Available online at www.pharmacie-globale.info ISSN 0976-8157

Research Article

PHARMACIE GLOBALE

INTERNATIONAL JOURNAL OF COMPREHENSIVE PHARMACY

FREE RADICAL SCAVENGING AND HEPATOPROTECTIVE ACTIVITY OF *Aegle marmelos* (Linn.) CORR LEAVES AGAINST CARBON TETRACHLORIDE

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Received: 24 May 2011; Revised: 23 July 2011; Accepted: 30 July 2011; Available online: 7 August 2011

ABSTRACT

Aegle marmelos L. belongs to the family Rutaceae; an important medicinal plant being used in the folk therapy. It is the most useful medicinal plant of India. Its medicinal properties have been described in the ancient medical literature. All parts of the tree (stem, bark, root, leaves and fruit at all stages of maturity) have medicinal virtues and have been used as medicine for a long time. A number of chemical constituents have been isolated from various plant parts including alkaloids, coumarin and steroids. The leaves contain skimianinc, sterol and aegelin. In the present investigation antioxidant and hepatoprotective activity of the methanolic extract of *A. marmelos* leaves (MEAML) was examined on carbon tetrachloride (CCl_4) intoxicated rats. MEAML hold considerable amount of phenolic (9.8367±0.0235 mg/kg) and flavonoid (8.248 ± 0.029 mg/kg) contents, which confirmed the antioxidant property of the leaves. The MEAML with different doses (50, 100, 200 mg/kg body weight) and standard silymarin (40 mg/kg body weight) were orally administered to CCl_4 treated rats and the effect was studied on serum enzymes [(aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and bilurubin, protein, albumin, thiobarbutiric acid (TBARS), reduced glutathione (GSH)]. In addition, *in-vitro* antioxidant activity of MEAML was also evaluated using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical. We noticed a significant hepatoprotective activity of MEAML on CCl_4 intoxicated rats which support folkloric utilisation of *A. marmelos,* and it was further confirmed by the histological investigation. The findings of the present investigation revealed that the MEAML possess significant hepatoprotective activity by suppressing CCl_4 induced cellular oxidative stress.

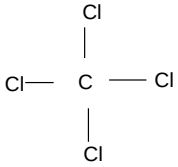
Keywords: *Aegle marmelos*, Antioxidant, Hepatoprotective activity.

INTRODUCTION

Carban tetrachloride (CCl_4), a well known toxicant, is metabolically activated by cytochrome P450 to form CCl_3 free radicals, which initiate lipid peroxidation in the cell. CCl_4 (Figure **1**) induces liver necrosis, and the Kupffer cells may possibly phagocytes the necrotic cell remnants. In addition, CCl_4 metabolites react with polyunsaturated fatty acids to propagate a chain reaction leading to lipid peroxidation / covalently bind with lipids and proteins; leading to the destruction of cell membrane and liver damage.[1,2] Hepatotoxicity or liver damage by CCl_4 can be measured by the analysis of several biochemical parameters including serum enzymes (ALT, AST, ALP), bilirubin and protein etc. The level of serum enzymes, bilirubin, TBARS increased in blood due to the administration of CCl_4 leading to cell membrane damage and necrosis. Serum enzymes are more specific to liver, and are a better marker for detecting liver injury.[3]

Carbon tetrachloride gives trichloromethyl radicals, which upon reacting with reactive oxygen species (ROS) yield trichloromethyl peroxide radicals and forms covalent bond with membrane lipids and disrupt the membrane integrity.[4,5] Antioxidants may guard against reactive oxygen species (ROS) toxicity by the prevention of ROS construction, disruption of ROS attack, scavenging reactive metabolites and converting them by enhancing the resistance of sensitive biological target to ROS attack.[6,7]

Figure 1. Chemical structure of carbon tetrachloride



About 20,000 deaths are found every year due to the liver disorders. More than 160 photochemical isolated from different plant species showed hepatoprotective activity.[8,9] Herbal drugs contain a variety of chemical constituents which showed potent liver protective activity.[10,11] Antioxidant activity might be due to the presence of phenolic compounds[12,13], which act as primary antioxidant or free radical scavengers[14]. In addition, several synthetic antioxidants like butylated hydroxy anisole (BHA),

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butylated hydroxy toluene (BHT), tertiary butylated hydroxy quinone and gallic acid esters have been reported to be carcinogenic.[15,16] Hence, strong limitations have been placed on their use and there is a trend to replace them with natural occurring antioxidants.[17]

A. marmelose (Linn.) belongs to the family Rutaceae, growing throughout the deciduous forest of India, and is commonly known as bael or koovalam in India. Its fruit and leaves are useful in indigenous medicine.[18,19] The plant has been employed for long time in folk therapy. The leaves are used in ophthalmic disorders, ulcers, lowering the blood glucose level[20,21], antifungal[22], antibacterial[23], antiprotozoal[24], antispermatogenic[25], cardiotonic and antioxidant [13], activities. The plant contained a number of phytochemicals[26-28,] had protective effect against oxidative stress for heart and brain[29,30], anti-inflammatory, histamine, allergic/inflammatory mediators, antioxidant, antitumor activity[31], and to prevent, prostatitis, cataracts, and respiratory diseases[32]. In this study, we have investigated and compared the antioxidant and hepatoprotective activity of different extracts from of the *A. marmelose* leaves to confirm its folk medicine claim.

MATERIALS AND METHODS

Plant material

Leaves of *A. marmelos* were collected from the Jamia Hamdard herbal garden (Hamdard University), New Delhi, India, and identified by Taxonomist (Department of Botany, Hamdard University). The voucher specimen (JHU/FP-1282) was deposited in Department of Pharmacognosy and Phytochemistry, faculty of Pharmacy, Hamdard University, New Delhi, India.

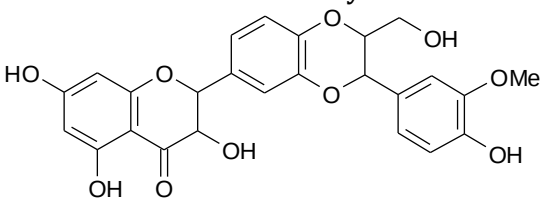
Preparation of extract

The leaves (100g) were shade dried, powdered and extracted in 200 ml of methanol using Soxhlet extractor for 4h at 65°C. The extract was filtered and evaporated to dryness under reduced pressure with rotary evaporator (Hahnshin Scientific Co, HS-2005 V-N, Korea) at controlled temperature.

Preparation of the test samples

The dried extract was suspended in 0.5% aqueous solution of carboxy methyl cellulose (CMC). It was orally administrated to animal at different doses (50, 100, 200 mg/kg body weight). Silymarin (Figure **2**) 40 mg/kg body wt. was used as reference drug.

Figure 2. Chemical structure of silymarin



Chemicals

The chemicals were purchased from different companies; silymarin (Sami Lab, Bangalore, India), carbon tetrachloride (Sigma Chemicals Co., St. Louis, USA), 1,1-diphenyl-2-picrylhydrazyl (E-Merck, Ltd., Mumbai, India), ascorbic acid (SD Fine Chem. Ltd.), thiobarbituric acid (Loba Chemie, Mumbai, India). All chemical used were of analytical grade.

Animals

Wistar-albino rats (150-200g) obtained from the animal house (Hamdard University) and were maintained as per the internationally accepted ethical guidelines. They were kept at standard environmental conditions (22 - 28°C, 60 - 70% relative humidity, 12 h light /dark cycle); fed with standard rat feed (Amrut Rat Feed, Nav Maharastra Chakan Oil Mills Ltd., Pune, India) and water *ad libitum*. Ethical clearance was obtained from institutional animal ethic committee (IAEC/CPCSEA-Project no 389/07).

Experimental protocol

Experimental protocol was designed according to the previous report.[3] In short, wistar albino rats were randomly divided into six groups (I, II, III, IV, V, and VI), and each group contained six animal. Animal of all the groups received concerned oral dose regularly up to five days. Group I (control) received CMC suspension (0.5%), Group II received CCl_4 plus CMC suspension (0.5%), Group III standard silymarin (40 mg/kg b.wt.) and group IV, V and VI received MEAML (50,100 and 200 mg/kg) respectively. Therefore, 50% CCl_4 in olive oil (1 ml/kg b.wt., s.c.) was administered on second and third day to all the treated groups**.** Animals were scarified on 6th day. Blood sample was collected, allowed to clot and serum was separated for biochemical assays. The liver was dissected out, stored at 20°C and finally used for biochemical and histopathological studies.

Estimation of total phenols

Total phenolic content was estimated by Folin Ciocalteu reagent.[4] A dilute leaf extract (0.5 ml of 1:10 g/ml) and gallic acid as a standard phenolic compound was mixed with 5 ml Folin Ciocalteu reagent (1:10 diluted with distilled water) and 4 ml of 1M Na_2CO_3. Total phenol content was measured by colorimetry at 765 nm. The standard curve was prepared using 25 to 300 mg/l solutions of gallic acid in methanol and water (50:50, v/v). The values are expressed in terms of gallic acid equivalent (mg/kg of dry mass).

Estimation of total flavonoids

Aluminium chloride colorimetric method was used for flavonoid estimation.[5] Leaf extract (0.5 ml of 1:10 g/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water, kept at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV visible spectrophotometer (Simadzu-1601, Tokyo, Japan). The calibration curve was prepared using quercetin solution at 12.5 to 100 g/ml concentrations in methanol. The values are expressed in term of quercetin equivalent (mg/kg of dry mass).

Free radical scavenging activity

It was carried out according to the method of Blois M.[33] The stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical was used for the estimation of free radical scavenging activity of MEAML. In short, 0.5 ml of different concentrations (25-35 µg/ml) of MEAML and standards (butylated hydroxytoluene, ascorbic acid and rutin) was mixed with 2.5 ml of methanol containing 75 µM DPPH radical. IC_{50} values denote the concentration of substances required scavenging 50% of DPPH free radicals and it was comparable with the used standard. The reaction mixture was kept in dark at room temperature for 90 min. Absorbance was recorded at 517 nm.

Assay of serum enzymes, bilirubin, albumin and protein

The estimation of serum enzymes, bilirubin, albumin and protein was carried out by the previous methods. The ALT, AST by Reitman and Frankel[34]; ALP and bilirubin by

Malloy and Evelyn[35]; protein[36], and albumin[37], were determined accordingly.

Estimation of Thiobarbutiric acid reactive substances (TBARS)

The thiobarbutiric acid was measured by the previous method.[38] The values of TBARS are expressed as "moles of malondialdehyde per mg of protein.

Estimation of reduced glutathione (GSH)

Glutathione was measured according to the previous method.[39] In brief; equal quantity of homogenate (w/v) and 10% trichloroacetic acid was mixed and centrifuged to separate the protein. 0.1 ml of the supernatant, 2 ml of phosphate buffer (pH 7.4), 0.5 ml of 5, 5-di-thiobisnitro benzoic acid (DTNB) and 0.4 ml of distilled water was added. The mixture was vortex and the absorbance was read at 412 nm. The concentration of reduced glutathione was recorded in terms of µmol/g of liver tissue.

Histological investigation

Liver tissues were fixed for 48 hours in 10% formalin solution and were processed for paraffin embedding following the standard micro technique.[40] Liver sections (5 µm in thickness) was stained with hematoxylin and eosin was observed microscopically for histopathological studies. The histopathological changes and microphotographs were taken using an Olympus BX50 microscope system (Olympus, Japan).

Statistical analysis

The statistic difference between the toxic and control was tested by one-way analysis of variance (ANOVA). Values are means of five replicates from two experiment and the presented mean values were separated using Duncan's multiple range test (DMRT) at $p < 0.001$ and $p < 0.05$.

RESULTS AND DISCUSSION

Estimation of total phenols and flavanoid: The total phenolic and flavanoid contents isolated from MEAML were (9.83±0.02 mg/kg) and (8.24± 0.02 mg/kg) respectively. Table **1** showing a comparative account of the total phenolic and flavanoid content isolated from MEAML.

Table 1. Total phenolic and flavonoid contents isolated from leaf extract of *A. marmelos*.

Sample	Total phenolic contents (mg/kg)	Total flavonoid contents (mg/kg)
Methanolic extract of leaf	9.8367 ± 0.023	8.248 ± 0.029

Values are mean of five replicates. Each value in the table was obtained by calculating the average of two experiments.

Free radical scavenging activity

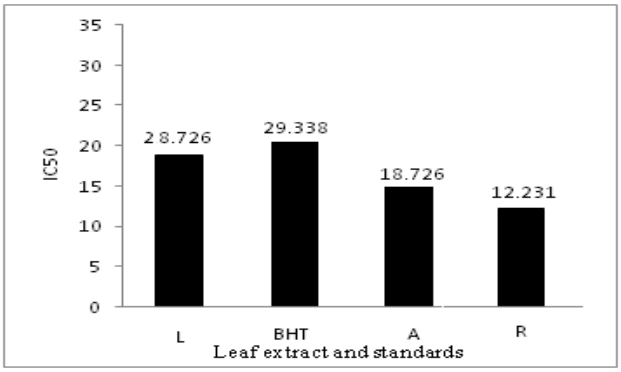
MEAML analyzed by the DPPH method showed potential antioxidant activity. Table **2** showing a comparison in percentage inhibition of MEAML with the tested standards. Maximum inhibition percentage was exhibited by rutin followed by BHT and ascorbic acid. No significant difference was noticed between the MEAML and BHT. In addition, Figure **3** Showed IC_{50} (µgml^{-1}) values of MEAML and standards for free radical scavenging activity by DPPH. Amongst the tested MEAML and standards (BHT, ascorbic acid, rutin), the lower IC_{50} value was exhibited by rutin which confirmed its higher antioxidant activity.

Table 2. Comparison of DPPH free radical inhibitory concentration of the leaf extract and those of BHT, ascorbic acid and rutin.

Plant extract & standard	Concentration (µg/ml)	% inhibition
Leaf	25	64.12 ± 0.01
BHT	35	65.09 ± 0.22
Ascorbic acid	35	52.163 ± 0.20
Rutin	35	72.686 ± 0.56

Each value in the table was obtained by calculating the average of five replicate with two experiments.

Figure 3. IC_{50} (µgml^{-1}) values of leaf extract for free radical scavenging activity by DPPH radical.



Lower IC_{50} value indicates higher antioxidant activity. L =Leaves, BHT = Butylated hydroxy toluene, A=Ascorbic acid and R=Rutin.

Assay of serum enzymes, bilirubin, albumin and protein

Effect of MEAML was investigated on the serum enzymes (AST, ALT and ALP) bilirubin, albumin and total protein in CCl_4 induced liver damage rats. The level of serum enzymes and bilirubin was found to be significantly increased; however, the protein and albumin content were decreased in CCl_4 induced liver damaged rats. The level of serum enzymes and bilirubin started to decrease from Group IV – VI respectively. The standard drug silymarin (40mg/kg b.wt.) decreased serum enzymes, bilirubin level and increased the level of protein and albumin content in CCl_4 treated group. Table **3**, showing a comparative account of serum enzymes, bilirubin, albumin and protein contents level in intoxicated and other groups.

Table 3. Effect of methanolic leaves extract of *A. marmelos* and silymarin on various biochemical parameters of the rats intoxicated with CCl_4.

Groups	AST (IU/ml)	ALT (IU/ml)	Alkaline Phosphatase (KAU/L)	Total Bilirubin (mg %)	Total Protein (gm/dl)	Total Albumin (gm/dl)
I	24.546±1.27	25.027±1.75	7.739±0.87	1.247±0.004	4.94±0.41	3.338±0.19
II	54.892±0.99†	56.555±1.26†	18.746±1.92†	2.853±0.007†	3.498±0.12†	2.612±0.22†
III	32.522±0.23**	37.375±1.50**	8.988±0.49**	1.44±0.008**	5.756±0.55**	4.468±0.14**
IV	46.366±1.74**	46.494±1.46**	13.061±0.45**	2.542±0.013**	4.301±0.25**	3.289±0.12**
V	38.059±0.37**	39.604±0.24**	11.828±0.22**	2.436±0.007**	5.282±0.08**	3.229±0.26**
VI	35.946±0.12**	36.489±067**	9.582±0.23**	2.024±0.017**	4.359±0.0**	3.463±0.11**

*Values are mean of five replicate with two experiments. **p<0.001, *p<0.05 vs. CCl_4, †p>0.001 Vs normal one way analysis (ANOVA) followed by DMRT test.*

Estimation of thiobarbutiric acid reactive substances

Effect of MEAM was studied on TBARS in rat liver homogenate (Table **4**). TBARS content in the liver homogenate was significantly increased in CCl_4 toxic group compared to the control (Group I). TBARS significantly decreased in IV, V and VI groups.

Estimation of reduced glutathione

A remarkable effect of MEAML was observed on GSH activity. A significant decrease in GSH level was noticed in CCl_4 induced liver damage rats when compared to those of other groups. Amongst the different doses of MEAML, 200mg/kg b.wt proved to be highly effective to restore the

GSH level (Table **5**).

Table 4. Effect of methanolic leaves extract of *A. marmelos* on tissue TBARS

Groups	Tissue TBARS (µmol MDA/mg protein)
I	0.785 ± 0.23
II	3.072 ± 0.32†
III	1.516 ± 0.16**
IV	2.567 ± 0.06**
V	1.931 ± 0.10**
VI	1.898 ± 0.05**

*Each value in the table was obtained by calculating the average of five replicate with two experiments. . **p<0.001, *p<0.05 vs. CCl_4, †p>0.001 vs normal one way analysis (ANOVA) followed by DMRT test.*

Table 5. Effect of methanolic leaves extract of *A. marmelos* on tissue GSH

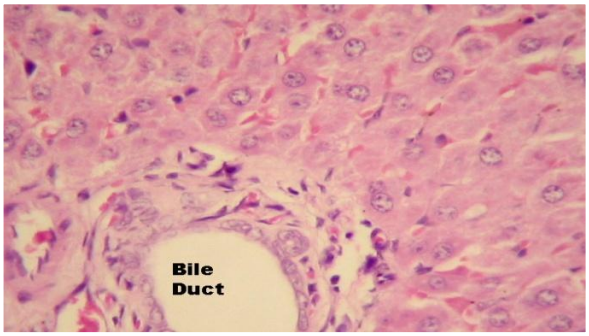
Groups	Tissue GSH (µmol/gm of tissue)
I	6.216 ± 0.257
II	3.116 ± 0.377†
III	6.516 ± 0.101**
IV	4.166 ± 0.135**
V	5.783 ± 0.249**
VI	6.083 ± 0.345**

*Each value in the table was obtained by calculating the average of five replicate with two experiments. **p<0.001, *p<0.05 vs. CCl_4, †p>0.001 Vs normal one way analysis (ANOVA) followed by DMRT test.*

Histological investigation

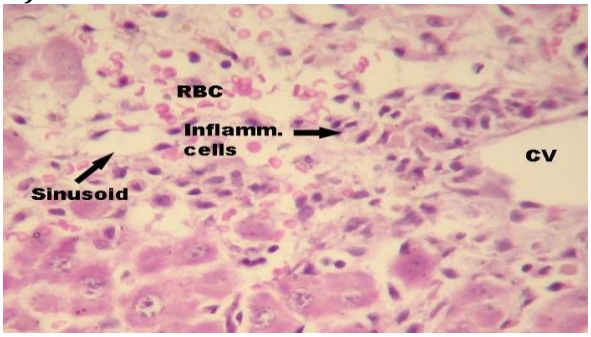
The liver sections of control group (I) showed normal liver architecture with well broad bile duct, prominent cytoplasm and nucleolus (Figure **4**).

Figure 4. High power photomicrograph of liver from control group animal showing normal hepatocytes in the periportal area. A large bile duct is also seen. (H&E x 400)



The liver sections of CCl_4 treated animals exhibited hepatic cell with severe toxicity characterized by inflammatory cell infiltration, scattered infiltration across liver parenchyma, focal necrosis and haemorrhage (Figure **5**).

Figure 5. High power photomicrograph of liver from animal treated with CCl_4 only showing loss of hepatocytes around the central vein (CV), inflammatory cell infiltration and haemorrhage (H&E x 400)



The standard drug silymarin completely restored the normal architecture of the liver at the respective dose of 40 mg/kg b.wt. (Figure **6**). Treatment with MEAML (50, 100, 200 mg/kg b.wt.) showed protective effect (Figure **7**, **8**, **9**) respectively. The MEAML at a dose of 200mg/kg b.wt. (Group VI) showed highly significant protective effect which is characterised by the presence of hepatic cell with well preserved cytoplasmic and normal nucleolus. It also caused significant decrease in inflammation.

Figure 6. High power photomicrograph of liver from animal treated with CCl_4 and Silymarine showing the centrizonal areas with presence of fat vacuoles in scattered hepatocytes. (H&E x 400)

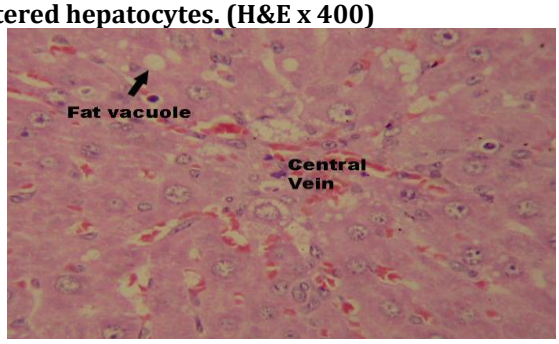


Figure 7. High power photomicrograph of liver from animal treated with CCl_4 and 50 mg/ Kg leaf extract showing sinusoidal dilatation and inflammatory cell infiltration (H&E x 400)

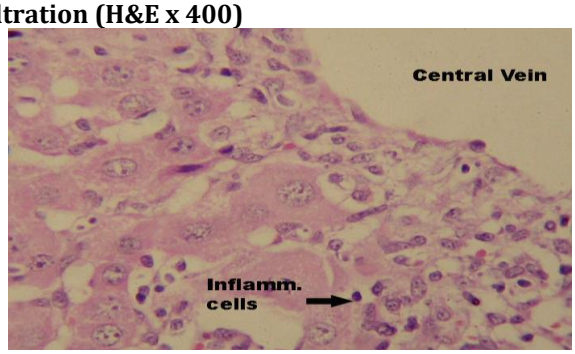


Figure 8. High power photomicrograph of liver from animal treated with CCl_4 and 100 mg/kg Leaf Extract showing centrizonal hepatocytes with sinusoidal dilatation and inflammatory cell infiltration. (H&E x 400)

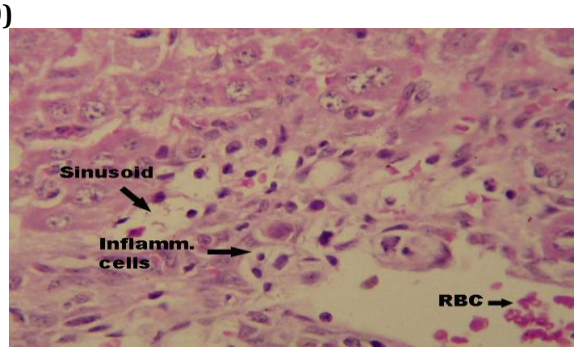
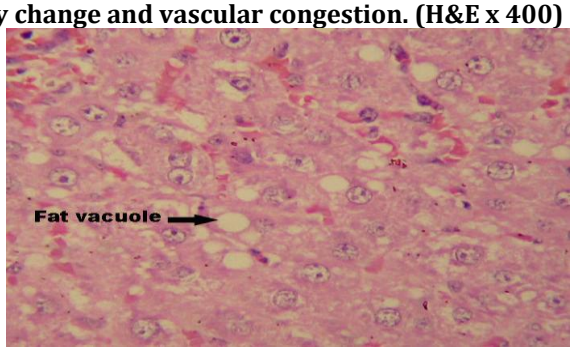


Figure 9. High power photomicrograph of liver from animal treated with CCl_4 and leaf extract 200mg/ kg bwt showing centrizonal hepatocytes with scattered fatty change and vascular congestion. (H&E x 400)



Liver is an important organ which involved in regulation of several physiological processes like metabolism, secretion and storage. Detoxification of varieties of drugs

occurs in the liver. Liver diseases mainly caused by the uptake of toxic chemicals, excess consumption of alcohol, infection and autoimmune disorder which damage liver cells by inducing lipid peroxidation and other oxidative damages.[41] Carbon tetrachloride (CCl_4) induced hepatotoxicity model is widely used for the study of hepatoprotective effect of drugs and plant extracts [42,43]. In the present investigation, antioxidant and hepatoprotective effects of MEAM was evaluated on carbon tetra chloride (CCl_4) intoxicated rats. The CCl_4 treatment elevated the level of serum enzymes and bilirubin which directly damaged to the liver. Treatment of the rats with different doses of MEAM showed a remarkable decrease in the level of AST, ALT, ALP and bilirubin, which might be due to the stabilisation of the cell membrane that prevent the release of serum enzymes in to the blood circulation. These results agreed with previous report.[41] Similarly, in our study a significant increase and decrease in the level of thiobarbituric acid reactive substances (TBARS) and gutathione (GSH) was observed in CCl_4 intoxicated rats's liver tissue. Treatment with MEAM caused significant decrease in the TBARS and increase level of GSH. The decreased level of endogenous antioxidant substance in liver tissue enhanced lipid per-oxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. It is well documented that antioxidants play a significant role in protecting living organisms from the toxic effects of chemical substances such as carbon tetrachloride and carcinogens.[34]

The Phenolic and flavanoid contents were estimated from the MEAML. The presence of phenolic and flavanoids confirmed the antioxidant activity[12,13], which act as primary antioxidant or free radical scavengers[14]. These antioxidant play a vital role in delaying, intercepting or preventing oxidative reaction, catalyse by free radical[25].

We have also observed a severe hepatotoxicity with inflammation, fatty changes and necrosis, haemorrhage, sinusoidal dilatation and vascular congestion in centerizonal area and periportal zone of the liver of CCl_4 intoxicated rats. Treatment with MEAML was found to reverse the CCl_4 induced histopathological changes[3,14,21,26,28].

CONCLUSION

The findings of the present investigation revealed that the MEAML possess significant hepatoprotective and antioxidant activity. Hepatoprotective activity of the test extract might be due to its antioxidant potential as MEAM exhibited DPPH free radical scavenging activity. Further, studies like isolation and characterization of the active constituents and evaluation of their pharmacological activity support the use of *A. marmelos*, in the treatment of liver disorders. The study suggested that *A. marmelos* suppress CCl_4 induced cellular oxidative stress and reversed the induction in hepatic disorder.

ACKNOWLEDGEMENT

The author (Nadeem Ahmad Siddique) highly grateful to the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard New Delhi for providing the necessary facilities to carried out the present research work.

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