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Research Article

HEPATOPROTECTIVE EFFECT OF WHOLE PLANT EXTRACT FRACTIONS OF *MARSILEA MINUTA* Linn

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ABSTRACT

Objective: The objective of the study is to separate and identify the most effective hapatoprotective fraction of methanolic extract of *Marsilea minuta* (MMME) by fractionating and evaluating its fractions for hapatoprotective activity in three mechanistically devised models viz., CCl_4, paracetamol and ethanol induced liver damage in rats. **Methods**: Acute toxicity study was carried out to the fractions according to the organization for economic corporation development (OECD)-420 guidelines. Liver damage was induced in different groups of rats by administering 1:1v/v CCl_4 in olive oil 1ml/kg.b.w.p.o, 3g/kg.b.w.p.o paracetamol and 5g/kg.b.w.p.o ethanol and the effect of fractions were tested for hepatoprotective potential by evaluating serum biochemical parameters, histology of liver of rats and the most effective bioactive fraction was screened for its effect on hepatic microsomal drug metabolizing enzymes (MDMA) and prothrombin time (PT). It was also tested for its antioxidant properties by DPPH method, lipid peroxidation method and for detection of different classes of chemicals present in it. **Results**: Pretreatment with fractions (toluene, 1-butanol, aqueous at 50, 100mg/kg.b.w) significantly reversed the changes in serum biochemical parameters and histology of liver caused by the three hepatotoxins namely CCl_4, paracetamol and ethanol indicating their hepatoprotective activity. The hepatoprotective activity of butanol fraction of MMME (BF-MMME) was well supported in MDMA, PT and DPPH studies. **Conclusion**: All the fractions of MMME exhibited significant hepatoprotective activity and BF-MMME (50mg/kg) was identified as the most effective fraction.

Keywords: *Marsilea minuta*, Hepatoprotective activity, CCl_4, Paracetamol, Ethanol, Silibinin, DPPH.

INTRODUCTION

Hepatic disease is a term for collection of conditions, diseases and infections that affect the cells, tissues, structures or functions of the liver. About 20000 deaths were found every year due to liver disorders [1]. Hepatic damage is a global metabolic and epidemic disease affecting essential biochemical activities in almost every age group [2]. Excess consumption of certain drugs like antibiotics, chemotherapeutic agents, acetaminophen, and exposure to some chemicals such as peroxidised oils, aflatoxin, CCl_4, alcohol etc make liver vulnerable to variety of disorders viz., jaundice, hepatitis etc which are the two major hepatic disorders that account for high death rate [3]. Treatment for these disorders is done by using drugs from different sources including traditional herbal medicines. These traditional herbal medicines are believed to be better than other in treating liver disorders without any scientific proof. Therefore it is necessary to explore and develop such herbal medicines scientifically.

Marsilea minuta is found throughout Africa, Madagascar and Comoros. In India it usually grows as a weed in wet rice fields and flooded low lands. Traditionally *Marsilea minuta* is used in treatment of diarrhea, bronchitis, epilepsy, hepatitis, kidney infection [4], blood purifier, in treatment of piles. *Marsilea minuta* was reported to contain steroidal/triterpenoid sapogenols, phenolic compounds, also flavonoids viz., quercetin-3-O-glucoside, kaempferol-3-O-glucoside, quercetin-3-O-galactoside, chalcone-3-O-glucoside [5], quercetin-3-rutinoside, and naringenin-7-O-glucoside [6] etc. the extracts and isolates of *Marsilea minuta* were reported to possess antibacterial activity[7], anti-inflammatory and analgesic activities [4], hypocholesterolemic activity[6], antianxiety activity [8] etc.

The preliminary investigation on MMME carried out in our labs revealed that it possesses significant hepatoprotective effect. The present investigation was aimed to screen the fractions of MMME for hepatoprotective activity in CCl_4, paracetamol and ethanol induced hepatotoxicity in rats and to identify the bioactive fraction.

MATERIALS AND METHODS

Drugs and Chemicals

Paracetamol was obtained as gift sample from Natco pharma Limited, Hyderabad, India. All other drugs and chemicals were purchased from various companies and the details are as follows: Silibinin- Sigma Aldrich, Spruce Street, St. Louis, China; Thiopentone sodium (Thiosol)- Neon Labs, Mumbai, India; Olive oil- Seven ships, Hyderabad, India; The biochemical analytical kits [Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Total Bilirubin (TBL), Direct Bilirubin (DBL), Triglycerides (TGL), Cholesterol (CHOL), Glucose (GLU), Creatinine (CRT), Albumin (ALB), and Total Protein (TP)] and Trichloro acetic acid- Merck Specialities Private Limited, Mumbai, India; 1,1 Diphenyl-1-picryl hydrazyl (DPPH), Thiobarbituric acid (TBA)- Himedia, Mumbai, India; 1,1,3,3- tetra ethoxy propane (TEP)- Sigma, Germany; Ethanol- Changshu yangyuan chemicals, China. All other chemicals and solvents used were of analytical grade.

Collection and preparation of extracts

The plant *Marsilea minuta* Linn was collected in the month of December 2010, from rice fields of Warangal, Andhra Pradesh, India, after the authentication of the plant by Prof. V.S. Raju, Department of Botany, Kakatiya University, Warangal. A voucher specimen of the plant (KU/UCPSC/45) is being maintained in the herbarium of Department of Pharmacognosy and Phytochemistry, University College of Pharmaceutical Sciences, Kakatiya University, Warangal.

The air dried whole plant material was coarsely powdered and macerated with methanol in a round bottom flask for 7days with intermittent stirring and filtered after seven days and concentrated under reduced pressure to yield a green semisolid mass. It was given a code MMME. The obtained MMME was suspended in water and fractionated with toluene and n-butyl

alcohol in succession. The obtained fractions were concentrated under reduced pressure to yield corresponding extracts. They were given the codes, as TF-MMME (Toluene fraction), BF-MMME (n-butyl alcohol fraction) and AF-MMME (Aqueous fraction- the residue left in the water after fractionation process).

Maintenance of animals

Wistar albino rats weighing 150-200 g were purchased from Mahaveera agencies, Hyderabad, India with a prior permission from our institutional animal ethical committee *(CPCSEA Reg.No. 146/2009)* and used for the studies. The animals were housed in standard polypropylene cages, and maintained under standard laboratory conditions (12:12 hour light and dark cycle; at an ambient temperature of 25 ± 5^{0}C ; 35- 60% of relative humidity). The animals were fed with standard rat pellet diet and water *ad libutum*.

Acute toxicity study

Acute toxicity study was carried out for the fractions, TF-MMME, BF-MMME and AF-MMME according to the Organization for economic co-operation and development (OECD) 420 guidelines (OECD, 2001). Wister albino rats were divided into groups comprising six animals in each group. The fractions were administered to different groups in doses ranging from 100-2000 mg/kg b.w.p.o. They were observed for signs of toxicity and mortality for 72 hrs.

Assessment of hepatoprotective activity

Carbontetrachloride induced hepatotoxicity

The screening of the fractions of MMME for hepatoprotective activity was done according to the procedure given in the literature [9] with minor modifications. The rats were divided into nine groups of six animals each. Group I (Control group), Group II (Toxic group) were treated with vehicle, (1 ml/kg b.w of 5% gum acacia in water) daily for seven days followed by toxic group treated with CCl_4 on the seventh day. Group III, IV, V, VI, VII, VIII, IX were treated with Silibinin (50 mg/kg b.w), TF-MMME (50 mg/kg.b.w), TF-MMME (100 mg/kg.b.w), BF-MMME (50 mg/kg.b.w), BF-MMME (100 mg/kg.b.w), AF-MMME (50 mg/kg.b.w), AF-MMME (100 mg/kg.b.w) daily for seven days followed by CCl_4 on the seventh day respectively. The blood was collected from the retro orbital plexus of the rats of all groups 24 h after administration of CCl_4 under ether anesthesia. The blood samples were allowed to stand for 30 min at room temperature and then centrifuged (Remi, model: RM-12C, India) at 3000 rpm for 15 min to separate the serum. The serum was analyzed for various biochemical parameters such as AST, ALT, ALP, TBL, DBL, CRT, GLU, TGL, CHOL, TP and ALB. The body weight (before and after treatment), liver weight and prothrombin time were recorded. The animals were then dissected and the livers were carefully removed and washed with 0.9% saline solution. A part of the liver sample was preserved in formalin solution (10% formaldehyde) for histopathological studies.

Effect of BF-MMME on barbiturate sleeping time in CCl_4 induced hepatotoxicity in rats

The effect of fractions of BF-MMME on thiopentone sodium induced sleeping time in CCl_4 intoxication in rats was determined according to the procedure described in literature [10]. Male Wistar rats were divided into five groups of six each. Group I was kept as normal and normal sleeping time was determined after injecting the thiopentone sodium (25 mg/kg b.w.i.p). Group II served as toxic control. A single dose of Silibinin (50mg/kg b.w.p.o.) was administered to group III, while BF-MMME (50 mg/kg b.w.p.o.) and BF-MMME (100 mg/kg b.w.p.o.) were given to group IV and V respectively. After 24 h, a dose of CCl_4 (1ml/kg b.w.p.o.) was given to II, III, IV and V groups. Then thiopental sodium (25mg/kg b.w.i.p.) was given to II, III, IV and V groups after 2 hrs of CCl_4 injection. The time between loss of righting reflex and its recovery were recorded in all the groups.

Paracetamol induced hepatotoxicity in rats

The experiment was performed according to the method given in the literature [11]. The rats were divided into five groups comprising six in each. 5% gum acacia was used as vehicle for suspending the standard drug and extract. Group I was kept as control received a single daily dose of vehicle (5% gum acacia 1 ml/kg.b.w.p.o.) for seven days. Groups II, III, IV and V were given orally a single daily dose of vehicle, Silibinin (50 mg/kg b.w.), BF-MMME (50 mg/kg b.w) and BF- MMME (100 mg/kg b.w) for seven days respectively. On 8th day a single dose of paracetamol (3 g/kg.b.w.p.o) was administered to the animals of all groups leaving group I. Then blood and liver samples were collected from the animals of all groups 24hrs after administration of paracetamol for estimation of various biochemical parameters and histopathological studies respectively

Ethanol induced hepatotoxicity in rats

The protective effect of BF-MMME against ethanol induced liver damage was done according to the procedure given in the literature with minor modifications [12]. The rats were divided into five groups consisting of six rats each. The total duration of study was for 10 days and the administration of fraction, standard, vehicle or ethanol was only once on the days specified. Group I (Control group): Treated with vehicle alone (1 ml/kg b.w.p.o of 5% gum acacia in water) daily for 10 days. Group II (Toxic group): Treated with vehicle (1 ml/kgb.w.p.o of 5% gum acacia in water) daily for ten days followed by ethanol intoxication on the 10th day. Group III, IV,V were treated with silibinin (50 mg/kg b.w.p.o), BF-MMME (50 mg/kg b.w.p.o), BF-MMME (100 mg/kg b.w) daily for ten days followed by ethanol intoxication on the 10th day. At the end of the 10th day, food was discontinued and the rats in all the groups except control group received, an acute oral dose of ethanol (5 g/kg.b.w) diluted with distilled water (6:4 v/v) by gavages. The blood and liver samples were collected 18hr after administration of ethanol under ether anesthesia, were analyzed for various biochemical parameters and histopathological studies respectively.

Histopathological studies

Histopathological studies could be carried out to assess the degree of damage. This is done by staining the fine section of liver isolates and examining under the microscope. After the animals were sacrificed, livers were taken out and washed with normal saline (0.9%). Then, 2-3 pieces of approximately 6cu.mm size were cut and fixed in phosphate buffered 10% formaldehyde solution. After embedding in paraffin wax, thin sections of 5 μm thickness of liver tissue were cut and stained with haemotoxylin-eosin stain.

Determination of antioxidant activity of BF-MMME by DPPH free radical scavenging assay

Free radical scavenging activity of test extract was measured by *invitro* method using DPPH [13]. 0.1 mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 2.5ml of test extract suspension in water at different concentrations (10, 20, 40, 60, 80,100μg/ml). The reaction mixture was then allowed to stand at room temperature in a dark chamber for 30 min. After 30 min, absorbance was measured at 517nm on a spectrophotometer (UV-Spectrophotometer; Elico-SL 159, Germany). The percentage inhibition of different concentrations was calculated by comparing the absorbance values of control and samples. The concentration of the fraction required to decrease the initial concentration by 50% (IC_{50}) was calculated.

Statistical analysis

The data obtained were analyzed by one-way of variance (ANOVA) followed by Student-Newman-Keul multiple comparison test for the significant interrelation between the various groups using Graph pad prism-3 instat computer software. $P<0.05$ was considered to be significant from the toxic.

RESULTS

Hepatoprotective effect of the fractions of MMME in CCl_4 induced hepatotoxicity in rats

The results of serum biochemical parameters of the study are presented in Table 1 and 2 and histopathology of liver sections of animals were shown in Fig. 1. CCl_4 intoxication in normal rats significantly elevated the levels of hepatospecific enzymes (AST, ALT, ALP), TBL, DBL, CRT, GLU, CHOL, TGL and decreased the levels of TP, ALB in serum, indicating acute hepatocellular damage and biliary obstruction which is supported by the histopathological studies in normal liver architecture of rats showing massive fatty changes, necrosis, degeneration and deformation of hepatocytes and hepatic cords, ballooning degeneration, dilatation in sinusoidal spaces, dilation in central vein, bleeding area in hepatic lobes. Pretreatment with all the fractions of MMME and Silibinin 50mg/kg b.w reversed the levels of these parameters in their respective groups and also reduced these histopathological changes in the liver associated CCl_4 induced hepatic damage. Among the fractions, percentage protection shown by BF-MMME with 50 mg/kg b.w.p.o. was more than that of the other fractions and was well comparable to that of reference drug, Silibinin (50 mg/kg).

Table 1: Effect of the fractions of MMME On different biochemical parameters in CCl_4 induced hepatotoxicity in rats

Groups	DOSE (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	TBL (mg/dl)	DBL (mg/dl)	CRT (mg/dl)
Control	-	31.65±2.27	40.00±4.48	186.65±8.38	0.54±0.18	0.321±0.01	0.85±0.22
Toxic	-	133.86±5.5 ^{d}	135.8±4.99^{d}	459.26±7.27^{d}	2.65±0.54^{d}	1.42±0.07^{d}	1.60±0.15^{d}
Standard	50	41.85±4.68^{c} (90.02%)	50.81±4.03^{c} (88.71%)	207.73±7.90^{c} (92.26%)	0.75±0.06^{c} (90.04%)	0.39±0.03^{c} (93.63%)	1.15±0.18^{c} (60.00%)
TF-MMME 50	50	73.96±5.20^{c} (58.60%)	76.58±5.07^{c} (61.81%)	357.83±7.78^{c} (37.20%)	1.38±0.12^{c} (60.18%)	0.69±0.05^{c} (66.36%)	1.29±0.05^{c} (41.33%)
TF-MMME 100	100	68.71±5.77^{c} (63.74%)	72.30±4.86^{c} (66.28%)	339.93±10.72^{c} (43.77%)	1.34±0.08^{c} (62.08%)	0.61±0.16^{c} (73.60%)	1.23±0.08^{c} (49.30%)
BF-MMME 50	50	39.35±1.57^{c} (92.46%)	50.71±4.01^{c} (89.38%)	264.30±5.63^{c} (71.51%)	0.74±0.02^{c} (94.78%)	0.33±0.03^{c} (99.09%)	1.12±0.18^{c} (64.00%)
BF-MMME 100	100	40.18±1.01^{c} (91.65%)	53.61±1.752^{c} (85.38%)	277.01±6.47^{c} (66.85%)	0.85±0.04^{c} (85.30%)	0.46±0.07^{c} (87.2%)	1.16±0.03^{c} (58.66%)
AF-MMME 50	50	74.06±10.76^{c} (58.50%)	84.5±6.75^{c} (53.54%)	355.3±3.15^{c} (38.13%)	1.36±0.17^{c} (61.13%)	0.63±0.05^{c} (71.81%)	1.29±0.09^{c} (41.33%)
AF-MMME 100	100	70.85±3.61^{c} (61.64%)	80.81±16.63^{c} (57.40%)	329.13±3.75^{c} (47.73%)	1.25±0.05^{c} (66.30%)	0.06±0.01^{c} (74.52%)	1.24±0.20^{c} (48.00%)

Data expressed as mean ± SD, n=6, values in parenthesis indicate percentage recovery.P value- CCl_4 Vs vehicle- ^{d} <0.001; P value-CCl_4 Vs treatments-^{a} <0.05;^{b} <0.01; ^{c} < 0.001

Table 2:Effect of the fractions of MMME on different biochemical parameters in CCl_4 induced hepatotoxicity in rats

Groups	DOSE (mg/kg)	GLU (mg/dl)	TG (mg/dl)	CHOL (mg/dl)	TP (g/dl)	ALB (g/dl)
Control	-	86.9±2.06	80.32±7.63	90.85±6.27	7.66±1.05	4.51±0.63
Toxic	-	189.4±6.78^{d}	132.51±4.85^{d}	133.55±6.90^{d}	3.7±0.65^{d}	2.34±0.22^{d}
Standard	50	97.06±2.30^{c} (90.08%)	88.61±6.09^{c} (84.11%)	92.11±3.33^{c} (97.04%)	7.31±0.29^{c} (91.17%)	3.91±0.38^{c} (72.36%)
TF-MMME 50	50	115.55±6.33^{c} (72.04%)	99.31±15.22^{c} (63.61%)	111.56±2.90^{c} (51.49%)	6.01±0.35^{c} (58.34%)	3.34±0.14^{c} (47.00%)
TF-MMME 100	100	123.1±6.90^{c} (64.68%)	97.25±13.34^{c} (67.56%)	108.60±7.91^{c} (41.56%)	6.39±0.20^{c} (67.93%)	3.40±0.19^{c} (48.85%)
BF-MMME 50	50	97.05±3.89^{c} (90.09%)	84.75±8.99^{c} (91.51%)	97.71±8.19^{c} (83.93%)	7.20±0.56^{c} (88.39%)	3.9±0.22^{c} (71.89%)
BF-MMME 100	100	101.75±3.84^{c} (85.51%)	87.31±3.15^{c} (86.60%)	99.51±8.19^{c} (79.71%)	7.16±0.54^{c} (87.38%)	3.76±0.16^{c} (65.50%)
AF-MMME 50	50	125.43±9.37^{c} (62.40%)	111.28±6.35^{c} (40.67%)	112.50±10.16^{c} (49.29%)	5.22±0.38^{c} (38.39%)	3.37±0.15^{c} (46.09%)
AF-MMME 100	100	104.61±2.22^{c} (82.70%)	102.00±5.44^{c} (58.40%)	101.20±7.47^{c} (75.76%)	5.91±0.28^{c} (55.81%)	3.54±0.06^{c} (55.30%)

Data expressed as mean ± SD, n=6, values in parenthesis indicate percentage recovery.P value- CCl_4 Vs vehicle- ^{d} <0.001; P value-CCl_4 Vs treatments-^{a} <0.05;^{b} <0.01; ^{c} < 0.001

Effect on other parameters

The results of effect on prothrombin time, body and liver weights were shown in Table 3. The CCl_4 intoxicated rats showed significant increase in prothrombin time, reduction in weight of the body and liver of the animals. In all the test and standard groups, there was a significant recovery in prothrombin time, weight of both the body and liver of the animals. Among the fractions of MMME, BF-MMME at 50mg/kg b.w was found to be more effective and was comparable to that of the standard, Silibinin (50mg/kg).

Table 3: Effect of the fractions of MMME on prothrombin time, body weight and liver weight in CCL_4 intoxicated rats

Groups	Prothrombin time (sec)	body weight (g)			Liver weight (g)
		Before treatment	After treatment	Difference	
Control	18.95±1.77	171±5	188±4	17±0.2	26.71±0.96
Toxic (ccl_4)	48.59±3.81^{d}	174±7^{d}	181±7^{d}	7±0.2^{d}	24.08±0.39^{d}
Standard (silibinin)	21.74±2.49^{c}(90.58%)	176±5^{c}	192±5^{c}	16±0.3^{c}(90%)	27.34±0.33^{c}
TF-MMME 50	41.08±1.81^{c} (25.33%)	173±4^{c}	182±4^{c}	9±0.2^{c} (20%)	24.77±0.28^{c}

TF-MMME 100	35.05±1.31[c] (45.68%)	174±3[c]	184±3[c]	10±0.3[c](30%)	25.02±0.29[c]
BF-MMME 50	22.94±2.21[c] (86.53%)	172±3[c]	187±3[c]	15±0.8[c](80%)	26.65±0.70[c]
BF-MMME 100	24.73±2.14[c] (80.49%)	176±2[c]	190±2[c]	14±0.4[c](70%)	26.06±0.53[c]
AF-MMME 50	39.95±0.84[c] (29.14%)	173±3[c]	182±3[c]	9±0.2[c] (20%)	25.35±0.22[c]
AF-MMME 100	33.46±3.89[c](51.04%)	175±2[c]	185±2[c]	10±0.7[c](30%)	26.08±0.57[c]

Data expressed as mean ± SD, n=6, values in parenthesis indicate percentage recovery.P value- CCl_4 Vs vehicle- [d] <0.001; P value-CCl_4 Vs treatments-[a] <0.05;[b] <0.01;[c] < 0.001

Assessment of effect of BF-MMME on thiopentone sodium induced sleeping time of rats in CCl_4 induced hepatotoxicity

The results are presented in Table 4. Thiopentone sodium at a dose of 25 mg/kg (i.p) caused sedation in rats of control group for a period of 61.33±3.48 min, whereas in toxic group, CCl_4 induced liver injury there was a significant (P<0.001) prolongation in the duration of thiopentone sodium induced sleeping time (136.59±4.50 min). Pretreatment of rats with the extract, BF-MMME at doses of 50 mg/kg b.w and 100 mg/kg b.w and Silibinin (50 mg/kg.b.w) significantly (P<0.001) shortened the thiopentone sodium induced sleeping time to 88.68±6.56, 78.18±5.08 and 91.73±6.29 respectively in CCl_4 intoxicated rats as compared to the toxic group. The ability of BF-MMME to reduce the prolongation of thiopentone sodium induced sleeping time in CCl_4 treated ratsubstantiates its hepatoprotective effect. Based on the percentage reduction in sleeping time of rats, the effect of BF-MMME at 50 mg/kg. b.w was comparable to that of reference drug, Silibinin (50 mg/kg).

Table 4: Effect of BF-MMME on thiopentone sodium induced sleeping time in CCl_4 intoxicated rats.

GROUPS	DOSE (mg/kg)	SLEEPING TIME IN MINUTES
CONTROL	--	61.33±3.48
TOXIC (CCl_4)	--	136.59±4.50[d]
STANDARD(SILIBININ)	50	78.18±5.08[c] (77.61%)
BF-MMME 50	50	88.68±6.56[c] (63.65%)
BF-MMME 100	100	91.73±6.29[c] (59.60%)

Data expressed as mean ± SD, n=6, values in parenthesis indicate percentage recovery.P value- CCl_4 Vs vehicle- [d] <0.001; P value-CCl_4 Vs treatments-[a] <0.05;[b] <0.01;[c] < 0.001

Assessment of hepatoprotecive effect of BF-MMME in paracetamol induced hepatotoxicity in rats.

The results of the study are presented in Table 5 and 6 and histopathological photographs of the liver sections of rats of the study were shown in Fig.2. Paracetamol intoxication in normal rats significantly (P<0.001) elevated the level of hepatospecific enzymes (AST, ALT, ALP), TBL, DBL, CRT, GLU, TGL, CHOL and decreased the level of TP , ALB in serum, indicating acute hepatocellular damage and biliary obstruction which was substantiated by the histopathological examination of the liver sections of rats showing necrosis, fatty changes, dilatation of sinusoidal space, and bleeding in hepatic lobes. The rats treated with BF-MMME 50, 100 mg/kg b.w.p.o. and Standard (Silibinin 50mg/kg b.w) showed a significant (*P*<0.001) protection, against paracetamol induced hepatic damage by normalizing serum biochemical parameters and by reducing the histopathological abnormalities.

Table 5: Effect of the fractions of MMME On different biochemical parameters in paracetamol induced hepatotoxicity in rats

GROUPS	DOSE (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	TBL (mg/dl)	DBL (mg/dl)	CRT (mg/dl)
CONTROL	-	50.59±6.12	46.6±6.11	148.21±8.56	0.39±0.10	0.25±0.05	0.66±0.05
TOXIC	-	159.46±9.22[d]	128.1±5.60[d]	375.68±5.21[d]	2.74±0.47[d]	0.59±0.06[d]	1.77±0.17[d]
STANDARD (Silibinin)	50	70.65±7.84[c] (81.57%)	53.55±4.80[c] (91.47%)	176.23±8.98[c] (87.68%)	0.75±0.06[c] (84.61%)	0.30±0.07[c] (85.29%)	0.82±0.05[c] (85.58%)
BF-MMME 50	50	68.33±5.21[c] (83.70%)	62.76±8.70[c] (80.17%)	191.70±4.47[c] (80.88%)	0.64±0.11[c] (89.31%)	0.33±0.03[c] (76.47%)	0.88±0.05[c] (80.18%)
BF-MMME 100	100	73.71±2.70[c] (78.76%)	63.42±5.66[c] (79.36%)	197.70±4.04[c] (78.24%)	0.67±0.09[c] (88.03%)	0.36±0.07[c] (67.64%)	0.91±0.06[c] (77.47%)

Data expressed as mean ± SD, n=6, values in parenthesis indicate percentage recovery.P value- CCl_4 Vs vehicle- [d] <0.001; P value-CCl_4 Vs treatments-[a] <0.05;[b] <0.01;[c] < 0.001

Table 6: Effect of the fractions of MMME On different biochemical parameters in paracetamol induced hepatotoxicity in rats

Groups	DOSE (mg/kg)	GLU (mg/dl)	TG (mg/dl)	CHOL (mg/dl)	TP (g/dl)	ALB (g/dl)
Control	-	100.90±10.91	70.31±2.07	62.19±4.46	8.43±0.45	3.97±0.76
Toxic	-	201.93±5.74[d]	125.70±3.07[d]	84.43±2.99[d]	4.51±0.68[d]	1.95±0.56[d]
Standard (silibinin)	50	112.81±10.72[c] (88.21%)	78.63±5.66[c] (84.97%)	63.63±3.05[c] (93.52%)	8.08±0.84[c] (91.07%)	3.74±0.29[c] (88.61%)
BF-MMME 50	50	120.86±8.24[c] (80.24%)	81.28±6.77[c] (80.19%)	67.9±4.25[c] (74.32%)	7.95±0.39[c] (87.75%)	3.63±0.13[c] (83.16%)
BF-MMME 100	100	124.35±5.63[c] (76.28%)	83.65±3.31[c] (75.91%)	69.40±4.74[c] (67.58%)	7.74±0.61[c] (82.39%)	3.58±0.12[c] (80.69%)

Data expressed as mean ± SD, n=6, values in parenthesis indicate percentage recovery.P value- CCl_4 Vs vehicle- [d] <0.001; P value-CCl_4 Vs treatments-[a] <0.05;[b] <0.01;[c] < 0.001

Assessment of hepatoprotecive effect of BF-MMME in ethanol induced hepatotoxicity in rats:

The results of the study are presented in Table 7 and 8 and histopathological photographs of liver section of rats of the study are shown in Fig.3. Ethanol intoxication in normal rats significantly (P<0.001) elevated the levels of hepatospecific enzymes (AST, ALT, ALP), TBL, DBL, CRT, GLU, TGL, CHOL and decreased the levels of TP and ALB in serum, and histopathological examination of the liver sections of ethanol intoxicated rats showed kupffer cell proliferation, dilatation of sinusoidal space and bleeding in hepatic lobes. The rats treated

with BF-MMME 50, 100 mg/kg b.w.p.o. and standard (Silibinin 50mg/kg b.w.) showed a significant (P<0.001) recovery from the ethanol induced hepatic damage both with serum biochemical parameters and histology of liver with little dilatation of sinusoidal space and no kupffer cell proliferation and bleeding areas. The extract, BF-MMME, at both the test doses exhibited a remarkable (P<0.001) hepatoprotective activity. However the percentage protection shown by BF-MMME 50 mg/kg b.w.p.o. against ethanol induced hepatotoxicity was well comparable to that of the reference drug, Silibinin (50 mg/kg).

Table 7: Effect of BF-MMME on different serum biochemical parameters in ethanol induced hepatotoxicity in rats

GROUPS	DOSE (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	TBL (mg/dl)	DBL (mg/dl)	CRT (mg/dl)
Control	-	55.26±4.84	40.91±4.54	180.58±8.08	0.43±0.06	0.18±0.03	0.6±0.03
Toxic(CCl_4)	-	127.11±4.62^d	101.26±7.87^d	432.08±6.70^d	1.70±0.17^d	0.25±0.06^d	1.42±0.07^d
Standard (Silibinin)	50	62.55±1.63^c (89.88%)	57.20±3.53^c (73.00%)	201.01±5.06^c (91.87%)	0.61±0.04^c (85.82%)	0.25±0.03^c (82.25%)	0.77±0.05^c (80.48%)
BF-MMME 50	50	67.16±2.23^c (83.43%)	63.42±5.66^c (62.70%)	225.51±3.11^c (82.13%)	0.67±0.09^c (81.10%)	0.29±0.02^c (70.5%)	0.66±0.11^c (92.60%)
BF-MMME 100	100	69.28±3.06^c (80.48%)	66.18±6.73^c (58.12%)	227.51±2.66^c (81.33%)	0.68±0.04^c (80.31%)	0.33±0.02^c (63.50%)	0.69±0.09^c (89.02%)

Data expressed as mean ± SD, n=6, values in parenthesis indicate percentage recovery.P value- CCl_4 Vs vehicle- ^{d} <0.001; P value-CCl_4 Vs treatments-^{a} <0.05;^{b} <0.01; ^{c} < 0.001

Table 8: Effect of BF-MMME on different serum biochemical parameters in ethanol induced hepatotoxicity in rats

Groups	DOSE (mg/kg)	GLU (mg/dl)	TG (mg/dl)	CHOL (mg/dl)	TP (g/l)	ALB (g/l)
Control	-	88.70±0.84	91.51±10.11	58.31±6.65	6.17±0.16	3.53±0.19
Toxic	-	178.60±9.71^d	133.76±7.61^d	140.60±4.33^d	3.17±0.64^d	1.55±0.45^d
Standard	50	93.36±3.22^c (94.81%)	98.08±3.52^c (84.44%)	66.33±2.25^c (90.25%)	5.43±0.45^c (69.91%)	3.42±0.19^c (94.44%)
BF-MMME 50	50	104.93±9.81^c (81.94%)	103.18±4.51^c (72.37%)	67.53±4.24^c (88.79%)	5.36±0.42^c (67.07%)	3.36±0.14^c (91.41%)
BF-MMME 100	100	111.83±6.34^c (74.27%)	106.81±9.44^c (63.78%)	69.26±4.15^c (86.69%)	5.26±0.25^c (63.00%)	3.26±0.41^c (86.36%)

Data expressed as mean ± SD, n=6, values in parenthesis indicate percentage recovery. P value- CCl_4 Vs vehicle- ^{d} <0.001; P value-CCl_4 Vs treatments-^{a} <0.05;^{b} <0.01; ^{c} < 0.001

DPPH free radical scavenging assay of BF-MMME

The 1,1 Diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging activity of the BF-MMME and ascorbic acid are summarized in Table 9. Both the extract and ascorbic acid exhibited a concentration dependant DPPH radical scavenging activity. The IC_{50} concentration for the standard, ascorbic acid and for BF-MMME were found to be 0.053 and 3.2 µg/ml respectively.

Table 9: DPPH free radical scavenging assay of BF-MMME

	CONCENTRATION (µG/ML)	%INHIBITION	IC_{50}VALUES (µG/ML)
ASCORBIC ACID	0.010	19.8	
	0.020	27.8	
	0.040	40.2	
	0.060	55.2	
	0.080	68.9	
	0.1	83.2	0.053
BF-MMME	1	22.1	
	2	38.6	
	4	58.9	
	6	76.4	
	8	88.5	
	10	99.8	3.2

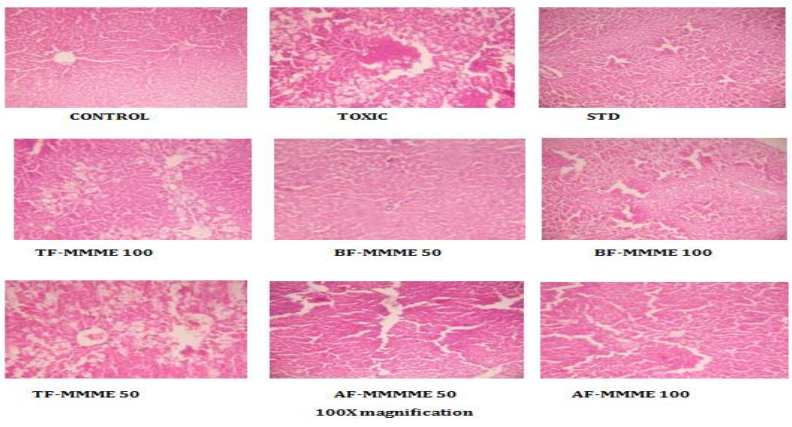

Figure 1: Effect of the fractions of MMME on histopathological changes in the liver of rats in CCl_4 induced hepatotoxicity

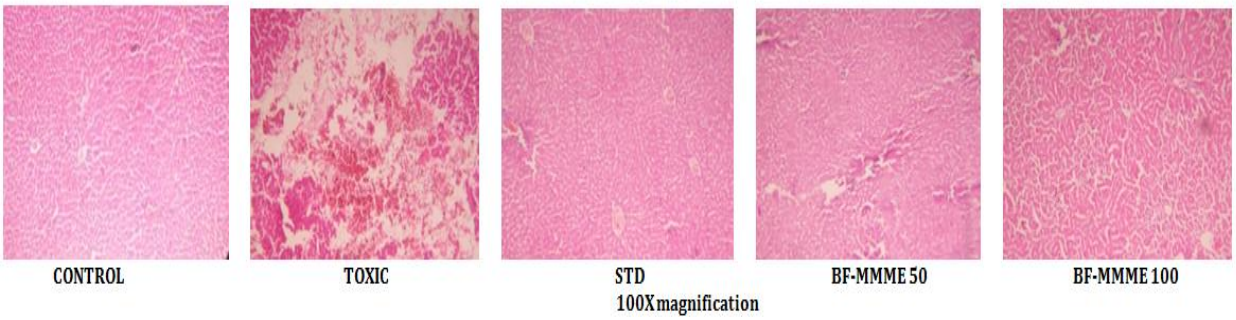

Figure 2: Effect of BF-MMME on histopathological changes in liver of rats in paracetamol induced hepatotoxicity.

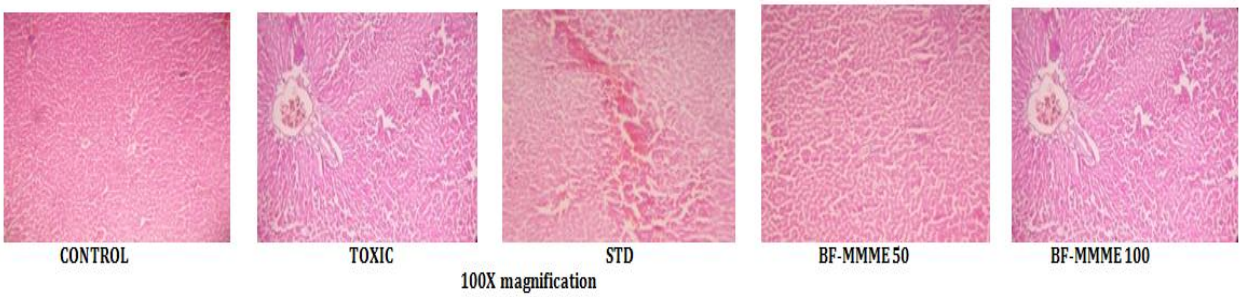

Figure 3: Effect of BF-MMME on histopathological changes in liver of rats in ethanol induced hepatotoxicity.

DISCUSSION

In view of hepatoprotective potential of MMME, the hepatoprotective activity of different fractions of MMME was tested in three mechanistically devised models viz., CCl_4, paracetamol and ethanol induced liver damage in rats. CCl_4 induced hepatotoxicity in rats causes a severe centrizonal necrosis, steatosis and damage to the structural integrity of liver is reflected by increase in the liver hepato-specific enzymes (ALP, ALT and AST) in the serum, because they are cytoplasmic in location and are released into circulation after cellular damage [14]. Treatment with the fractions, TF-MMME, BF-MMME and AF-MMME at 50 and 100 mg/kg b.w significantly reduced the level of these marker enzymes may be a consequence of stabilization of plasma membrane as well as repair of damaged hepatic tissue [15]. Elevations in serum bilirubin level (TBL and DBL) also occur in CCl_4 induced hepatotoxicity due to defective excretion of bile by the liver [16]. TF-MMME, BF-MMME and AF-MMME at 50 and 100 mg/kg b.w.p.o showed a significant decrease in serum bilirubin level suggesting the possibility of the extract's ability to repair the damage of the hepatocytes caused by CCl_4 in prophylactic studies. A depression in total protein is observed due to the disruption and disassociation of polyribosomes from endoplasmic reticulum following CCl_4 administration [17]. The increase in serum TP and ALB level by the fractions of MMME indicates their hepatoprotective effect, which may be due to stimulation of protein synthesis by stabilization of endoplasmic reticulum causing the acceleration of the regeneration process of liver cells [18]. Hepatotoxicity caused by CCl_4, results in defective biosynthesis of bile acids from cholesterol and defective excretion of creatinine by the liver, which leads to increase in serum cholesterol and creatinine respectively. Hepatocellular damage also causes a modest hypertriglyceridemia due to slow uptake and removal of triglycerides from the blood [19], hyperglycemia from reduced glucogenesis process and increase in serum MDA level resulting from the peroxidation of biological membrane polyunsaturated fatty acid (PUFA) [20]. The reversal of serum level of aforesaid biochemical parameters in the study indicates that the fractions have exhibited hepatoprotective activity by restoring the normal architecture of the liver .

In CCl_4 intoxicated rats the PT was drastically increased which may be attributed to decreased synthesis of prothrombin due to hepatocellular damage [21]. Treatment with fractions of MMME showed significant decrease in prothrombin time, indicating their recovery effect on the liver. In CCl_4 intoxicated rats, the loss of body weight and liver weight occur due to decrease in the number of hepatocytes which results in the decreased hepatic capacity to synthesize protein and glycogen leading to decrease in the weight. Treatment with fractions of MMME showed significant increase in the weight of liver and body which is most probably through the hepatic cell regeneration [20]. The results of the test extract BF-MMME at 50mg/kg was well comparable to that of Silibinin (50mg/kg), a reference hepatoprotective drug.

The histopathological profile of the liver of CCl_4 administered rats revealed drastic alterations in its histoarchitecture showing centrilobular necrosis, fatty changes, dilation of sinusoidal spaces, kupffer cell proliferation, ballooning degeneration and bleeding area in hepatic lobes. The fractions of TF-MMME, BF-MMME and AF-MMME at both test doses (50 and 100mg/kg) showed a definite sign of protection and recovery against CCl_4 injury. Of the three test fractions, BF-MMME at 50mg/kg exhibited a remarkable recovery, towards normalization of histological architecture of liver of the rats, which was almost similar to that of Silibinin (50mg/kg). The significant hepatoprotective activity shown by the fractions indicate their ability to bring a remarkable recovery in the damaged architecture of the liver.

The hepatoprotective effect of extract BF-MMME was substantiated in thiopentone sodium sleeping time experiment in rats. It has been established that since the barbiturates are metabolized almost exclusively in the liver by hepatic microsomal drug metabolising enzymes (MDME) and duration of sleeping time in intact animals is considered as a reliable index for the activity of hepatic MDMEs. In CCl_4 intoxication, the sleeping time after a given dose of the barbiturate will be prolonged, because the amount of hepatic metabolism/min will be less [22]. The treatment of BF-MMME, at 50 and 100 mg/kg b.w significantly reduced the sleeping time in CCl_4 intoxicated rats. Ability of the BF-MMME to reduce the prolongation of thiopentone sodium induced sleep in CCl_4 poisoned rats is further indicative of its hepatoprotective effect. The effect of the

BF-MMME at 50mg/kg was well comparable to that of reference drug, Silibinin (50 mg/kg).

The hepatoprotective effect of BF-MMME was also evaluated in the drug induced (Paracetamol) and ethanol induced hepatotoxicity in rat models. Pretreatment of rats with BF-MMME at 50 and 100mg/kg b.w showed a significant protection against both Paracetamol and ethanol induced alterations in serum level of ALT, AST, ALP, TB, DB, TP, CRT, GLU, TGL, CHOL and ALB. The histopathology of liver of rats treated with BF-MMME at 50 and 100 mg/kgb.w showed a remarkable protection against these hepatotoxins viz., paracetamol and ethanol induced changes in normal liver architecture of rats. Thus results of biochemical and histopathological parameters of the study are complementing each other the hepatoprotective effect of BF-MMME. Further, it is evident from the results that the hepatoprotective effect of the test extract BF-MMME at 50mg/kg was well comparable to that of the reference drug Silibinin (50mg/kg).

The antioxidant study was conducted for BF-MMME, to assess whether the antioxidant mechanism is involved in its hepatoprotective activity in CCl_4 induced hepatotoxicity in rats. It has been shown that protective agents exert their action against CCl_4 induced liver injury by impairment of CCl_4 mediated lipid peroxidation, either through decreased production of free radical derivatives or due to the antioxidant activity of the protective agent itself. In the present investigation, BF-MMME exhibited a concentration dependant DPPH radical scavenging activity, indicating that the hepatoprotective activity of the extract may be due to its antioxidant effect by inhibiting lipid peroxidation [23]. Hence, the hepatoprotective activity of the extract BF-MMME may be attributed to its antioxidant effect.

It is well known that plants or their extracts contain a number of chemicals belonging to different classes. Most of them are of pharmacological importance such as flavonoids, steroids, triterpenoids and their glycosides, alkaloids etc. Hence, the extract BF-MMME was subjected to preliminary chemical analysis to detect the different classes of compounds present in it. The results of the study revealed that the steroidal/triterpenoidal and flavonoidal glycosides, and phenolic compounds are present in BF-MMME. The literature reveals that the plants containing steroid and triterpenoids and flavonoids can control liver diseases [24]. Therefore the hepatoprotective activity of BF-MMME may be attributed to aforesaid classes of compounds present in it. The findings of the study substantiated the ethnomedicinal value of the plant *Marsilea minuta* used in the treatment of hepatitis. However, a comprehensive investigation of bioactive fraction BF-MMME is required to identify the active principle(s), to evaluate the efficacy and toxicity in different models with the mechanism of action for developing it as a safe and effective herbal hepatoprotective drug.

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