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HEPATOPROTECTIVE ACTIVITY OF THE AQUATIC FERN *MARSILEA QUADRIFOLIA*

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ABSTRACT

The aim of the study is to investigate the hepatoprotective activity of the methanolic extract of the plant *Marsilea quadrifolia* against acetaminophen induced hepatotoxicity in rats. The animals used in this study showed no mortality or symptoms of toxicity upto a dose level of 3000mg/kg b.w for 1hr. Hence two test doses 200 and 400mg/kg b.w were taken in the study. Silymarin 100mg/kg b.w was used as reference standard drug. Liver damage was achieved by the administration of acetaminophen suspension (2gm/kg) orally. It significantly elevated the levels of Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Serum Alkaline Phosphatase (ALP) and Total Bilirubin (TB) and decreased the levels of Total Protein (TP) and albumin (ALB), indicating acute hepatocellular damage and biliary obstruction. The rats treated with silymarin, test extracts of 200 and 400mg/kg doses significantly reversed the level of these parameters as compared to the toxic group. Methanolic extracts of *M. quadrifolia* at a dose 200mg/kg exhibited a significant hepatoprotective activity. These biochemical observations were substantiated by histopathological examination of liver sections. These findings suggest that the plant *M. quadrifolia* possesses hepatoprotective potential against acetaminophen induced hepatotoxicity in rats.

INTRODUCTION:

Herbal drugs play a major role in the treatment of hepatic disorders. In India, a number of medicinal plants and their formulations are used to cure hepatic disorders in traditional systems of medicine. Hepatitis is a general term which refers to 'Inflammation of the liver' is used to describe diseases resulting in hepatocellular damage. Recent pharmacological and clinical experiments have shown that herbal medicines are beneficial against liver disorders, as measured by standard liver function tests. They support or promote the process of healing or regeneration of liver cells with fewer side effects. The majority of plants described as being used for liver disorders increase the bile flow and reverse cholestasis [Farnsworth *et al.,* 1985]. The plant *Marsilea quadrifolia (M. quadrifolia)* is a creeping perennial herb with a slender long branching rhizome belongs to the family marsileaceae. It is widely distributed

in India, China, Japan and North America. Based on the ethnomedicinal information, the *M. quadrifolia* leaves are used in the treatment of hypertension, sleep disorders and headache. Entire fresh plant is used in the treatment of cough and convulsion. A juice of the leaves is used for diuretic snakebite and febrifuge. The plant also posses anti-inflammatory, diuretic, depurative and refrigerant property [Longman.1997, Khare.2004]. The phytoconstituents reported on *M. quadrifolia* such as marsilin (1-triacontanol-cerotate), 3-hydoxy-triacontan-11-one, beta-sitosterol, flavonol-O-mono-and-diglycoside[Asha *et al.*, 2013]. It is well documented that flavonoids have antioxidant property [Makari *et al.*, 2008] which plays a vital role in the treatment of liver damage. Anti-inflammatory property of the plant may be useful inducing liver protection activity [Vogel *et al.*, 1975] and the plant has not been screened pharmacologically for its traditional claim for the treatment of hepatitis. In view of above claims and facts, the present investigation was undertaken with a view to provide scientific evidence for its traditional use in the treatment of liver disorders. The crude methanolic extract of the plant *M. quadrifolia* was evaluated for hepatoprotective activity against acetaminophen induced hepatic damage.

MATERIALS AND METHOD

Collection and Preparation of Extract

The plant materials were collected from Kanyakumari Dist. The plant *Marsilea quadrifolia* was washed thoroughly, it was shade dried and then coarsely powdered. The powder was passed through sieve no.40 and stored in an air tight container for further use. The powder was then extracted with methanol using Soxhlet apparatus for 72 hrs. The extract was dried and stored in dessicator.

Caging of Animals: Adult rats were collected and caged under hygienic conditions. Animals were fed with hygienic feed, pure water and kept under temperature 22 ± 2°C.

Acute toxicity studies:-

Albino mice (Swiss strain) were divided into five groups six animals in each group. One group was set as control group and the remaining four groups received (800, 1000, 2000 and 3000 mg/kg b.w) the extract orally. The mice were observed continuously for 1 hr for any gross behavioral changes and death, if any, intermittently for the next 6 hrs and then again for 24 hrs after dosing.

PROCEDURE

The total number of 30 rats was used in the study. These 30 animals were divided into five groups of six animals in each. All groups of animals were fed with the acetaminophen suspension (2gms/kg) orally for first three days, except for Group I. The Group I animals received 1% CMC for seven days. The Group II animals were treated with 1%CMC (1ml/kg) for the remaining four days. The Group III animals were treated with silymarin (100mg/kg) for the remaining four days. The Group IV and V and animals received 200 mg/kg and 400 mg/kg methanol extract of *Marsilea quadrifolia* in 1% CMC (1ml/kg) for the remaining four days.

ASSESSMENT OF LIVER FUNCTION TEST

Blood sample collection

The blood samples were collected separately on1st, 4th, 10th day by retro-orbital plexus into sterilized dry centrifuge tubes and allowed to coagulate for 30 min at 37°C. The clear serum was separated at 3000 rpm for 10 min and biochemical investigations were carried out to assess liver function viz., total protein, albumin, total bilirubin, SGOT, SGPT, and serum alkaline phosphatase. Then the animals were sacrificed by cervical dislocation (ketamine) and liver was dissected out. The isolated organs were sliced and histopathologically examined.

Estimation of serum glutamic oxaloacetic transaminase (SGOT):

The serum aspartate aminotransferase was estimated by the method of Reitman and Frankel (1957) using AST test kit (Span Diagnostics Ltd.). 0.25 ml of buffered aspartate - α-KG substrate was added to the

0.05 ml of serum and incubated at 37ºC for 5 minutes. Thereafter, 0.25 ml of DNPH (2,4-Dinitrophenyl hydrazine) was added to samples, mixed well and allowed to stand at room temperature for 20 minutes. After that 2.5 ml of 4 N sodium hydroxide (dilute 1 ml of sodium hydroxide up to 10 ml with purified water) was added to the above mixture and mixed well, allowed to stand at room temperature for 10 minutes. The absorbance of blank, standard and test were read at 505 nm. Working pyruvate standard was used as a standard.

Estimation of serum glutamate pyruvate transaminase (SGPT):

The serum acid phosphatase activity was estimated by the method of King and Jagatheesan (1959) using ALT test kit (Span Diagnostics Ltd.). 0.5 ml of buffered substrate, (pH 4.9) was added in control (C) and test (T). 0.5 ml of purified water was added in control (C) and test (T). 0.6 ml of purified water was added in standard (S) mixed well and incubated at 37ºC for 3 minutes. 0.1 ml of serum was added in test (T), 0.5 ml of working standard was added in standard (S). All the tubes were mixed well and incubated at 37ºC for 60 minutes. 0.5 ml of reagent II (Sodium hydroxide, 0.5 N) was added in all the tubes. 0.1 ml of serum was added in control (C). 0.5 ml of sodium bicarbonate, (0.5 N), 0.5 ml of solution (Dissolve 4-aminoantipyrine, 0.6% in 25 ml of purified water) and 0.5 ml of solution III (Dissolve potassium ferricyanide, 2.4% in 25 ml of purified water) was added in all the tubes. All the tubes were mixed well and absorbance was read at 510 nm. Serum acid phosphatase activity is expressed as KA units.

Estimation of serum alkaline phosphatase (ALP)

Alkaline phosphatase activity was estimated by the method of Kind and King (1954) using ALP test kit (Span Diagnostics Ltd.). 0.5 ml of working buffered substrate and 1.5 ml of water was mixed well and incubated at 37°C for 3 minutes. 0.05 ml of serum was added in test (T), 0.05 ml of reagent III (10 mg% Phenol standard) was added in standard (S) and 0.05 ml of purified water was added in blank (B) tubes. All the tubes were mixed well and incubated at 37°C for 15 minutes. 1 ml of reagent II (Chromogen reagent) was added in all the tubes. 0.05 ml of serum was added in control (C). All the tubes were mixed well and absorbance was read at 510 nm. Serum alkaline phosphatase activity is expressed as KA units.

Serum alkaline phosphatase in KA units =

O.D. of test – O.D. of control/ O.D of standard – O.D. of blankX100

Estimation of total bilirubin:

The serum bilirubin was estimated by the method of Jendrassik and Grof (1938) using globulin test kit (Beacon Diagnostics Ltd). 1.0 ml total bilirubin reagent was added 0.5 ml serum mixed well and incubated at room temperature for 5 minutes. The absorbance was read at 550 nm. Direct bilirubin reagent was used as a standard.

Total protein

The serum total protein was estimated by modified Biuret method (Yatzidis, 1977) using total protein test kit (Span Diagnostics Ltd.). 3 ml of biuret reagent (Copper sulphate - 7 mM/L; sodium hydroxide – 200 mM/L; sodium potassium tartrate - 20 mM/L) was added to 0.03 ml of serum mixed well and incubated at 37ºC for 5 minutes. The absorbance was read at 578 nm. Bovine serum albumin was used as a standard.

Albumin

The serum albumin was estimated by the method given by Corcoran and Durnan (1977) using albumin test kit (Span Diagnostics Ltd.). 3.0 ml of albumin reagent (Succinic acid - 37 mM/L; bromocresol green - 0.15 mM/L; sodium hydroxide - 1 mM/L; buffer pH - 3.68) was added to 0.03 ml serum mixed well and incubated at room temperature for 1 minute. The absorbance

was read at 630 nm. Bovine serum albumin was used as a standard.

Isolation of Liver:

The animals were sacrificed by cervical dislocation (ketamine anesthesia) and liver was dissected out. The isolated organ was sliced into 5mm pieces and fixed in neutral formalin (10 % solution) for 3 days. Standard method for the fixation of liver section was followed. Liver pieces were washed under running water for about 12hrs. This was followed by dehydration with alcohol of increasing strength (70%, 80%, and 90%) for 12hrs each. Final dehydration was carried out using absolute alcohol with about 3 changes at 12min interval. Using xylene with changes at 15 – 20 min interval did cleansing. After cleansing the pieces they were subjected to paraffin infiltration in automatic tissue processing unit. The pieces were washed under running water to remove formalin completely.

Embedding in paraffin:

Hard paraffin was melted and poured into L-shaped block. The liver pieces were then dropped into the liquid paraffin quickly and allowed to cool.

Sectioning:

The blocks were cut using microtone to get sections of thickness of 5microns. The sections were fixed on a glass-using albumin and allowed to dry.

Staining:

Eosin, an acid stain and hematoxylin, a basic stain were used for staining the liver sections.

RESULT AND DISCUSSION

Biochemical parameters

The toxicity study did not cause any gross behavioral changes and mortality upto the dose level of 3000 mg/kg b.w and was considered as safe. Hence two graded doses of the extract i.e., 200 and 400 mg/kg b.w were chosen for the studies. In hepatoprotective study it was observed that administration of acetaminophen elevated the levels of serum SGOT, SGPT, TB, ALP and decreased the levels of TP and albumin (Table 1) indicating acute hepatocellular damage and biliary obstruction. The rats treated with standard drug Silymarin (100mg/ kg), MEMQ 200 and 400 mg/ kg between significantly reversed the level of these parameters as compared to toxic group. The SGOT in the control group was 130 ± 6.54IU/L and the SGPT level was 31 ± 5.29 IU/L, administering of acetaminophen, the SGOT level was raised to 222.5 ± 11.12 IU/L and the SGPT level was raised to 55.75 ± 7.85IU/L. The rise in serum levels of SGOT and SGPT has been attributed to the damaged structural integrity of the liver, because they are cytoplasmic in location and released into circulation after cellular damage (Sallie *et al.,* 1991). By inducing 200 and 400 mg/kg of the methanolic extract of *M. quadrifolia* the SGOT level was reduced to 182 ± 7.05 IU/L and 168.6 ± 4.63 IU/L and the SGPT level was reduced to 48.6 ± 7.05 IU/L and 43.3 ± 4.63 IU/L. The decrease in the levels of these enzymes may be a consequence of stabilization of plasma membrane as well as hepatic tissue damage (Kamalakkannan *et al.*, 2005). The total protein present in control group of rat was 5.6 ± 0.31 g/dl, when acetaminophen was induced the protein level was decreased to 2.8 ± 0.23 g/dl. The albumin level in the control group rat was 3.9 ± 0.2 g/dl it was decreased to 1.4 ± 0.03 g/dl when acetaminophen was induced. A depression in total protein is observed due to the disruption and disassociation of polyribosomes from endoplasmic reticulum following CCl_4 administration [Vetriselvan *et al.,* 2011]. Administering of 200 and 400mg/kg of methanolic extract of *M. quadrifolia*, the protein level was increased to 4.8 ± 0.2g/dl and 5.2 ± 0.6 g/dl, the albumin level was increased to 2.8 ± 0.32 g/dl and 3.3 ± 0.28 g/dl. The increase in serum TP and ALB level by the methanolic extract of *M. quadrifolia* 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 [Clawson. 1989].

Table 1: Hepatoprotective activity of the methanolic extract of the plant *Marsilea quadrifolia*

Parameters	(Group-I) Control	(Group-II) Acetaminophen	(Group-III) Standard	(Group-V) (200mg/kg) Extract	(Group-VI) (400mg/kg) Extract
Total Bilirubin (mg/dl)	0.61±1.08	2.39±1.16	0.94±1.21	1.2±0.115	1.1±0.145
SGOT (IU/L)	130±6.54	222.5±11.12	144±17.34	182±7.05	168.6±4.63
SGPT (IU/L)	31±5.29	55.75±7.85	36.75±5.34	48.6±7.05	43.3±4.63
ALP (IU/L)	82±7.82	133±12.83	106.5±10.43	139.6±7.44	124.6±12.13
TP (g/dl)	5.6 ± 0.31	2.8±0.23	6.2±0.15	4.8±0.2	5.2±0.6
Albumin (g/dl)	3.9 ±0.2	1.4±0.03	3.5±0.4	2.8±0.32	3.3±0.28

SECTION OF CONTROL GROUP

Figure. 1 Normal

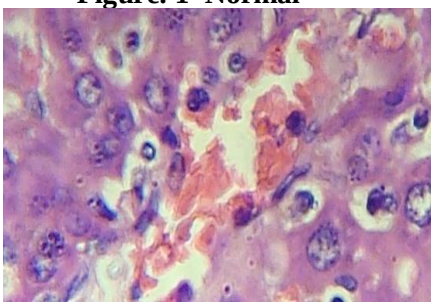

Figure. 2 Toxic (Acetaminophen)

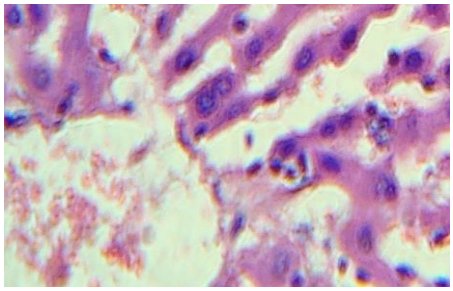

Figure. 3 Standard (Silymarin)

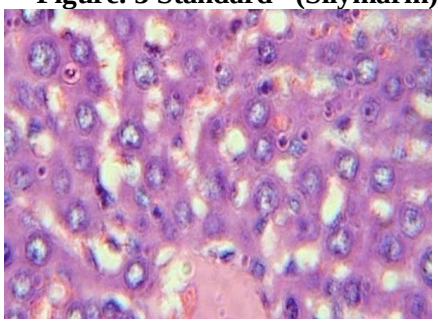

Figure. 4 200mg/kg

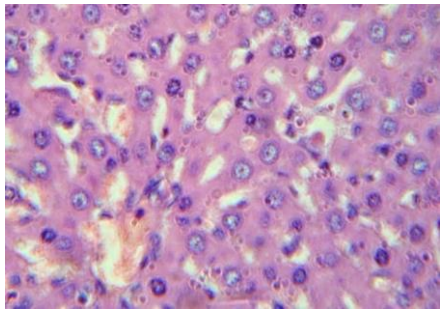

Figure. 5 400mg/kg

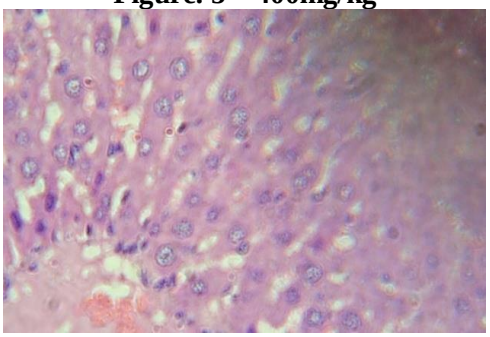

Fig 1: The parenchymatous tissue showing the regenerative changes by emptying of cytoplasm of Hepatocytes and nucleus was centrally located. **Fig 2**: Liver showing mild congestion, increased space of canaliculi moderate vacuolation and foci of necrosis **Fig 3:** showed less vacuole formation, reduced sinusoidal dilation, and less rearrangement and degeneration of hepatocytes indicating marked **Fig 4:** Hepatocytes were regenerative and showed a milder degree of vacuolation but prominent nuclei, indicating returning to normalcy, with normal canalicular space.

Fig 5: Hepatic parenchyma revealing a large area of necrosis and serve degenerative changes. Normal space of canaliculi was observed.

The results indicate the MEMQ at 400 mg/ kg between afforded better protection as compared to MEMQ at 200 mg/kg and is comparable to that of the reference drug silymarin(100mg/kg). The elevated levels of serum enzymes are indicative of cellular leakage and loss of functional intregrity of cell membrane in the liver. Thus lowering of enzyme contents in serum is a definite indication of hepatoprotective action of drug. High level of SGOT indicates the liver damage due to viral hepatitis. SGPT catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore the SGPT is more specific to the liver and a better parameter for detecting liver damage. Serum ALP and bilirubin levels are also related to the status and function of hepatic cells. Increase in serum ALP is due to increased synthesis in presence of increasing biliary pressure. The result of present study demonstrates that the methanolic extract of *M. quadrifolia* has potent hepatoprotective activity against Acetaminophen induced liver damage in rats. It is also showed that, it has great influence of liver and blood parameters (SGOT, SGPT, total bilirubin, ALP, Total protein and albumin). Thus it is proved that *M. quadrifolia* extract has potent hepatoprotective activity.

HISTOPATHOLOGICAL EXAMINATIONS

Further the results were substantiated by the histopathological estimation of liver reactions (Figure1). The Histopathological profile of the control group rats were treated with saline solution, liver section (Figure-1) showed the parenchymatous tissue showing the regenerative changes by emptying of cytoplasm of hepatocytes and nucleus was centrally located. The second groups of animals were treated with acetaminophen (2gm/kg, per oral). Liver section (Figure-2.) showed mild congestion, increased space of canaliculi moderate vacuolation and foci of necrosis and sinusoidal hemorrhage and dilatation. There was chronic inflammatory cell infiltrate in the portal tract. The third group of animals treated with silymarin liver section (Figure-3) was showed less vacuole formation, reduced sinusoidal dilation, and less rearrangement and degeneration of hepatocytes indicating marked regenerative active. The fourth group of animals were treated with plant extract (200 mg/kg b.w. p.o.) with acetaminophen, liver section (Figure-4) showed hepatic parenchyma revealing a large area of necrosis and serve degenerative changes. Normal space of canaliculi was observed. The intensity of centrilobular necrosis was less. The fifth group of animals was treated with plant extract (400 mg/kg b.w. p.o.) with acetaminophen, liver section (**Figure-5**) showed hepatocytes were regenerative and showed a milder degree of vacuolation but prominent nuclei, indicating returning to normalcy.

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