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ANTIOXIDANT AND HEPATOPROTECTIVE POTENTIAL OF *MAMMEA SURIGA* AQUEOUS EXTRACT AGAINST CCL_4 INDUCED HEPATOTOXICITY IN RAT MODELS

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ABSTRACT

Mammea suriga is an endemic tree of the family Calophyllaceae, which has important traditional usage as medication. A detailed study was performed on the antioxidant activity of *M. suriga* flower bud aqueous extract by *in vitro* chemical analyses and carbon tetrachloride (CCl_4) induced hepatotoxicity in Albino rats. The extract was subjected to preliminary phytochemical screening for various constituents which revealed the presence of flavonoids, phenols and terpenoids. The total phenolic contents (79.97 µg/mg) and total flavonoid contents (113 µg/mg) of extract were found to be higher. 200 and 400 mg/kg doses of aqueous extract, CCl_4, water and ascorbic acid (100 mg/kg) were used as treatment groups. The blood samples were analyzed for levels of biochemical markers, tissue samples were subjected for estimation of liver antioxidant and histopathological studies. Analysis of the extract treated rats (400 mg/kg) showed an elevation of lipid peroxidation (0.68±0.01 nmol mg^{-1} of protein). Moreover, the biochemical parameters in serum like SGOT, SGPT, total cholesterol, total creatinine, total protein (TP) and urea were also improved in treated groups compared to the control. The oral administration of doses of *M. suriga* extract significantly protected the hepatic cells from damage. This study proved that *M. suriga* could be taken as a good natural source of the antioxidant agent.

KEYWORDS: *Mammea suriga*, antioxidant activity, hepatotoxicity.

1. INTRODUCTION

Oxidant-free radicals destruct the cells and cause many health issues. Longer the oxidants attacking the cells, the more chance that we can suffer from cancer, damaging our immune system, clogging our arteries, nervous system malfunction.[1] Antioxidant enzymes present in the body system normally neutralizes the free radicals.[2]

Many investigators explored the antioxidant potency of the phyto or microbial originated antioxidants.[3,4] Synthetic drugs in use have shown harmful side effects compared to natural antioxidants; therefore, there is a need for more effective, less toxic and cost effective antioxidants from natural sources. Several medicinal plants with ethnobotanical uses have been used traditionally in the treatment of diseases and have been exploited for these desired traits. Consequently, there has been a growing interest to recognize natural antioxidants from plants.[5]

Mammea suriga Kosterm. is an aromatic tree of the Central Western Ghats of India, belongs to the family Calophyllaceae. Flowers are cauliflorous, highly fragrant and dry flowers maintain the fragrance for 3 to 4 months. The essential oil of the flowers known as punnaga oil is being used in perfumery industry and Silky red dye extracted from its dried flowers is used in textile industries. The flower buds have good medicinal value and are used against disorders of blood such as abscess, skin disorders, bleeding disorders such as menorrhagia, nasal bleeding hepatocyte diseases, cardiac problems and useful in Pitta imbalance disorders such as gastritis, burning sensation, etc. useful in psychiatric disorders. A special collyrium prepared using the seed oil is used in ophthalmic disorders.[6,7] In a previous study, we also reported the antimicrobial property of the flower bud oil.[8]

However, the various parts of the tree have been traditionally used in the treatment of many disorders, no any systematic documents to confirm its folklore uses. Besides, the plant has not yet undergone any chemical or pharmacological investigation except for the antimicrobial activity of root extracts, stem bark extract and flower bud extract, which have been reported earlier. Hence, to defend the traditional medicinal claims, the present study was performed to evaluate the antioxidant property and hepatoprotective property of the aqueous extract of flower buds of the plant using carbon tetrachloride (CCl_4) induced liver damage in rats.

2. MATERIALS AND METHODS

2.1. Collection and extraction of plant material

Flower buds of *M. suriga* were collected during the month of April from the evergreen forests of Sirsi, Uttara Kannada District, Karnataka. The plants were identified and authenticated by Dr. Y. L. Krishnamurthy, Professor, Post graduate studies and research in Botany, Kuvempu University and the voucher specimen was maintained at Kuvempu University herbaria (KUYLK4364). The flower bud aqueous extract (FBAE) was prepared using soxhlet apparatus and water as a solvent. The extract was concentrated over a water bath, dried at vacuum and desiccated until the usage.

2.2. *In-vitro* antioxidant activity

2.2.1. Total antioxidant capacity

Total antioxidant activity of FBAE was carried out by Phosphomolybdenum method (Prieto *et al*.). Extract of concentrations 50,100,150 and 200 µg was added into different test tubes, followed by addition of 3 ml of Molybdenum reagent mixture (4 mM Ammonium molybdate, 0.6 M sulfuric acid and 28 mM of Sodium phosphate). The reaction mixture was kept for incubation at 95˚c for 90 min then allowed to cool. Absorbance was measured at 695 nm against blank using ascorbic acid as standard.[9]

2.2.2. Total reductive capacity

Total reductive capacity was determined according to the method of Oyaizu *et al* and FBAE was tested at the concentrations of 50,100,150 and 200 µg followed by addition of phosphate buffer (2.5 ml, 0.2m. p H-6.5) and Potassium ferricyanide (2.5 ml,10%) into all tubes, kept for incubation at 50˚c for 20min. then Trichloro acetic acid (2.5 ml,10%) was added and centrifuged at 3000 rpm for 10min. 2.5ml of the supernatant was taken then 2.5 ml of milli pore water and ferric chloride (0.5 ml,0.1%) was added. The absorbance of the reaction mixture was measured at 700 nm against a blank, using quercetin as standard.[9]

2.2.3. DPPH assay

DPPH radical scavenging activity of FBAE was conducted as per the method of Braca *et al*. 3 ml of 0.004% DPPH in 95% ethanol was added to 1ml of each of 100 µg, 200 µg and 300 µg FBAE and kept for incubation in dark place at room temperature for 30 min. The absorbance of the reaction mixture was measured at 517 nm against Ascorbic acid (Vitamin-C) used as a standard. The DPPH radical scavenging activity (%) is calculated and expressed as I=[(Ao-Ax)/Ao] X 100%.[9]

2.2.4. Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of FBAE was evaluated following the method of Marcocci *et al*. 250 µl of FBAE extract of different concentration (100, 200, 300 µg) was added into different test tubes along with 2 ml of Sodium nitroprusside (10 mM in 0.5 M phosphate buffer, PH 7.4). The reaction mixture was kept for incubation at 25˚ c for 150 min .0.5ml of the reaction mixture was added to test tubes containing 1ml of Sulfanilamide (1% in 5% phosphoric acid) and incubated at 25˚c for 5 min, then added 1ml of 0.1% of α-napthyl to the test tubes. The reaction mixture was incubated at 25˚c for 30min and absorbance was measured at 546 nm using ascorbic acid as standard. Scavenging activity (%) is calculated and expressed as I=[(Ao-Ax)/Ao] X 100%.[10]

2.2.5. Metal chelating activity

Metal chelating activity of FBAE was conducted by the method of Dinis et al. 1ml of std EDTA with concentrations of 100, 200 and 300 µg and 1ml of 100, 200,3 00 µg of each FBAE extract were added into different test tubes followed by the addition of $FeCl_2$ (50 µl of 2 mM) and the reaction was initiated by addition of Ferrozine (0.2 ml, 5 mM) solution. The reaction mixture was kept for 10 min at room temperature and the absorbance was measured at 562 nm. Metal ion chelating activity scavenging effect =[(Ao-Ax)/Ao] X 100%.[11]

2.3. *In vivo* antioxidant study

Adult male albino Wistar rats (180 ± 20 g) were obtained from the College of Pharmacy, Shimoga. They were kept under a temperature of (23 ± 2) ˚C, humidity of 50% and light and dark cycles of 12 h:12 h. They were fed with commercial pellet diet (Krish Scientific Shop) and water was provided ad Libitum. The protocol was approved by Institutional Animal Ethics Committee and the lab was approved by CPCSEA, Government of India (NCP/IAEC/CL/213/01/2012-13).

2.3.1. Acute toxicity Study

Acute toxicity of FBAE was determined using albino rats as per the OECD guidelines. By using water as a vehicle the extracts were administered orally. The animals were observed individually for changes in their behavioral pattern and mortality. The LD_{50} of the FBAE was found to be 2000 mg/kg. As per the toxicology guidelines, $1/10^{th}$ of the dosage (200 mg/kg) and $1/5^{th}$ of the dosage (400 mg/kg) was administrated to experimental rat models.[12]

2.3.2. *In-vivo* hepatoprotective activity

The *in-vivo* hepatoprotective activity of *M.suriga* aqueous extract was determined by employing carbon tetra chloride induced hepatotoxic rat models. Rats were divided into 5 groups comprising 6 rats each. FBAE was dissolved in sterile distilled water, two different concentrations of FBAE was administrated orally. Group 2 to 5 received CCl_4 on 1st and 7th day.

Group 1: Normal control, fed with distilled water normally every day

Group 2: Negative control, received 0.5 ml/kg of CCl_4 (1:1 ratio, CCl_4: Olive oil)

Group 3: Standard control, received 100 mg/kg of Ascorbic acid for 7 days

Group 4: Test, received 200 mg/kg of FBAE for 7 days

Group 5: Test, received 400 mg/kg of FBAE for 7 days

All the rats were sacrificed by cervical decapitation; blood samples were collected and allowed to clot for 30 min at room temperature. The clear serum was separated by centrifugation at 4000 rpm for 10 min and serum samples were stored at -40°C until use for the determination of biochemical parameters. 1 gm of hepatic liver tissues were carefully excised, cleaned and homogenized in cold 1.15% KCl and10 Mm phosphate buffer with EDTA (pH 7.4) and centrifuged at 10,000 rpm for 10 min. the supernatant of the liver homogenate was collected and subjected to enzyme assays. Remaining hepatic liver samples were stored for the histopathological studies.[13]

2.3.3. Assessment of liver function serum markers

The functional state of the liver was determined by estimating the levels of biochemical markers such as Serum Glutamic Oxalo acetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT), Total protein, Creatinine, Urea, Cholesterol by using auto bioanalyzer.[12]

2.3.4. Assessment of Lipid Peroxidation activity

LPO was assayed as stated by Okhawa, the tissues are homogenized in 0.1 M buffer pH 7.4 with a Teflon-glass homogenizer. LPO in the homogenate is determined by measuring the amount of Malon di aldehyde (MDA) produced primarily. Tissue homogenate (0.2 ml), 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid and1.5 ml of 8% TBA are added. The volume of the mixture made up to 4 ml with distilled water and heated in water bath at 95^0 C for 60 min. After incubation, the tubes were cooled to room temperature and the volume was made up to 5 ml. 5 ml of butanol: pyridine (15:1) mixture is added,

vortexed thoroughly and centrifuged at 3000 rpm for 10 min. The upper organic layer is taken and OD is measured at 532 nm against an appropriate blank. The levels of lipid peroxide are expressed as n moles of TBARS/mg protein using an extinction coefficient of 1.56 x 10^5 ML cm^{-1}.[11]

2.4. Histopathological studies

The liver tissue was dissected out from the animals of each group after draining the blood and washed with the normal saline and fixed in 10% formalin, dehydrated in gradual ethanol grades (50–100%), cleared in xylene and embedded in paraffin. Sections of 5 µm thickness were prepared, processed in alcohol-xylene series and were stained with alum-haematoxylin and eosin (H–E) dye for photomicroscopic observation for the evaluation of histological changes, including cell necrosis, fatty change, tissue architecture. Fig. 2 shows photomicrographs of hematoxylin–eosin-stained liver tissues.[14]

2.5. Statistical analysis

The result was statistically analyzed using one-way ANNOVA using a software ezANNOVA followed by Tukey's *t*-test. The difference in values at $P \leq 0.01$ was considered as statistically significant.

3. RESULTS

3.1. Extraction yield and preliminary phytochemical analysis

The extraction of flower Bud yields 1.9% (dark brown solid). The results of the qualitative analysis presented in Table: I showed the presence of Flavonoids, Phenolic compounds, Terpenoids and Saponins. The quantitative analysis was presented in Table: II and the result revealed the presence of phenolic and flavonoids compounds of each at the concentration of 74.97µg/1mg and 113µg/1mg respectively.

Table I: Qualitative phytochemical analysis of extracts of *M. suriga*

Test	FBAE
Alkaloids	-
Flavonoids	+
Phenolic compounds	+
Terpenoids	+
Glycosides	-
Tannins	-

FBAE-Flower Bud Aqueous Extract, (+) indicate the presence and (–) the absence of the phytochemicals.

Table II: Quantitative phytochemical analysis of extracts of *Mammia suriga*

Tests	FBAE
Phenolic	74.97µg/mg
Flavonoids	113µg/mg

FBAE-Flower Bud Aqueous Extract.

3.2. *In-vitro* Antioxidant activity

The total antioxidant activity and total reductive capacity of the *M. suriga* aqueous extract were determined and shown in Fig. 1 A and Fig. 1 B respectively.

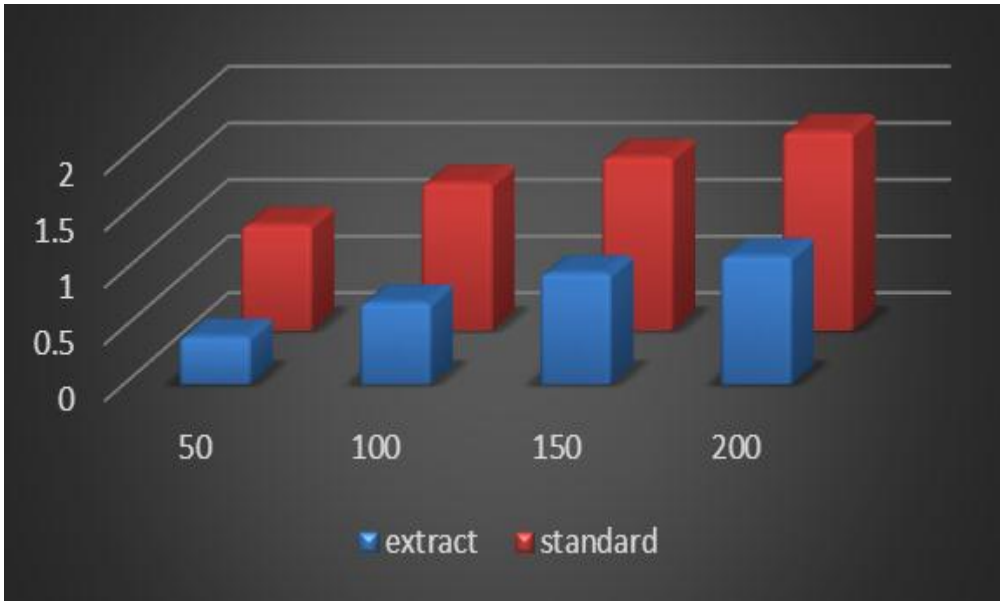

Fig. 1 A: Total antioxidant capacity

x-axis showing concentration, y-axis showing absorbance

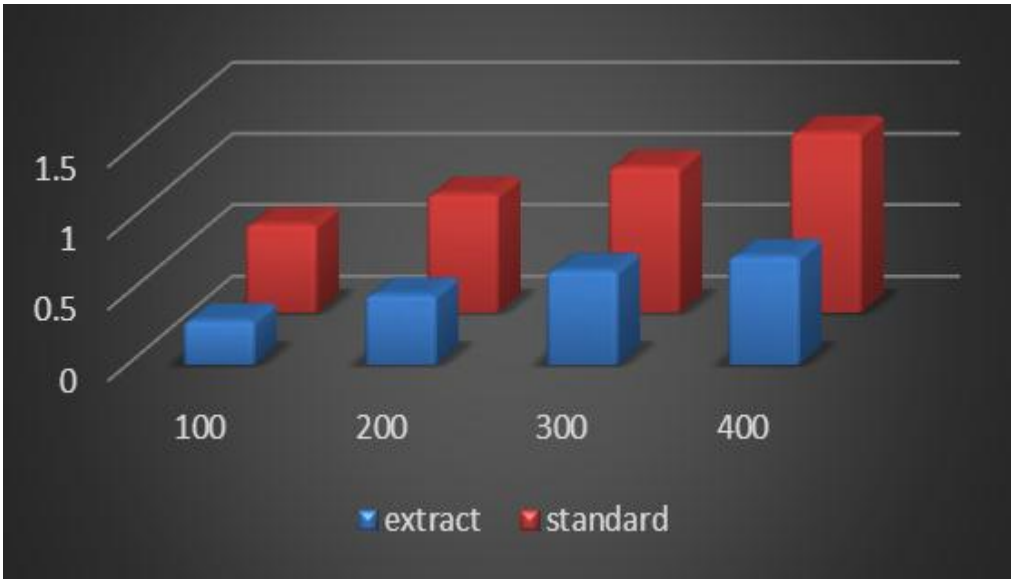

Fig. 1 B: Total reductive capacity

x-axis showing concentration, y-axis showing absorbance

Figure 1 A and 1 B shows the dose response increase in the reducing power of extract at all concentration 50 to 400 µg/ml. It was found that the reducing power increased with the concentration of the sample. Reducing power at 100 µg/ml was evident in *M. suriga* extract.

3.2.1. Free radical scavenging activity

The free radical scavenging property of *M. suriga* aqueous extract against DPPH, Nitric oxide and metal chelating radicals is shown in Table III. The extract and ascorbic acid standard showed antioxidant activity in a dose-dependent manner in the range of 68.15±0.21 to 85.09±0.20% respectively. The IC_{50} values for extract and ascorbic acid were 129.02 and 114.93 mg/mL, respectively. In Nitric oxide radical scavenging activity dose-dependent range was 48.71±0.29 to 66.64±0.39% and the IC_{50} values were 144.45 and 172 mg/ml, respectively. In the metal chelating activity, antioxidant activity expressed was 45.58±0.27 to 61.96±0.27% and the IC_{50} values were 142.95 and 180.96 mg/mL respectively.

Table III: *In vitro* antioxidant assays

Sl. No.	In vitro antioxidant assays	Sample (FBAE)	
		Extract Concentration (µg/ml)	% of activity
1	DPPH assay	1)100 2)200 3)300	68.15±0.21 77.34±1.29 85.09±0.20
2	Nitric oxide scavenging assay	1)100 2)200 3)300	48.71±0.29 58.84±0.39 66.64±0.39
3	Metal chelating activity	1)100 2)200 3)300	45.58±0.27 58.09±0.46 61.96±0.27

Values are the mean ± S.E.M.

3.3. *In vivo* hepatoprotective activity

3.3.1. Assessment of liver function by the level of biochemical markers

The results presented in Table IV indicated the level of biochemical markers namely SGOT, SGPT, total protein, creatinine, cholesterol and urea.

Table IV:

Groups	SGOT (U/L)	SGPT (A/L)	Total Protein (g/dL)	Creatinine (mg/dL)	Cholesterol (mg/dL)	Urea (mg/dL)
Normal Control	55.38±0.37	57.74±0.6	5.89±0.08	2.89±0.1	20.16±0.03	59.32±0.13

Negative Control	313.04±0.2	355.37±3.43	6.32±0.09	3.39±0.16	23.54±0.23	42.36±0.1**
Standard Control	66.3±0.48**	77.7±0.64**	5.7±0.05*	2.55±0.13*	20.61±0.11*	58.08±0.03**
Test Group (200 mg/kg)	192.01±0.96**	163.36±0.96**	**5.72±0.06***	1.73±0.05*	**21.83±0.12***	**60.38±0.2****
Test Group (400 mg/kg)	**92.01±0.17****	**81.68±0.5****	5.69±0.13*	**1.87±0.02***	27.28±0.08**	54.11±0.07**

Values are the mean ± S.E.M. of six rats. Symbols represent statistical significance. *P < 0.0005, ** P < 0.0001, as compared to corresponding control group.

The levels of liver enzymes showed significant results when compared to standard control groups, SGOT; 92.01±0.17 U/L at 400 mg/kg, SGPT; 81.68±0.5 A/L at 400 mg/kg, Total protein; 5.69±0.13 g/dL at 200 mg/kg, Creatinine; 1.87±0.02 mg/dL at 400 mg/kg, Cholesterol; 21.83±0.12 mg/dL at 200 mg/kg, Urea; 60.38±0.2 mg/dL at 200 mg/kg. Reduction in the toxic effect was found in extract treated rats when compared to control groups.

3.3.2. Enzyme assay in liver

Lipid Peroxidation (LPO) Assay

Lipid Peroxidation is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. Malondialdehyde (MDA) is one of the end product in the lipid peroxidation process. Malonaldehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals, which is accepted as an indicator of lipid peroxidation.

The results of Lipid Peroxidation assay were presented in Table V.

Table V: LPO Assay

Groups	LPO Assay
Normal Control	0.62±0.01
Negative Control	1.28±0.01
Standard Control	0.66±0.01
Test Group (200 mg/kg)	0.76±0.01
Test Group (400 mg/kg)	0.68±0.01

The lipid peroxidase levels have been significantly decreased in the animal groups treated with the different doses of aqueous extract (Table 5). Whereas, CCl_4 treated group showed an increase in the level of antioxidant enzymes when compared to the control group. However, among the treated groups, animals treated with 400 mg/kg of aqueous extract showed maximum protection than 200 mg/kg treated animals.

3.3.3 Histopathological studies

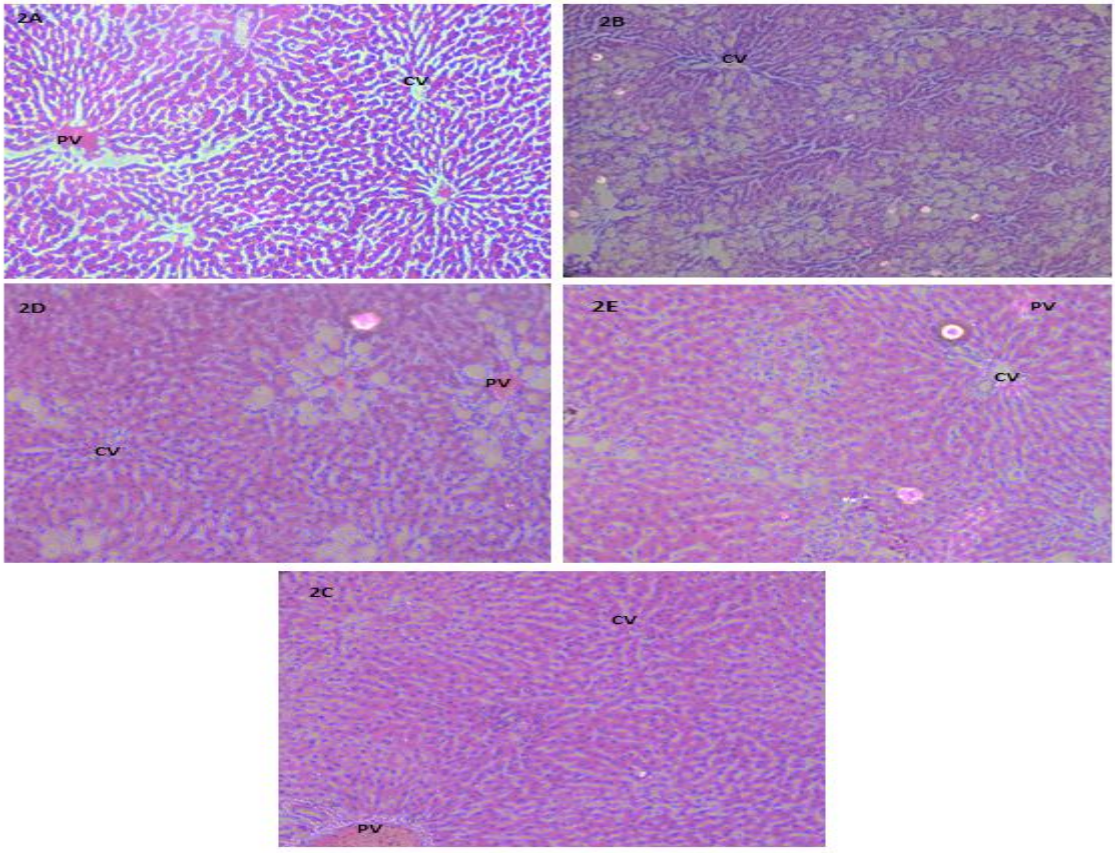

Fig: 2A: Normal healthy rats, showing normal arrangements of the hepatocytes with well-preserved cytoplasm and central vein and portal vein. 2B: Hepatocytic necrosis and evident vacuolation of hepatocytes in CCl_4 treated rats. 2C: Liver sections of animals treated with CCl_4 and standard drug showing mild sinusoidal dilatation in the centrizonal area. 2D: Animal treated with CCl_4 and 200 mg of extract showing very mild sinusoidal dilatation in the centrizonal area, decrees in fatty lobules and also the normal arrangement of hepatocytes with well-preserved cytoplasm. 2E: Animal treated with CCl_4 and 400 mg extract showing normal architecture in the centrizonal area and also the normal arrangement of hepatocytes with well-preserved cytoplasm.

In the case of control, hepatocytes had normal architecture. Severe hepatocyte necrosis, fatty degeneration, vacuolation were found in rats 24 h after CCl_4 treatment Fig 2B. Pretreatment of FBAE at 200 and 400 mg/kg body weight reduced the severity of CCl_4 induced liver intoxication Fig 2D & E. These results clearly indicate the protection provided by FBAE when compared with control groups.

4. DISCUSSION

Reactive oxygen species (ROS) and free radicals, in general, are involved in cell signaling and other vital biological functions; but, an excessive amount can cause variation in cellular reduction-oxidation (redox) balance, and disrupt normal biological functions. When there is an imbalance between activities of ROS and antioxidant/scavenging defense systems, oxidative stress (OS) occurs. A good number of studies have shown OS is involved in the development of several disease conditions, including male infertility.[15] The results of our study revealed that *M.suriga* aqueous extract had an effective capacity of scavenging for free radical and associated with total flavonoid content, hence suggesting its antioxidant potential.

The preliminary phytochemical analysis of the plant extract showed the presence of various phytocomponents[8] and also revealed the presence of a rich quantity of flavonoids in flower bud aqueous extract of *M. suriga* (113µg/mg).

The antioxidant capacity of the extract was measured spectrophotometrically through phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption.[13] The present study proved that extract showed the highest antioxidant capacity for phosphomolybdate reduction. Various studies like R Bharathi *et al*., Naima Saeed *et al.*, and Sutha Devaraj *et al.* have shown that many flavonoid and related polyphenols contribute significantly to the scavenging activity of medicinal plants.[16,17]

Reducing power assay showed that, the yellow color of the test solution turns to green depending on the reducing power of the test sample. The presence of the reductants in the solution causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, Fe2+ can be monitored by absorbance measurement at 700 nm. Previous reports suggested that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain.[13] Increase in absorbance indicates an increase in reducing property. The antioxidants present in the aqueous extract of *M. suriga* flower bud caused their reduction of Fe3+ / ferricyanide complex to the ferrous form and thus proved the reducing power.

In the present study, *M.suriga* showed effective scavenging activities for DPPH, Nitric oxides, and metal ions in dosage dependent manner, suggesting that it could scavenge the free radicals generated. This finding is consistent with earlier studies that the antioxidant activity

of *M.suriga* extract comes from rich sources of bioactive components, such as flavanoids, which quench ROS and regenerate membrane-bound antioxidants.

The liver has to detoxify various toxic constituents hence liver failure is very common. There are much more chemicals which are known to induce hepatotoxicity by producing the reactive oxygen species which form covalent bonds with the lipids of the tissue.[18,19] Liver injury due to CCl_4 in rats was first stated in 1936[20] and has been widely used for study.[21,22] Carbon tetrachloride is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of CCl_3O^-, a reactive oxidative free radical, which initiates lipid peroxidation.[23,24]

Most toxic chemicals damage liver by inducing, directly or indirectly, lipid peroxidation. MDA is one of the end products in the lipid peroxidation process.[25] In our *in vivo* study, elevation in levels of the end product of lipid peroxidation in liver of rats treated with CCl_4 were observed. The increased MDA levels in liver suggest higher lipid peroxidation leading to tissue injury. Treatment with *M. suriga* extract significantly upturned these changes. Hence it may be possible that the mechanism of hepatoprotection of the extract is due to its antioxidant effect.[26]

Histopathological studies revealed that among all the treated groups, animals treated with *M. suriga* extract showed significant liver protection against CCL_4 induced hepatotoxicity as evident by the normal hepatic tissue architecture, absence of fatty infiltration, less number of necrosis, presence of normal hepatic cords and well preserved cytoplasm, which was almost comparable to the ascorbic acid treated groups.

5. CONCLUSION

The replacement of synthetic with natural antioxidants may be beneficial for human health. In the present study, analysis of *in vitro* antioxidant property and preliminary phytochemical screening showed that extract of *M. suriga* can be the potent source of natural antioxidants. The results of *in vivo* studies suggest that aqueous extract of *M. suriga* may be useful in defense against CCl_4 induced liver damage, possibly due to its antioxidant properties.

6. ACKNOWLEDGEMENT

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