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Hepatoprotective and antioxidant activity of methanolic extract of flowers of *Nerium oleander* against CCl_4−induced liver injury in rats

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ABSTRACT

Objective: To investigate the antioxidant and hepatoprotective activity of methanolic flower extract of *Nerium oleander* against CCl_4−induced hepatotoxicity in rats. **Methods:** *In vitro* antioxidant activity of methanolic extract of flowers of *Nerium oleander* (MENO−F) was evaluated by various assays, including reducing power, lipid peroxidation, DPPH, ABTS, superoxide anion, hydroxyl radicals and metal chelation. The hepatoprotective and *in vivo* antioxidant activity of MENO−F were evaluated against CCl_4−induced hepatic damage in rats. The MENO−F at dose of 100, 200 and 400 mg/kg were administered orally once daily for seven days. Serum enzymatic levels of serum glutamate oxaloacetate transaminase (AST), serum glutamate pyruvate transaminase (ALT), serum alkaline phosphatase (ALP) and total bilirubin were estimated along with estimation of superoxide dismutase (SOD) and malondialdehyde (MDA) levels in liver tissues. Further histopathological examination of the liver sections was carried out to support the induction of hepatotoxicity and hepatoprotective efficacy. **Results:** The extract showed potent activities on reducing power, lipid peroxide, DPPH, ABTS, superoxide anion, hydroxyl radical and metal chelation. The substantially elevated serum enzymatic levels of AST, ALT, ALP and total bilirubin were found to be restored towards normalization significantly by the MENO−F in a dose dependent manner with maximum hepatoprotection at 400 mg/kg dose level. The histopathological observations supported the biochemical evidences of hepatoprotection. Elevated level of SOD and decreased level of MDA further strengthen the hepatoprotective observations. **Conclusions:** The results of the present study strongly reveal that MENO−F has potent antioxidant activity and hepatoprotective activity against CCl_4−induced hepatic damage in experimental animals.

1. Introduction

The liver demonstrates a major role in metabolism of xenobiotics by regulating the synthesis, secretion and metabolism of xenobiotics. Various physiochemical functions of the body including oxidation, reduction, hydroxylation, hydrolysis, conjugation, sulfation, acetylation *etc* are well balanced by the liver alone. Injury to liver and damage to the hepatic parenchyma are always proved to be associated with distortion of different metabolic functions of liver[1,2]. Etiologically various infectious agents including viruses and different hepatotoxic chemicals along with environmental pollutants are thought to be responsible for different type of liver damage and hepatic injury. Recent research in free radical biology also suggested the pathophysiological role of free radicals and oxidative stress in liver damage and injury. Revealing the mechanism of actions of potent hepatotoxin such as CCl_4, paracetamol *etc* also indicated the role of oxidative stress and free radicals in the pathophysiology of hepatic injury[3,4].

The free radicals normally generated during the normal body metabolic pathways and also they can be acquired from the environment also. Free radicals contain unpaired electrons. The oxygen radicals, such as superoxide radical (O_2^-), hydroxyl radical ($\bullet OH$) and non free radical species, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($\bullet O_2$), are generated in many redox processes of normal physiochemical pathways[5,6]. Antioxidant defense system comprising different enzymes such as superoxide

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dismutase, catalase and glutathione peroxidise *etc* trap and destroy these free radicals. Vitamin deficiency together with overproduction of free radicals and a reduced level of above mentioned enzymes is considered as the main culprit for producing oxidative stress[6].

Research on oxidants and antioxidants over the past few years has shown a link between most diseases like cardiovascular diseases, cancer, osteoporosis, degenerative diseases *etc* and production of reactive oxygen species (ROS) along with oxidative stress[3,7,8]. Free radicals mainly act by attacking the unsaturated fatty acids in the biomembranes which causes membrane lipid peroxidation (a hallmark sign of hepatotoxicity), decrease in membrane fluidity and reduction of enzyme and receptor activity and damage to membrane protein which finally triggers the cell inactivation and death[5,6]. Therefore, antioxidants can be used to reverse the harmful and pathological action of free radicals. These antioxidants generally restore the normal physiological system by scavenging the free radicals. The antioxidants in use are either derived naturally from plants or synthetically. Due to carcinogenic probability, synthetic antioxidants are not the preferred type of antioxidants[6]. Current research in the field of free radical biology therefore accentuates the use of antioxidants from natural origin and in view of this more and more antioxidants of natural origin are being investigated.

CCl$_4$ is one of the most common hepatotoxin used for experimental induction of liver injury in animal studies[9,10]. Impoverishment of modern system of medicine in terms of a reliable liver protective drug switched on the exploration of traditional systems of medicine including Ayurveda, Siddha, Unani *etc* for a probable answer to hepatotoxicity[11,12]. Numerous medicinal plants are being researched for an effective hepatoprotective remedy. A number of medicinal preparations in the Indian system of medicine (Ayurveda) have been used as effective hepatoprotective. In view of this several medicinal preparations and a number of medicinal plants mentioned in Ayurveda for treatment of liver disorders are being investigated[11,13]. Moreover traditional folklore and indigenous knowledge of medicinal uses of plants are also now being explored and documented for possible bioactive molecules to be future drugs.

Nerium oleander Linn. (Family: Apocynaceae) is a traditional Ayurvedic herb and also known as Kaner in Hindi and Surkh Kaner in Unani. In Ayurveda there are several other names indicating this plant such as Karavira, Viraka, Ashvamaaraka, Hayamaaraka, Gauripushpa, Divyapushpa, Shatakumbha, Siddhapushpa (white–flowered variety) Raktapushpa, Raktaprasava, Ravipriya (red–flowered variety)[12]. In English it is known as red oleander or rose ray. The common oleander is an ornamental evergreen shrub, omnipresent in temperate and subtropical regions[14]. The plant is also widely grown in Indian gardens. The plant is also widely found in humid and coastal areas including Assam, Arunachal Pradesh, Himachal Pradesh, Rajasthan, Uttar Pradesh and Tamil Nadu[12]. Preparations of oleander

have been used for centuries as folk and indigenous remedies for various ailments including indigestion, malaria, leprosy, mental or venereal diseases and as aborifacient[15]. *Nerium oleander* L. is also used indigenously as a cardiac tonic, diuretic, molluscicide and insecticide and for the treatment of epilepsy, snake bites and skin conditions[16]. Previous studies reported the use of oleander extracts for cardiac insufficiency which was mainly attributed to the cardiac glycosides within this plant[17]. Oleandrin is one of the most prominent secondary compounds of *Nerium oleander*. The plant is also known to be toxic against a wide range of tumor cells[18]. The plant is also documented as a potential vegetable source of antioxidants[19]. According to the previous phytochemical works, the leaves of *Nerium oleander* L. contain two novel cytotoxic pentacyclic triterpenoids *cis*–karenin (3 β –hydroxy–28–Z–*p*–coumaroyloxy–urs–12–en–27–oic acid) and *trans*–karenin (3– β –hydroxy–28–E–*p* coumaroyloxy–urs–12–en–27–oic acid)[20] as well as two new cardiac glycosides, kancroside and neriumoside[21]. Another study also revealed the presence of oleandrin, folineriin , adynerin, digitoxigenin cardiac glycosides in oleander[22]. Seeds of *Nerium oleander* L. were reported to contain about 12% of Δ9–hydroxy–18:1$^{\Delta 12}$ (isoricinoleic acid)[23]. Methanolic extract of the leaves of this plant was found as anticonvulsant, central nervous system depressant and analgesic[24]. As far our literature survey is concerned, extracts from different parts of this plant have been reported to possess biological activity and various phytochemicals have been isolated from different parts of this plant including some potential hepatoprotective constituents (Oleanolic acid)[25], while no report has been published in reference to the flowers of this plant. Preliminary screening also suggested potent lipid peroxidation inhibitory activity of the methanolic extract of the flowers of this plant under investigation. Therefore in this present study an attempt has been made to evaluate the hepatoprotective activity as well as antioxidant profile of *Nerium oleander* L. flower extract with a view towards elucidating the probable mechanism of action by employing various *in vitro* and *in vivo* methods.

2. Materials and methods

2.1. Chemistry

Malondialdehyde (MDA) was obtained from Sigma Chemicals Company, St Louis, MO, USA. Silymarin was obtained from Ranbaxy Laboratories, Delhi, India. CCl$_4$ was obtained from E Merck, Mumbai, India. All other reagents and chemicals used in the experiments were of analytical grade and available commercially via reputed vendors.

2.2. Preparation of plant extract

The flowers of *Nerium oleander* L. were collected from

Kota, Rajasthan, India, during the months April and May. The flowers of *Nerium oleander* L. were dried in shade and coarsely powdered. Coarsely powdered flower (5 kg) was successively extracted in the Soxhlet apparatus using petroleum ether, chloroform, ethyl acetate, methanol and water as solvent for the complete extraction of the phytochemicals. The five extracts were dried in rotary evaporator at 45 ℃ and the dried extracts were stored in vacuum desiccators containing anhydrous silica gel. All the five extracts were subjected to acute toxicity studies as per the OECD guidelines.

2.3. Acute toxicity studies

An acute oral toxicity study was performed according to the OECD guidelines for the testing of chemicals, Test No. 423 (OECD, 2001; Acute oral toxicity–acute toxic class method). Wistar rats ($n = 3$) of either sex were selected by a random sampling technique for the acute toxicity study. The animals were fasted overnight prior to the experiment and maintained under standard laboratory conditions. Each extract was administrated orally in increasing dose up to 2 000 mg/kg.

2.4. Determination of total phenolic compounds

Total soluble phenolics in the leaf methanolic fraction of the plant were determined by using Folin–Ciocalteau method using quercetin as a standard phenolic compound[6]. The concentration of total phenols was expressed as mg/g of extract. The concentration of total phenolic compounds in the extract was presented as gram of quercetin equivalent (QE) using an equation obtained from the equation of regression line of standard quercetin graph:
$Y = 0.05 x + 0.054 5$, $r^2 = 0.987 3$
Where, y was the absorbance and x was the concentration.

2.5. In vitro antioxidant activity

2.5.1. Reducing power
The reducing power of the methanolic fraction of flowers of NO, BHT and ascorbic acid were determined according to the method described elsewhere[26]. The absorbance was measured at 700 nm.

2.5.2. Total antioxidant activity
The total antioxidant activity of the extract was determined according to the thiocyanate method[6].

2.5.3. DPPH radical scavenging activity
The free radical scavenging activity of the extract was measured by DPPH• using the method described previously[6,27]. BHT was used as a standard free radical scavenger.

2.5.4. ABTS radical scavenging activity
Antioxidant activity of this plant was measured using an improved ABTS method as described previously[27].

2.5.5. Superoxide anion scavenging activity
The effect of scavenging superoxide radical was determined in a riboflavin–light–nitroblue tetrazolium (NBT) system by the nitroblue tetrazolium reduction method[28].

2.5.6. Hydroxyl radical scavenging activity
The effect of extracts on hydroxyl radical was assessed by using the deoxyribose method[28].

2.5.7. Metal chelating activity
The chelating of ferrous ions by the methanolic extract, BHT and EDTA were measured by the method described previously[29].

2.6. In vivo hepatoprotective activity

2.6.1. Test animals
Wistar rats (180－240 g) of either sex procured from the central animal house, ASBASJSM College of Pharmacy, Bela (Ropar)–140111 (Punjab), were used for the study. The animals were housed in large, clean polypropylene cages in a temperature–controlled room (22 ± 2) ℃ with relative humidity (44%–55%) under 12–h light and dark cycles. All the animals were acclimatized to laboratory environment for a week prior to experiments. Animals were provided with a standard rodent pellet diet and clean drinking water *ad libitum*. The care and use of laboratory animals were strictly in accordance with the guidelines prescribed by the Institutional Animal Ethical Committee constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

2.6.2. Experimental design
A total of 36 rats were divided into 6 groups of 6 rats each.
•Group I served as normal control and received only the vehicle (1 mL/kg/day of 1% CMC; *p.o.*).
•Group II received CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) *i.p.* once daily for 7 days.
•Group III received CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) *i.p.* and silymarin 100 mg/kg orally (*p.o.*) for 7 days.
•Groups IV, V, VI were administered methanolic extract of flowers of *Nerium oleander* (MENO–F) at 100, 200, and 400 mg/kg body weight *p.o.*, respectively and dose of 1 mL/kg *i.p.* of CCl_4 (1:1 of CCl_4 in olive oil) for 7 days.
All rats were sacrificed by cervical dislocation 24 h after the last treatment. Just before sacrifice, blood was collected from the retro–orbital sinus plexus under mild ether anesthesia. Collected blood was allowed to clot and serum was separated at 3 500 rpm for 15 min for carrying out further biochemical investigations. One part of liver was dissected out and used for biochemical and histopathological studies[4, 30].

2.6.3. Measurement of serum biochemical parameters

The activities of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and total bilirubin were determined using the Hitachi 912 clinical chemistry automatic analyzer (Roche Diagnostic GmbH, Mannhei m, Germany).

2.6.4. Assessment of lipid peroxidation and superoxide dismutase (SOD)

In chilled normal saline excised livers were perfused to remove all the blood cells. Then they were cut down into small pieces, placed in 0.1M phosphate buffer (pH 7.4), and homogenized using remi homogenizer to obtain 20% homogenate. The homogenate thus obtained was centrifuged at 3 000 rpm for 15 min and the supernatant was collected in an Eppendorf tube. This supernatant was again centrifuged at 12 000 rpm for 30 min. The final supernatant was used for the determination of MDA as a lipid peroxidation marker[31,32]. SOD was also assayed by the method described previously[32,33].

2.6.5. Histopathology

The liver tissue was dissected out and fixed in 10% formalin solution. It was then dehydrated in ethanol (50%–100%), cleared in xylene and embedded in paraffin wax. Afterwards thick sections (5–6 mm) were made and then stained with hematoxylin and eosin dye for photomicroscopic observation. Scoring on scale of 1–4 was done for the liver sections under microscope as given below[34–36].

0= Normal liver histology; 1 = Tiny and short septa of connective tissue without influence on the structure of hepatic lobules; 2 = Large septa of connective tissue, flowing together and penetrating into the parenchyma. Tendency to develop nodules; 3 = Nodular transformation of the liver architecture with loss of structure of hepatic lobules; 4 = Excessive formation and deposition of connective tissue with subdivision of the regenerating lobules and with development of scars.

2.7. Statistical analysis

The data were expressed as mean ± SD. Statistical differences at $P < 0.001$ between the groups were analyzed by one–way ANOVA followed by Turkey as *post hoc* using GraphPad Instat software package. The IC_{50} values were calculated graphically by linear regression analysis.

3. Results

3.1. Acute toxicity studies

All the extracts of *Nerium oleander* flowers except chloroform

extract did not cause any mortality up to 2 000 mg/kg dose level. Hence 1/20 th, 1/10th and 1/5 th of the maximum dose (*i.e.*, 100, 200 and 400 mg/kg, *p.o.*) were selected for the present study. At dose level of 2 000 mg/kg chloroform extract was highly toxic and it produced sever toxic symptoms and lethality (LD_{100}). The maximum tolerable dose for the chloroform extract was found to be 500 mg/kg. Results of the acute toxicity studies revealed that the toxic nature of this plant belongs to the phytochemicals extracted in chloroform fraction only.

3.2. Determination of total phenolic compounds

Mostly antioxidant activities of plant sources are due to the presence of phenolic–type compounds. Due to their hydroxyl groups, phenols are very important plant constituents with scavenging ability. But the antioxidant effects do not necessarily always correlate with the presence of large quantities of phenolics[6]. The methanolic extract (MENO–F) was evaluated for total phenolic content. The amount of total phenolics was found to be (289.5±5.0) mg QE/g extract. The present study did not try to establish any correlation between phenolic content and biological activity.

3.3. In vitro antioxidant activity

3.3.1. Reducing power

The reducing power of the extract compared to BHT and ascorbic acid is shown in Figure 1. In the reductive ability measurement, $Fe^{3+}-Fe^{2+}$ transformation in the presence of extract samples was investigated using the method described elsewhere[26]. With the increasing concentration of the extract the reducing power of the extract was increased. The extract disclosed an equivalent reducing power to BHT but reductive capability was lower than ascorbic acid.
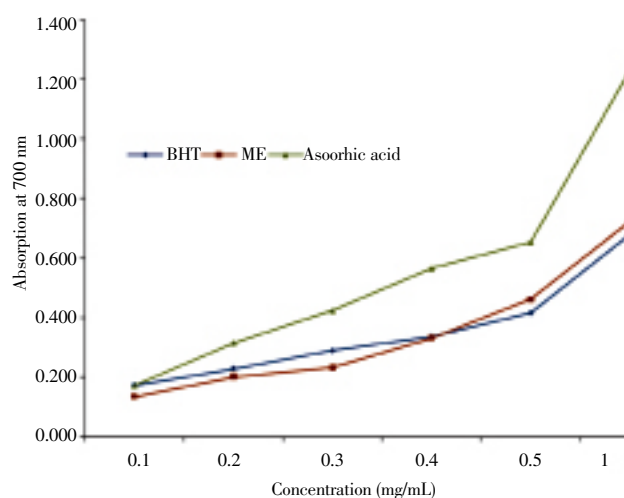

Figure 1. Reductive ability of MENO–F.

3.3.2. Total antioxidant activity

Thiocyanate method was used to evaluate the total antioxidant activity of the plant extract. The extract exhibited effective and powerful antioxidant activity at a concentration of 500 μg/mL. The effect of 500 μg/mL concentration of the extract on peroxidation of linoleic acid emulsion is shown in Figure 2. The antioxidant activity of the plant extract initially was increased with an increasing time of incubation and then it showed a decrease in activity further with increasing time of incubation. The studied concentration of the extract exhibited higher antioxidant activity than 500 μg/mL concentration of α-tocopherol but lower antioxidant activity than same concentration of butylated hydroxyanisole (BHA). The percentage inhibition of peroxidation of the extract in linoleic acid system was found to be 62.34%. And percentage inhibition of 500 μg/mL concentration of BHA and α-tocopherol was found as 95.13% and 32.58%, respectively.
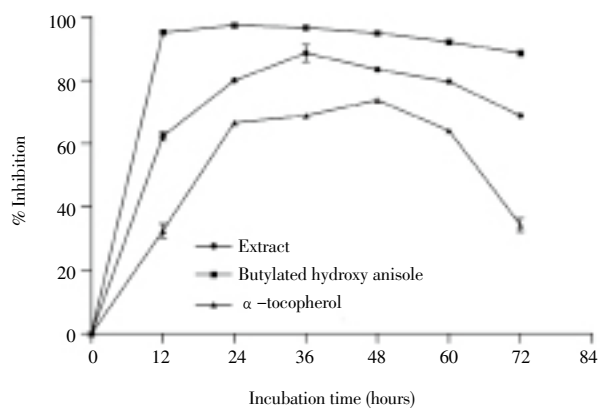

Figure 2. Total antioxidant activity of MENO–F, BHA and α-tocopherol at 500 μg/mL concentration.

3.3.3. DPPH radical scavenging activity

Figure 3 shows the decrease in concentration of DPPH radical due to scavenging capability of the extract and standard compound (BHT) at different studied concentrations (10–500 μg/mL). The scavenging ability of the extract on DPPH radical was found to be equivalent to BHT. The percent DPPH scavenging effect of the extract and the standard were found to be 95.64% and 93.69%, respectively at the concentration of 500 μg/mL. The radical scavenging effect of the extract at 200 μg/mL was similar to BHT at the similar concentration. The results indicated the plant as a strong scavenger of DPPH radical comparable to standard BHT. The IC$_{50}$ values of the extract and BHA were calculated using the equation obtained from linear regression analysis. The calculated IC$_{50}$ values of the extract and the standard compound (BHT) were found to be 193.37 μg/mL and 189.57 μg/mL,

respectively. When free radical formation exceeds the body's ability to protect itself, oxidative stress occurs and forms the biological basis of chronic condition[6,37]. Data from this present study indicate that the plant extract is powerful free radical scavenger, which can reduce or reverse the damage caused by free radicals in the human body.
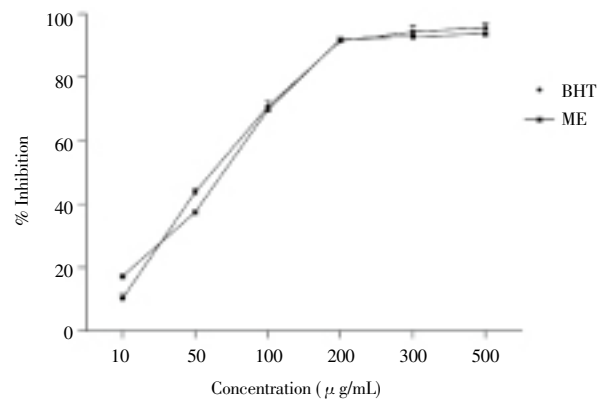

Figure 3. Free radical scavenging activity of different concentrations of MENO–F and BHT by DPPH radicals.

3.3.4. ABTS radical scavenging activity

Figure 4 shows the decrease in concentration of ABTS$^{•+}$ radical due to scavenging capability of the extract and standard compounds at different studied concentrations (50–500 μg/mL). The methanolic fraction produced a concentration dependent scavenging of ABTS$^{•+}$ radical. The calculated IC$_{50}$ values of the extract, quercetin and vitamin C were found to be 156.05 μg/mL, 85.63 μg/mL and 164.78 μg/mL, respectively.
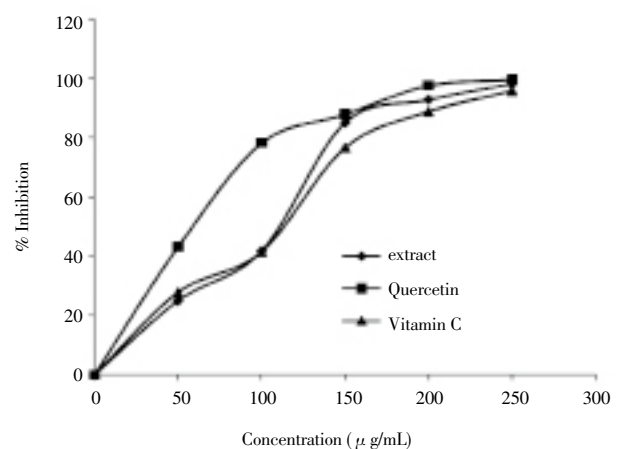

Figure 4. Free radical scavenging activity of different concentrations of MENO–F, Quercetin and vitamin C by ABTS radicals.

3.3.5. Superoxide anion scavenging activity

Superoxide anion is a highly toxic species and generated by different biological reactions in the physiological system.

In the present study, the decrease in absorbance at 590 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Figure 5 shows the increase in percentage inhibition of superoxide radical generation with increasing in concentration of the extract and standard compounds (Quercetin and ascorbic acid). The plant extract showed good superoxide radical scavenging activity but the scavenging activity of the extract was found to be lower than the standard ascorbic acid. The percentage inhibition by the extract, quercetin and ascorbic acid at were found as 89.87%, 98.17% and 91.35%, respectively at the concentration of 500 μ g/mL. The calculated IC_{50} values of the extract, quercetin and ascorbic acid were found to be 210.61 μ g/mL, 89.50 μ g/mL and 142.92 μ g/mL, respectively.
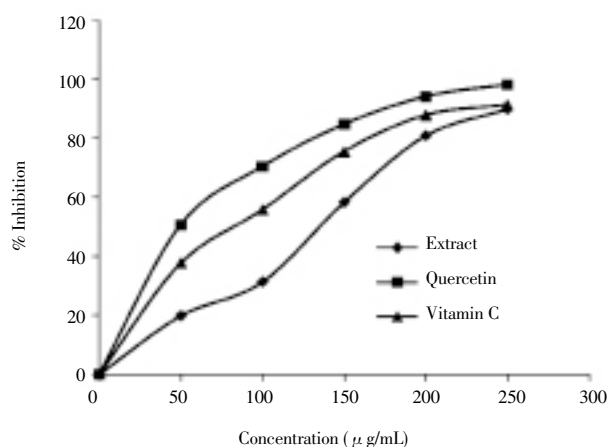

Figure 5. Superoxide anion scavenging activity of MENO–F and same doses of ascorbic acid (vitamin C) and quercetin in PMS–NADH–NBT method.

3.3.6. Hydroxyl radical scavenging activity

Hydroxyl radical is the most reactive of the ROS that attacks almost every molecule in the physiological system. It initiates the peroxidation of cell membrane lipids yielding malondialdehyde, which is mutagenic and carcinogenic. Hydroxyl radicals are formed *in vivo* from water by high-energy irradiation or from H_2O_2 in a metal-catalyzed process. *In vitro*, methanolic extracts (MENO–F) were able to scavenge the hydroxyl radical (Figure 6) in a concentration-dependent manner (50–500 μ g/mL). The IC_{50} values were found to be 211.29 μ g/mL, 138.22 μ g/mL, 179.71 μ g/mL and 107.30 μ g/mL for MENO–F, quercetin, ascorbic acid and sodium metabisulphite, respectively.

3.3.7. Metal chelating activity

The method of determination of metal chelating ability is based on chelation of Fe^{2+} ions by the ferrozine reagent[29]. A complex with Fe^{2+} ions is formed in the reaction which gives absorbance. This formation of the complex is disturbed in the presence of other agents with metal chelating property and absorbance decreases with the reduction of formation of red coloured complex. Measurement of the rate of reduction of the colour, therefore allows estimation of the chelating activity of the co–existing chelator. In this present experimental setup, the formation of ferrous complex with ferrozine reagent was interfered by both extract and standard compounds (EDTA). Figure 7 illustrates the chelating activity of the extract and standard compounds in terms of percentage of metal chelating activity with increasing concentration (50–500 μ g/mL). From the results it was evident that the metal chelating activity of the extract was concentration dependent. The percentage metal chelating activity was increased with increasing concentration of the extract. The IC_{50} values were found to be 333.46 μ g/mL and 198.42 μ g/mL for MENO–F and EDTA, respectively.
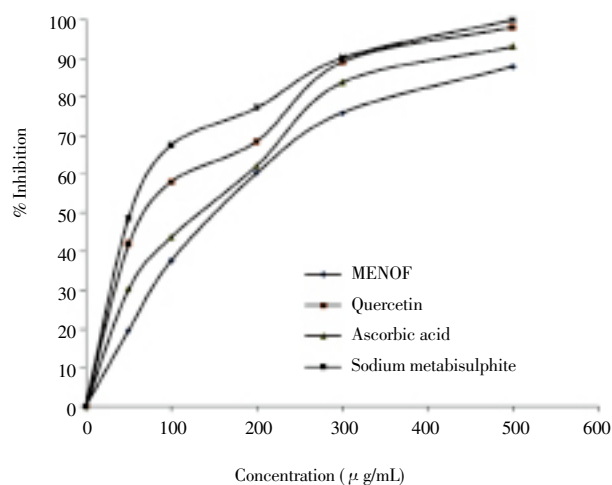

Figure 6. Hydroxyl radical scavenging activity of MENO–F and same doses of quercetin, ascorbic acid and sodium metabisulphite.
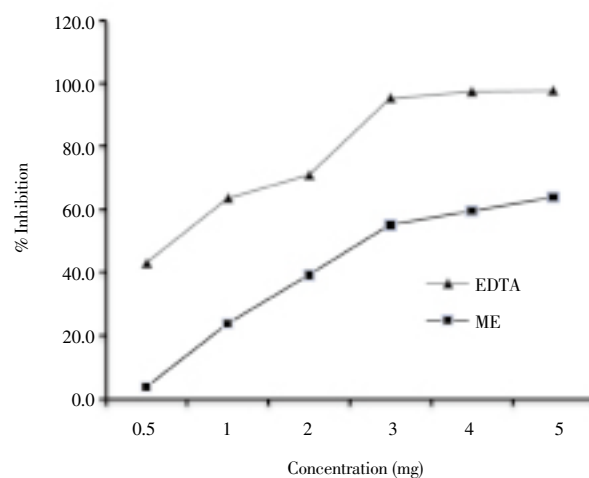

Figure 7. Metal chelating activity of MENO–F and EDTA.

3.4. In vivo hepatoprotective activity

3.4.1. Effect of MENO–F on the measurement of serum biochemical parameters

The hepatoprotective effects of MENO–F on serum biochemical parameters in CCl$_4$–intoxicated rats are shown in Table 1. Rats treated with CCl$_4$ (Group II) showed a significant increase in serum AST, ALT, ALP and total bilirubin levels compared to control animals (Group I). Pre–treatment with MENO–F at 100, 200 and 400 mg/kg for 7 days (Groups IV, V and VI) showed significant hepatoprotection in terms of serum AST, ALT, ALP and total bilirubin levels compared to the toxic control group (Group II). Pretreatment with the standard hepatoprotective agent–Silymarin (Groups III) also decreased all measured serum biochemical activities towards normalness.

3.4.2. Effect of MENO–F on MDA and SOD levels

Lipid peroxidation was increased in the toxic control group, as revealed by elevated MDA levels, when compared with the normal control group. Pre–treatment with MENO–F at 100, 200 and 400 mg/kg significantly decreased the MDA levels, which were almost similar to those of rats receiving the standard drug silymarin. A level of antioxidant enzyme, SOD was significantly increased in MENO–F treated groups. The extract at the dose of 400 mg/kg demonstrated maximum hepatoprotection as shown in Table 1.

3.4.3. Histopathology

Histopathological observations revealed that the normal architecture of liver was completely lost in rats treated with CCl$_4$ with the appearance of vacuolated hepatocytes and degenerated nuclei (Figure 8). Vacuolization, fatty changes and necrosis of hepatocytes were severe in the central lobular area. CCl$_4$ intoxication led to excessive formation of deposition of connective tissue and development of scars (score of 4). MENO–F at 100 mg/kg dose did show significant hepatoprotective activity. Liver sections of rats treated with MENO–F (100 mg/kg) revealed nodular transformation of liver architecture with loss of structure of hepatic lobules (score of 3). Large septa of connective tissue flowing together and penetrating into the parenchyma (score of 2) were observed in liver sections of rats treated with MENO–F 200 mg/kg. Liver sections of rats treated with MENO–F 400 mg/kg bear witness more or less normal lobular pattern with short septa of connective tissue and a mild degree of fatty change, and necrosis (score of 1) almost comparable to the control and silymarin treated groups.
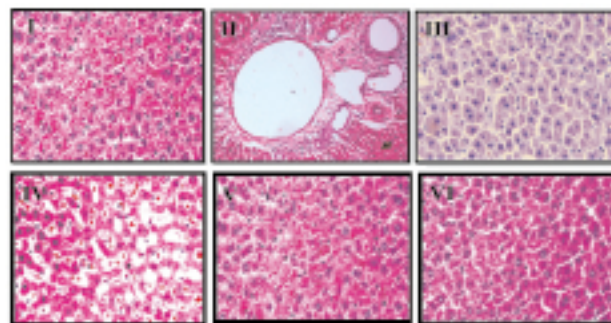

Figure 8. Representative photomicrographs of liver sections.
I. Liver section of normal control rats showing normal architecture. II. Liver section of CCl$_4$ treated rats showing: massive fatty changes, necrosis, ballooning degeneration, and severe infiltration of the lymphocytes and the loss of cellular boundaries. III. Liver section of rats treated CCl$_4$ and 100 mg/kg of silymarin showing: signs of inflammatory cascade around central vein indicating a mild degree of fatty change, and necrosis and focal necrosis (dilatation). IV. Liver section of rats treated CCl$_4$ and 100 mg/kg of MENO–F showing: less inflammatory cells around central vein, absence of necrosis. V. Liver section of rats treated CCl$_4$ and 200 mg/kg of MENO–F showing: minimal inflammatory cellular infiltration, large septa of connective tissue flowing together and penetrating into the parenchyma. VI. Liver section of rats treated CCl$_4$ and 400 mg/kg of MENO–F showing: minimal inflammatory cellular infiltration with almost near normal liver architecture. There is regeneration of hepatocytes evident.

Table 1
Effects of MENO–F on serum biochemical parameters in CCl$_4$–intoxicated rats.

Group	Treatment	AST (SGOT) (IU/L)	ALT (SGPT) (IU/L)	ALP(IU/L)	Serum Billirubin (mg/dL)	MDA	SOD
Group I	Control	180.00±3.30	65.00±5.50	200.00±3.80	0.50±0.04	100.00±0.00	9.00±0.07
Group II	CCl$_4$ 1 mL/kg (i.p.)	620.00±3.60	130.00±6.30	550.00±8.20	0.97±0.05	130.00±1.10	4.20±0.07
Group III	Silymarin 100 mg/kg+CCl$_4$ (prophylactic)	240.00±17.00*	71.00±1.90*	200.00±3.90*	0.42±0.03*	110.00±1.20*	9.00±0.08*
Group IV	MENO–F 100 mg/kg+CCl$_4$ (prophylactic)	430.00±3.30*	85.00±3.50*	260.00±7.40*	0.68±0.04*	130.00±1.40*	6.00±0.14*
Group V	MENO–F 200 mg/kg+CCl$_4$ (prophylactic)	320.00±11.00*	78.00±2.70*	210.00±2.80*	0.70±0.04*	120.00±0.84*	6.40±0.08*
Group VI	MENO–F 400 mg/kg+CCl$_4$ (prophylactic)	250.00±7.30*	65.00±4.80*	210.00±6.20*	0.58±0.03*	130.00±0.84*	8.10±0.13*

Data are expressed as mean±SD ($n = 6$). One–way ANOVA followed by Tukey's *post hoc*: * $P< 0.001$ compared with group II.

4. Discussion

The present study demonstrates the hepatoprotective, prophylactic and antioxidant effects of MENO–F against CCl_4–induced liver injury in rats. The liver mainly detoxifies toxic chemicals and drugs and becomes the main target organ for all possible toxic xenobiotics. Being a potent hepatotoxin, CCl_4 is the most extensively used chemical agent to investigate hepatoprotective activity on various experimental animal models. CCl_4 is known to cause hepatotoxicity. The experimental hepatic damage caused by CCl_4 histologically also resembles viral hepatitis[38,39]. CCl_4 is biotransformed in liver by cytochrome $P4_{50}$ enzymes to CCl_3 radical which is a very active radical. This active CCl_3 radical reacts with oxygen to produce trichloromethylperoxyl radical ($CCl_3O_2·$), which is then covalently binds with cellular macromolecules and biomembranes to cause lipid peroxidation of the lipid membranes of the adipose tissue. Peroxide products finally trigger production and leakage of biomarkers like MDA. This whole cascade of biochemical events ultimately causes loss of cellular integrity and hepatic damage[31,39–48]. Lipid peroxidation is an important parameter of oxidative stress along with other free radical damage occurred in the biochemical cascade. Therefore, antioxidant efficacy is regarded as one of the utmost important parameter indicative of the possible mechanism of hepatoprotection.

AST, ALT and ALP are the serum hepatobillary enzymes present normally in the liver in high concentrations. Upon necrosis or hepatic damage these enzymes will be leaked into the circulation; raising serum concentration of these enzymes[31]. Elevated serum AST, ALT and ALP levels in CCl_4 treated animals indicated cellular breakage and loss of functional integrity of cell membranes in liver[31].

In the present study, increased MDA levels in liver indicated increased lipid peroxidation induced by CCl_4 (Group II animals). This enhanced lipid peroxidation finally triggered hepatic tissue damage. Reduced estimation of SOD in CCl_4 treated animals also suggested failure of antioxidant defense mechanism to block peroxidation damage.

In the present experimental setup, antioxidant activity of the methanolic extract of the flowers of *Nerium oleander* and the possible mechanism had been investigated by evaluating DPPH and ABTS radical scavenging activity. Reducing power assay, superoxide radical scavenging and hydroxyl radical scavenging were also assessed. Reducing power assay is one of the most investigated significant indicators of antioxidant potential. Mainly capability of bioactive compounds to donate hydrogen and electron reflects reducing power[6,27,49]. In this assay, the extract showed a good concentration–dependent increase in reducing power. ABTS and DPPH radicals are very popular and well established free radicals used to investigate free radical scavenging power of components *in vitro*. The present study indicated a strong ABTS and DPPH radicals scavenging activity of the extract. Degree of lipid peroxidation was also found to be inhibited by the extract as suggested by results of thiocyanate method. Superoxide anion radical has been known as relatively weak oxidant but the ability to generate more toxic and dangerous singlet oxygen, hydroxyl radical and peroxynitrile radical made superoxide radical a dangerous reactive species[3]. The results of the present study revealed a good significant hydroxyl radical and superoxide radical scavenging activity of the extract under evaluation.

In view of this, the increased serum level of AST, ALT and ALP enzymes in CCl_4 treated animals (Group II) confirmed hepatic damage. As a breakdown product of heme in red blood cells, bilirubin is regarded as a clinical and pathophysiological indicator of necrosis of liver tissues. Pretreatment with *Nerium oleander* extract in different animal groups (Group IV/V/VI) resulted a significant decrease in serum AST, ALT, ALP and total bilirubin levels as compared to CCl_4 treated group (Group II). Prophylactic use of the extract resulted in an inhibition of the degree of hepatic necrosis and concomitantly decreased the leakage of intracellular enzymes by stabilizing hepatic cellular membranes. The results are further confirmed by the histopathological observations. Inhibition of lipid peroxidation to a significant degree is also a predominant mechanism of hepatoprotection as suggested by the significant decrease in MDA levels. Increase in the SOD level was also suggestive of repairment of antioxidant defense system, which plays an important role in hepatoprotection. Based upon the results of this present study, it can be concluded that the methanolic flower extract of *Nerium oleander* L. has proven itself as a significant hepatoprotective as well as a considerable antioxidant.

The present study clearly reflected the 'in vitro' effectiveness of the extract in 'in vivo' in terms of lipid peroxidation inhibitory capacity and further confirmed the significant hepatoprotective activity of the methanol extract of flowers of *Nerium oleander* L. along with its antioxidant mechanisms of action.

Conflict of interest statement

The authors declare no conflict of interest.

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