental 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 hours of NDMA administration. The dose of OTE and its administration time was selected on the basis of pilot studies [Suresh Kumar et al., 2006]. A set of animals from each group were anesthetized with diethyl ether and 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 hours prior to sacrifice.

3.2.5. Assessment of liver injury

The process of liver injury and toxicity was assessed through the progression of hepatic fibrosis 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: 80i) and photographed.

3.2.6. Staining of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA)

The degree of hepatic fibrosis was monitored by following activation of hepatic stellate cells as indicated by immunohistochemical (IHC) staining of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) filaments. For detection of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA), serial liver sections (5µm) were stained immunohistochemically using \(\alpha\)-SMA monoclonal antibodies. Peroxidase activity was quenched by incubating the sections with 3% hydrogen peroxide for 15-20 min. The sections were washed with
Phosphate Buffer Saline (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 10-20 min. Developed slides were rinsed with PBS, counter stained with Mayer’s hematoxylin and mounted with DPX.

3.2.7. Biochemical parameters

The commercial kits of Span diagnostics Ltd. (India) were used for determination of liver enzymes like alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and bilirubin levels in rat sera. The enzyme activity levels were again verified by using other routine laboratory techniques. Hydroxyproline (HP) levels were also estimated in sera and urine samples of control and treated group of rats as per previously described protocols [Woessner, 1961].

Levels of Lactate dehydrogenase (LDH) isoenzymes were analyzed in sera and liver homogenates of experimental rats. Livers were homogenized in ice cold Tris-HCl buffer (pH, 7.5), centrifuged at 12,000 rpm and ~10-15 µl of clear supernatants were loaded and resolved on native 7.5% vertical slab polyacrylamide gel electrophoresis [Ahmad and Hasnain, 2005]. LDH isoenzymes were visualized by histochemical staining using L-lactate-βNAD and phenazine methosulphate (PMS)-tetrazolium (NBT) protocol [Ahmad et al., 2009]. LDH isoenzyme bands developed as purple to dark blue bands at 37°C within 25-30 min.
3.2.8. Densitometry

Quantitative estimates of LDH isoenzyme were made by densitometry of enzyme gel-scans using Scion Imaging (Scion Corporation: Beta release-4.0) software programs and presented as fractions in arbitrary units (AU) per gram wet weight of the tissue.

3.2.9. Statistical analysis

The 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 levels of various liver enzymes.

3.3. Results

3.3.1. Microbial

The results obtained for antimicrobial screening of *Operculina turpethum* extract (OTE) are shown in table-3.1. The antimicrobial activity has been tested against one gram-negative bacterium, one gram-positive bacterium and two fungi. The results of the screening indicate that OTE possessed good antimicrobial activity against the entire tested microorganism. The *Operculina turpethum* extract (OTE) was also observed to inhibit the growth of fungus, *Candida albicans* at the lowest concentration of 6.25µg/ml.

3.3.2. Hematoxylin and eosin staining

3.3.2.1. Histological observations 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 shown in Fig. 3.1. The control liver sections showed normal lobular architecture (Fig. 3.1A). On day-7
disintegration of liver parenchyma, hepatic necrosis and severe centrilobular congestion were observed (Fig. 3.1C). Massive hepatic necrosis along with neutrophilic infiltration and multifocal collapse of parenchyma were the prominent features in liver specimens (sections) of day-14 (Fig. 3.1E). 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. 3.1G). At certain places sign of early cirrhosis was also visible.

3.3.2.2. OTE treatment

The hematoxylin and eosin staining of liver sections during amelioration by OTE is also displayed in Fig. 3.1. The control liver specimens showed normal cellular and lobular architecture with radiating hepatic cords (Fig. 3.1B). 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. 3.1D). On day-14, moderate infiltration of mononuclear cells and regeneration of hepatocytes was observed in liver sections of OTE treated rats (Fig. 3.1F). Remarkable decrease in the hepatic fibrosis was visualized on day-21 of OTE treatment (Fig. 3.1H). The liver architecture restored to normalize with more number of regenerating hepatocytes.

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

3.3.3.1. Histological observations 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. 3.2. In control liver specimens, the α-SMA staining was absent (Fig. 3.2A). A large number of α-SMA positive stained stellate cells were observed in the necrotic zone on day-7 of treatment with NDMA (Fig. 3.2C). 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. 3.2E). 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. 3.2G). The number of positive stained cells in the fibrotic area 
was much higher when compared with normal areas of the liver sections.

3.3.3.2. OTE treatment

The α-SMA staining in liver specimens of rats treated with NDMA+OTE is 
also shown in Fig. 3.2. Similar to the saline control, the α-SMA staining was also 
absent in OTE control liver samples (Fig. 3.2B). On day-7, focal staining of α-SMA 
around the central vein indicated the ameliorative action of OTE (Fig. 3.2D). 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. 3.2F). 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. 
3.2H).

3.3.4. Sera ALP, GOT, GPT and bilirubin levels

Hepatotoxicity due to NDMA in rats was assessed by the levels of liver 
function enzymes (SALP, SGOT, SGPT) 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-3.2). Time and dose dependent protection 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 noted (Table-3.2).

3.3.5. 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 and 57.84% was noted in sera and urine hydroxyproline levels, respectively (Table-3.2). Noticeable 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.

3.3.6. 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 control. The increase in total LDH 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 observed in the sera (Fig. 3.3A). 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-3.3).

In liver samples of NDMA injected 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. 3.3B). 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 exactly similar to control values (Table-3.4).

3.4. Discussion

We obtained encouraging results of antimicrobial activity of *Operculina turpethum* extract (OTE), with its activity against all the tested bacteria and fungi. Evidence suggests that even in the alcoholic extracts of fresh roots of *O. turpethum* antibacterial activity is present [The Wealth of India, 2001]. The extract was observed to inhibit the growth of *S. typhimurium* and *L. monocytogenes* at a concentration of 50µg/ml, while that of *C. albicans* successful inhibition was obtained at 6.25µg/ml. The growth of *C. neoformans* was inhibited at a higher concentration (>100 µg/ml) of OTE. It is presumed that compared with other routine methods being used to ameliorate the common ailments, method of treating with effective natural compounds is preferred [Ahmad et al., 2008 a].

The biochemical and molecular data obtained during the present study strongly support to hepatic fibrosis and cirrhosis-inducing potential of *N*-Nitrosodimethylamine (NDMA) in rats [George and Chandrakasan, 1996 a; George and Chandrakasan, 1996 b; George and Chandrakasan, 2000; George et al., 2001; George, 2003; Das and Vasudevan, 2007]. The consequent histological and biochemical alterations include increased synthesis and deposition of connective tissue proteins, changes in hepatic architecture and concomitant impairment of several biochemical processes [Jenkins et al., 1985; Savolainen et al., 1988] leading to shrunken liver. For the first time we report that aqueous extract of *O. turpethum* root (OTE) can protect NDMA-induced toxic effects on liver. OTE treatment restored normal lobular architecture of the liver through extensive regeneration of hepatocytes and recovery of biochemical processes.
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 (IHC) staining for the expression of α-smooth muscle actin (α-SMA). H&E stained liver sections 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 [Brenner et al., 2000; Das and Vasudevan, 2007; George, 2008]. 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 day-14 and 21.

The expression of α-smooth muscle actin (α-SMA) by the stellate cells (SCs) is considered as the most reliable marker for activated hepatic stellate cells which add to collagenous connective tissue [Rockey et al., 1992]. The presence of high number of activated SCs on day-7 of NDMA administration indicates that the fibrosis had started much before day-7 [George and Chandrakasan, 1996 b; George and Chandrakasan, 2000; George et al., 2001]. The late stage of hepatic fibrosis and cirrhosis was characterized by more enhanced expression of α-SMA in the activated SCs in the fibrotic area on day-14 and 21, respectively. OTE treatment caused constant decline in the density of activated SCs on day-7 and 14. Antifibrotic and hepatoprotective potential of OTE is evident by a decline in the α-SMA staining and increase hepatocytes regeneration on day-21 indicating the elimination of activated hepatic SCs.
Similar to previously published reports [George and Chandrakasan, 1997; El-Zayat, 2007], we also recorded significant increases in total LDH activity in sera with major contribution of LDH-4 and LDH-5 isoenzymes. These changes begin on day-7 and become more intense on day-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 [George and Chandrakasan, 1997]. 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 [George and Chandrakasan, 1997; Ahmad et al., 2008 b; Ahmad et al., 2009]. That the enzymatic changes may actually be the result of cellular injury is supported by our histopathological data with the changes in enzyme and isoenzyme levels. While the induction of fibrosis by NDMA disrupts cellular architecture of liver tissue, which is also collateral to 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 [Suresh Kumar et al., 2006] and in other clinical and pathological conditions has been recognized [Austin, 1982; The Wealth of India, 2001]. According to the present data, the protection provided by OTE against NDMA-induced hepatotoxicity was time as well as 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 made earlier [George and Chandrakasan, 2000; George et al., 2001].

The present study demonstrates that liver detoxification and the prevention of collagen accumulation are simultaneous processes. This is evident from the correspondence between histopathological observations of OTE treated rat livers 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 collagensesis. An increase in hepatic collagenolytic enzymes and liver function test enzymes has been recorded in cases of carbon tetrachloride-induced early fibrosis [Murawaki et al., 1990; Hall et al., 1991] and the patients with chronic liver injuries [Murawaki and Hirayama, 1980].

3.5. Conclusion

The present study thus, suggests that *O. turpethum* (roots) aqueous extract (OTE) shows hepatoprotective effects by decreasing collagen contents, restoring α-SMA activity and enzyme markers of the liver injury. Therefore it is advisable that OTE in its maximum evaluated dose may be potentially useful in preventing liver injuries in particular NDMA induced fibrosis.

Moreover, the extract also shows remedial effect against *Salmonella typhimurium*, *Listeria monocytogenes*, *Candida albicans* and *Cryptococcus neoformans*. Therefore may also be used to combat tested pathogenic borne infection.
Table 3.1 Antimicrobial activity of *Operculina turpethum*.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Minimum inhibitory concentration in µg/ml</th>
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<tbody>
<tr>
<td></td>
<td>OTE</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>50</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>50</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>6.25</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

OTE = *Operculina turpethum* extract, positive control; Gentamicin (*Salmonella typhimurium*, *Listeria monocytogenes*) and Amphotericin B (*Candida albicans*, *Cryptococcus neoformans*)
Table 3.2 Biochemical markers in the sera of rats treated with NDMA and different doses of *O. turpethum* extract for 21 days.

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control group</th>
<th>Treated groups</th>
<th>OTE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A)</td>
<td>NDMA (10 mg kg⁻¹ b.wt.)</td>
<td>Per cent increase (B-A/B × 100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75 mg kg⁻¹ b.wt.</td>
<td>150 mg kg⁻¹ b.wt.</td>
</tr>
<tr>
<td>Hydroxyproline (µg/ mL serum)</td>
<td>11.63±1.3</td>
<td>14.59**±1.57</td>
<td>20.28</td>
</tr>
<tr>
<td>Hydroxyproline (µg/ mL urine)</td>
<td>28.17±1.49</td>
<td>66.82*±3.11</td>
<td>57.84</td>
</tr>
<tr>
<td>Serum ALP (U/L)</td>
<td>317±42.06</td>
<td>716*±40.64</td>
<td>55.72</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>126±21.19</td>
<td>190*±22.9</td>
<td>33.68</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>56±8.95</td>
<td>65**±9.53</td>
<td>13.84</td>
</tr>
<tr>
<td>LDH (AU/g wet weight of liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH-1</td>
<td>0.596±0.2</td>
<td>0.92±0.23</td>
<td>35.21</td>
</tr>
<tr>
<td>LDH-2</td>
<td>0.596±0.17</td>
<td>0.976*±0.20</td>
<td>38.93</td>
</tr>
<tr>
<td>LDH-3</td>
<td>0.617±0.17</td>
<td>0.742**±0.14</td>
<td>16.84</td>
</tr>
<tr>
<td>LDH-4</td>
<td>0.627±0.14</td>
<td>1.05±0.22</td>
<td>40.28</td>
</tr>
<tr>
<td>LDH-5</td>
<td>0.755±0.2</td>
<td>0.86**±0.17</td>
<td>12.2</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.316±0.05</td>
<td>1.49±0.18</td>
<td>78.79</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dL)</td>
<td>0.13±0.02</td>
<td>0.15**±0.04</td>
<td>13.33</td>
</tr>
</tbody>
</table>

Values are significant at *P*<0.05 * and *P*<0.001**. Note: ALP = Alkaline phosphatase; AU = Arbitrary units; LDH-1 to -5 = Lactate dehydrogenase isoenzyme-1 to -5; OTE = *Operculina turpethum* extract; SGOT= Serum glutamic oxaloacetic transaminase; SGPT= Serum glutamic pyruvic transaminase.
Table-3.3 Apparent ranking and percent 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).

<table>
<thead>
<tr>
<th>LDH Isoenzymes</th>
<th>NDMA</th>
<th>NDMA+OTE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Day-7</td>
</tr>
<tr>
<td>LDH-1</td>
<td>18.32±2.01</td>
<td>12.63±1.11</td>
</tr>
<tr>
<td>LDH-2</td>
<td>13.66±1.5</td>
<td>7.45±0.74</td>
</tr>
<tr>
<td>LDH-3</td>
<td>8.09±0.78</td>
<td>7.23±0.69</td>
</tr>
<tr>
<td>LDH-4</td>
<td>11.53±1.03</td>
<td>8.45±0.98</td>
</tr>
<tr>
<td>LDH-5</td>
<td>48.34±2.33</td>
<td>64.17±3.08</td>
</tr>
<tr>
<td>Rank of LDH isoenzymes</td>
<td>5&gt;1&gt;2&gt;4&gt;3</td>
<td>5&gt;1&gt;4&gt;2&gt;3</td>
</tr>
</tbody>
</table>

Values are mean ± s.d. of 5 samples
* Treated with OTE (200 mg kg⁻¹ body weight)
Values are significant at $P<0.05$ * and $P<0.001$**
Table-3.4 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).

<table>
<thead>
<tr>
<th>LDH Isoenzymes</th>
<th>NDMA</th>
<th>NDMA+OTE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Day-7</td>
</tr>
<tr>
<td>LDH-1</td>
<td>11.72±1.1</td>
<td>6.65±0.88</td>
</tr>
<tr>
<td>LDH-2</td>
<td>13.05±1.4</td>
<td>8.98±0.79</td>
</tr>
<tr>
<td>LDH-3</td>
<td>17.94±1.2</td>
<td>15.26±1.05</td>
</tr>
<tr>
<td>LDH-4</td>
<td>25±2.3</td>
<td>30.41*±2.9</td>
</tr>
<tr>
<td>LDH-5</td>
<td>32.3±2.57</td>
<td>38.16±3.1</td>
</tr>
<tr>
<td>Rank of</td>
<td>5&gt;4&gt;3&gt;2&gt;1</td>
<td>5&gt;4&gt;3&gt;2&gt;1</td>
</tr>
<tr>
<td>LDH isoenzymes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.d. of n=5.

† Treated with OTE (200 mg kg⁻¹ body weight)
Values are significant at $P<0.05$ * and $P<0.001^{**}$
Fig. 3.1 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.
Fig. 3.2 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.

Fig. 3.3 Polyacrylamide gel electrophoretic (PAGE) pattern 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.
References


Husain et al., (1992). *A Dictionary of Indian Medicinal Plants*. Published by Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India.


Yoganarasimhan, S.N. (2000). *Medicinal Plant of India, Tamil Nadu* vol. II.