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Hepatoprotective effect of standardized antioxidant phenolic fractions of *Hibiscus mutabilis* Linn.

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ABSTRACT

Defatted polar phenolic fraction of Hibiscus mutabilis Linn. from leaf, stem and flowers was evaluated against carbon tetrachloride induced hepatic injuries in rats. In this test, markers of liver injuries (ALT, AST, ALP) were measured analytically after treatments confirmed by histophatological consideration in rat livers. These fractions were standardized by HPTLC quantitatively and evaluated for antioxidant potential using DPPH and nitric oxide models. Administration of carbon tetrachloride significantly increased the release of ALT, ALP and AST. Treatment of rats with ethanol fractions significantly modulate these enzymes in blood serum to normal values. Also, histo-pathological analysis of liver biopsies and antioxidant potential of standardized fractions was consistent with the biochemical findings. Experimental work led to conclude the curative potential of defatted antioxidant ethanolic fractions of H. mutabilis leaf, stem and flower against chemical (carbon tetrachloride) induced hepatic injuries.

Keywords: *Hibiscus mutabilis* Linn., antioxidant, phenolic, hepatoprotective, serum enzymes

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INTRODUCTION

Hibiscus mutabilis is native to southern China and is favorite landscape plant in mild winter climates [1]. *Hibiscus mutabilis* is also known as the Confederate rose or the cotton rosemallow. This plant is recommended for persistent cough, menorrhagia, dysuria and wound caused by burn and scalds. *Hibiscus mutabilis* also reported for its antiphlogistic, depurative, febrifuge and stimulant properties and also used for burns, pectoral and pulmonary complaints, swellings and other skin problems in folklore medicine [2]. The plant also bears edible parts and used as fodder. The plant possesses Anthocyanines [3,4], Rutin [5], Naringenin, Quercetine [6]. This review indicate presence of much phenolic compound specially flavonoids in the parts of plant. Flavonoids are phenolic substances isolated from a wide range of vascular plants, act in plants as antioxidants [7]. Flavonoids, has ability to reduce free radical formation and to scavenge free radicals. The capacity of flavonoids to act as antioxidants in vitro has been the subject of several studies in the past years, and important structure-activity relationships of the antioxidant activity have been established. Many studies have suggested that flavonoids exhibit biological activities, including hepatoprotective activity. Hepato toxicity is generally produced by hazardous chemicals, alcohol intake and drugs like paracetamol. Chemical treatment significantly increased the serum enzyme levels, namely Alanine Transaminases (ALT), Aspartate transaminases (AST) and Alkaline phosphatase (ALP) indicating chemical induced hepatocellular toxicity[8]. Serum levels of these enzymes are very sensitive markers employed in the diagnosis of liver diseases. When the hepatocellular plasma membrane is damaged, the enzymes normally present in the cytosol are released into the blood stream. This can be quantified to assess the type and extent of liver injury [9].

Above all statements potentiate us to study and evaluate hepatoprotective activity of the plant. Hence we have studies different polar extracts of *H. mutabilis* leaf, stem and flowers for presence antioxidant activity of phenolic

compounds which were evaluated for hepatoprotective potential against chemical (CCl_4; carbon tetrachloride) induced hepatotoxicity. Different extracts were quantitatively standardized with quercetin and kaemferol.

MATERIALS AND METHODS

Collection and identification of the plant

Plant material was collected from Sangamner area of Ahmednagar district (Maharashtra, India) in the month of November. The plant was previously identified by Dr. S. C. Pal, Head of Department, Pharmacognosy, MVP College Nashik. Specimen herbarium was submitted to Botanical Survey of India, Pune for authentication.

Extraction of plant material

Shade dried and coarsely powdered Leaves, stem and flowers (250gm each) were defatted with petroleum ether in soxhlet apparatus followed by maceration with ethanol [10]. Ethanol extracts of leaf (HML), stem (HMS) and flowers (HMF) were prepared by filtration of macerated solvents followed by concentration and dying under vacuum [11]. Solid mass were taken for further study.

Determination of total phenolic content of *H. mutabilis* Linn.

Total phenolic content of extracts were determined using the Folin–Ciocalteau assay according to a previously described method [12].

Evaluation of antioxidant activity

DPPH scavenging activity:

Preparation of extracts: Free radical scavenging activity determination Different concentrations of extracts (25, 50, 75, 100, 200, 500 μg/ml, in methanol) were added at an equal volume (2.5 mL) to methanol solution of DPPH (0.3 mM, 1 mL). The method was carried out according to Nikolova and Katalinic [13, 14]. The IC50 values were calculated by sigmoid non-linear regression model using plots IC50 values denote the concentration of sample required to scavenge 50% of DPPH radical. Ascorbic acid is used as standard (std.) for comparison of IC 50 value and antioxidant activity. The experiment was performed in triplicate.

Nitric acid scavenging activity:

Sodium nitroprusside 10 mM was prepared in phosphate buffer pH 7.4. To 1 ml of various concentrations of test compound, sodium nitroprusside 0.5 ml was added. The test tubes were incubated at 25 ºC for 5 hrs after which, 0.5 ml of Griess reagent was added. The absorbance of the chromophore was read at 546 nm. The experiment was performed in triplicate [15].

Standardization of extracts:

Estimation of quercetine and kaemferol

Test solution: For each sample about 100 mg of sample extracts were dissolved in 10 ml of methanol and sonicated for 15 minutes. The resulting solution is used for the further analysis by Camag HPTLC instrument.

Standard solution: 10 mg of standard (quercetin/kaemferol) was dissolved in 10ml methanol (Stock solution). 1 ml of stock solution was diluted to 10 ml (100 ppm).

Stationary phase: TLC Aluminum sheets coated with silica gel 60F254 (Merck, Germany). Solvent system: Toluene:Ethyl acetate:formic acid (7:3:0.5 v/v/v) for quercetin and Toluene:Ethyl acetate:formic acid (5:5:1 v/v/v) for kaemferol.

Technique of development: Ascending

Procedure: 15 μl (microliter) of the test solution HML, HMS, HMF as 1, 2, 4, 6, 8, 10 μl standard solutions were applied separately on a precoated silica gel 60 F254 TLC plate (E. Merck) of uniform thickness of 0.2 mm. The plate was developed in the solvent system in a twin trough chamber to a distance of 8.5 cm. The plate was scanned at 254 nm at Band width 6 mm and Chamber saturation 20 minute using Camag HPTLC insrutment.

Acute toxicity studies: The acute toxicity study was carried out according to OECD test guideline 420 (Fixed Dose Procedure) in Wistar albino rats with approval from AEC of the institution. All extracts fall under class 4 hence; One-tenth of this dose (200 mg/kg body wt.) was selected as the maximum therapeutic dose for the evaluation [16, 17].

Hepatoprotective activity:
Wistar albino rats weighing in the range of 150–200 g body weight are grouped into six groups containing six animals in each. Group I served as the control (normal saline, 10 ml/kg b.w. p.o. for seven days). Groups II–V received equal mixture of CCl_4 and olive oil (50%, v/v) 2ml/kg, i.p. Groups III–V received the ethanolic extracts HMF, HMS and HML at 200 mg/kg b.w. doses p.o. respectively for seven days. The standard drug Liv 52 (25mg/kg b.w./day p.o.) was administered to Group VI animals for seven days. All the animals were anaesthetized and then sacrificed on day 8. The blood samples were collected, clear serum was separated and used for the biochemical investigations to assess for Alanine Transaminases (ALT), Aspartate transaminases (AST) and Alkaline phosphatase (ALP).

For histopathological studies the liver sections were prepared in formaline and sections were studies for change in cell or tissue microscopy [18].

Statistical analysis
Data are presented as means ± Standard Error of Mean (SEM). The results were subjected to one-way ANOVA followed by Dunnet test, Significant levels were defined as $^{**}P < 0.01$ and $^{*}P < 0.05$.

RESULTS

Collection and identification and extraction of plant
The plant was collected in Sangamner area, Ahmednagar District, Maharashtra. Herbarium was prepared and fresh flowers, leaves and stems were collected in the month of October. Shade dried parts are powdered coarsely, deffated and extracted with ethanol. The plant specimen was identified and authenticated as *Hibiscus mutabilis* L. by Dr. S. C. Majumdar, Scientist D., Botanical Survey of India, Pune (specimen voucher number RDNHMPL1 (letter number- BSI/WC/Tech/2008/497).

Determination of total phenolic content of *H. mutabilis* Linn.
Ethanol fraction of leaf stem and flowers designated as HML, HMS and HMF respectively, which were subjected to determination of total phenolic content by Folin–Ciocalteau assay method. Gallic acid was used as standard hence, different dilutions of Gallic acid solutions were subjected to prepared calibration curve. From the trial (data not shown) concentrations 0 microgram per ml to 10 ug/ml solutions established straight line graph and hence selected for determination of phenolic content as shown in Figure 1. HML, HMS and HMF showed 0.528, 0.512 and 0.660 as absorbance which further calculated mg of Gallic acid equivalence per gm of extract as 15.85, 15.38 and 19.89 respectively by dilution factor as shown in Table 1.

Figure 1: calibration curve for Gallic acid showing equation of line and R^2 value

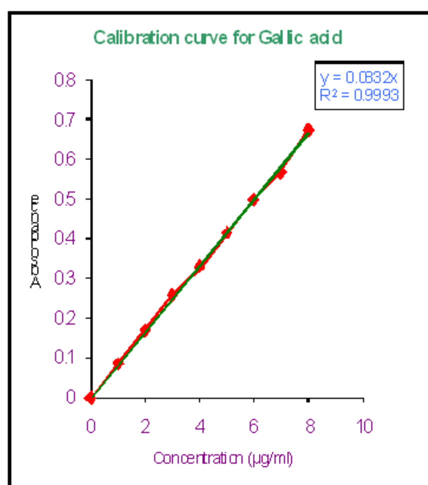

Table 1: Absorbance of extracts and phenolic content expressed in mg of Gallic acid equivalence/gm of extract

Sample	Absorbance	Phenolic content
Leaf Extract(HML)	0.528	**15.85**
Stem Extract(HMS)	0.512	**15.38**
Flower Extract(HMF)	0.660	**19.89**

Further ethanol fractions were standardized using Quercetine and Kaemferol by modern technique of plant drug analysis using HPTLC. All the fractions showed presence of Quercetin with concentrations 0.24 %, 0.07 %, 0.25 % of HML, HMS and HMF. HML and HMF also showed presence of Kaemferol in the concentration of 0.06 % and 0.28 % respectively (Table 2). Calibration curve for Quercetin and Kaemfero were prepared and from the area under peak were taken for determination of concentrations.

Table 2: quantification of Quercetin and kaemferol

Sample	Quercetin		kaemferol	
	Area	**Quantification** (Gram per cent)	**Area**	**Quantification** (Gram per cent)
HML	2566	0.24 %	1631.7	0.06 %
HMS	669	0.07 %	--	--
HMF	2719	0.25 %	4771.5	0.28 %
Equation	Y=7.386x – 114.8		Y=27.55x- 1120	
R^2 value	0.989		0.990	

Evaluation of antioxidant activity

Three fractions of *H. mutabilis* were subjected to evaluation of in vitro antioxidant activity by valid methods of determination. Scavenging of free radicals generated by DPPH in the solution was shown by all the three extracts which were compared with scavenging carried out by ascorbic acid standard. In this in vitro study, HML showed highest percent scavenging of 94.35±0.03 at concentration of 500ug/ml where as HMF and HMS showed it as 82.32±0.03 and 68.17±0.10 respectively (Table 3).

Table 3 Antioxidant activity in terms of percent scavenging activity by DPPH method

Sample	% Scavenging (mean ± SEM)					
	25µg/ml	**50µg/ml**	**75µg/ml**	**100µg/ml**	**200µg/ml**	**500µg/ml**
HMS	6.44 ±0.05	18.66±0.02	26.55±0.12	37.11±0.21	51.44±0.16	68.17±0.10
HML	26.44±0.05	48.22±0.12	72.22±0.16	83.44±0.09	90.44±0.13	94.35±0.03
HMF	14.23±0.11	29.22±0.17	39.22±0.05	47.77±0.01	53.65±0.06	82.32±0.03
Std.	15.64±0.09 (5µg/ml)	34.51±0.11 (10µg/ml)	51.45±0.04 (15µg/ml)	73.87±0.01 (20µg/ml)	91.93±0.03 (25µg/ml)	-

The percent scavenging are concentration dependant and goes to increase from concentration 25ug/ml to 500ug/ml.

In support to DPPH model, the nitric oxide scavenging model also showed comparable results. HML, HMFand HMS showed percent scavenging 85.89±0.12, 68.35±0.14 and 58.66±0.03 respectively in the concentration of 500ug/ml where ascorbic acid taken as standard as shown in Table 4.

Table 4: Antioxidant activity in terms of percent scavenging activity by Nitric oxide method

Sample	% Scavenging (mean ± SEM)					
	25µg/ml	**50µg/ml**	**75µg/ml**	**100µg/ml**	**200µg/ml**	**500µg/ml**
HMS	6.35±0.13	15.79±0.01	26.65±0.12	31.95±0.12	38.91±0.08	58.66±0.03
HML	20.32±0.12	26.85±0.18	45.36±0.016	65.55±0.11	80.32±0.17	85.89±0.12
HMF	15.62±0.05	20.76±0.13	35.62±0.23	41.51±0.06	55.36±0.02	68.35±0.14
STD	25.17±0.04 (5µg/ml)	48.29±0.1 (10µg/ml)	63.18±0.05 (15µg/ml)	78.19±0.02 (20µg/ml)	93.78±0.05 (25µg/ml)	--

Hepatoprotective activity

Effect of different ethanolic fractions of *H. mutabilis* on ALP, AST and ALT level in serum

Animals in the group II, III, IV, V and VI were treated with Carbon tetrachloride to induce hepatotoxicity. Effect of extracts in the concentration of 200 mg/kg BW in group III, IV and V were observed which showed enzyme level in blood serum as shown in Table 6.

Table 6: Estimation of ALP, AST and ALT in animals of different groups

Group	Description	ALP (Mean ± SEM)	AST (Mean ± SEM)	ALT (Mean ± SEM)
I	Control	14.22 ± 3.1*	61.65 ± 2.4*	19.2 ± 4.5**
II	CCl4 control	156.63±3.8**	236.14±16.69*	62.42±14.92
III	Standard (Liv52)	24.55±3.67**	53.85±3.4**	28.07±5.61**
IV	HMF	39.04±12.3**	147.3±6.4**	39.86±9.5**
V	HMS	59.04±12.8**	83.25±2.6*	43.21±7.21**
VI	HML	15.38±2.8**	66.2±7.2**	24.37±2.11**

*Values are expressed in mean ± S.E.M.; n = 6 in each group. **P < 0.01, *P < 0.05*

Group II showed elevated enzyme level ALP, AST and ALT due to CCl_4 as 156.63±3.8, 236.14±16.69 and 62.42±14.92 respectively. This elevated level found to be lower in animals treated with Liv 52 as standard for Liver protection which showed values as 24.55±3.67, 53.85±3.4 and 28.07±5.61 for ALP, AST and ALT respectively. The results from group III, IV and V were compared with CCl_4 treated Group and showed significant decrease in level of enzyme. The data indicated that the ALP, AST and ALT activities were reduced significantly after treatment of rats with the ethanol fractions of *H. mutabilis* leaf, Stem and flower (200 mg/kg b.w) and Liv 52 as compare to control groups ($P < 0.05$).

Further liver sections were studied for change in liver cell histology. These changes in treatment groups were studied by centrilobular necrosis, vacuolization and macro-vesicular fatty change.

DISCUSSION

The present study indicated that standardized antioxidant ethanol fractions of leaf, flower and stem of *Hibiscus mutabilis* Linn. significantly inhibits hepatic injuries induced by Carbon tetrachloride. Oral administration of defatted ethanol fractions at the dose of 200mg/kg lowers the enzyme levels in blood serum of rats which found to be increased in hepatotoxin treated group. It is well established that CCl_4 is metabolized in the liver to the highly reactive trichloromethyl radical and this free-radicals leads to auto-oxidation of the fatty acids present in the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane [19, 20]. Herbal drugs inhibit the CYP2E1 enzyme activity in hepatic microsomes and give protection against hepatotoxicity induced by CCl_4 [21]. CCl_4 treatment significantly increased the enzyme levels, namely ALT, AST and ALP in serum indicating chemical induced hepatocellular toxicity. These enzymes are very sensitive markers employed in the diagnosis of liver diseases. When the hepatocellular plasma membrane is damaged, the enzymes normally present in the cytosol are released into the blood stream [9].

The liver injury due to toxins can result in defective excretion of bile by hepatocytes which are reflected as their increased ALP levels in serum [22]. Ethanol fraction treated groups restored the liver enzyme parameters at a given dose. ALT is found to be significant which, is a specific marker of liver injury due to toxic drugs, alcohol and virus [23]. The protective effect may be the result of stabilization of plasma membrane thereby preserving the structural integrity of cell as well as the repair of hepatic tissue damage caused by CCl_4 [24]. In this connection, ethanol fractions of *H. mutabilis* at dose of 200 mg/kg b.w. decrease the activities of CYP450 and serum transaminases (ALT and AST) elevated in the liver of CCl_4 treated rats (Table 3). Results of histopathological studies provided supportive evidence for biochemical analysis.

Histology of liver section of normal control animal exhibited normal hepatic cells each with well-defined cytoplasm, prominent nucleus and nucleolus and well brought out central vein, whereas that of Group II intoxicated group animal showed total loss of hepatic architecture with centrilobular hepatic necrosis, fatty changes, toxicity vacuolization.

The sections of liver taken from the animals treated with standard drug Liv 52 showed hepatic architecture, which was similar to that of control. When compared to the extract treated animals, the animals treated with extracts of flower exhibited significant liver protection against the CCl_4 as evident by presence of less fatty infiltration, hepatic cords found normal and no necrosis of the cell.

In support to validate the extract and activity, standardization of extracts was carried out using HPTLC and evaluate for antioxidant activity. The fact that antioxidant agents inhibit carbon tetrachloride-induced liver damage [25] prompted us to study the antioxidant effect of the isolated compounds. Trichloromethyl radicals formed from CCl_4 converted to trichloromethyl peroxy radical. This radical form covalent bond with sulfhydryl groups of several membrane molecules like GSH leading to their depletion and causes lipid peroxidation. Lipid peroxidation initiates number of reactions leading to tissue necrosis [26]. It is apparent from the present result that the antioxidant property of *H. mutabilis* extracts prevent the formation of trichloromethyl peroxy radicals thereby reducing tissue damage. Therefore the hepatoprotective activity of Ethanol fractions are due to antioxidant chemical present in the plant especially more due to flavonoid and other phenolic content.

CONCLUSION

In the present study, defatted ethanol fractions of *Hibiscus mutabilis* Linn. Possess strong hepatoprotective activity in a rat model of CCl4-induced hepatotoxicity and antioxidant activity. The hepatoprotective activity of these extracts may be due to its free radical-scavenging and antioxidant activity, resulting from the presence of some flavonoids and phenolic compounds in the extracts. Additional studies are in progress to better understand the

mechanism of action of these extracts that is responsible for the hepatoprotective and antioxidant effects. Earlier different flavonoids are identified in the plant which support new finding of the medicinal use. Further additional studies are in progress to better understand the mechanism of action of fractions that is responsible for the hepatoprotective and antioxidant effects.

List of abbreviation
Microgram (µg), milligram (mg), mililiter (ml), gram (gm), liter (l)

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