EVALUATION OF ANTIOXIDANT PROPERTIES OF LEAVES OF *HIBISCUS SYRIACUS L.*

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Summary

Hibiscus syriacus L. (Malvaceae) well known drug in the system of Ayurveda system of Medicine. In the present study, antioxidant activity of Methanolic extract (ME) and its chromatographic methanolic fraction (CMF) of leaves of *H. syriacus* was evaluated in several *in vitro* and *ex vivo* models. Further, preliminary phytochemical analysis and TLC fingerprint profile of the extract was established to characterize the extract which showed antioxidant properties. The *in vitro* and *ex vivo* antioxidant potential of leaves of *H. syriacus* was evaluated in different systems viz. Hydrogen donating activity by DPPH reduction, superoxide radical scavenging activity in NBT system, reducing power and inhibition of lipid peroxidation induced by TBARS in liver homogenate. The CMF was found to have different levels of antioxidant properties in the models tested. In scavenging DPPH and superoxide radicals, its activity was intense (EC_{50} = 248.00 and 105.00 μg/ml respectively) while in reducing ability by ferric radical, it was 15 mg/ml comparable to ascorbic acid was moderate. It also inhibited lipid peroxidation of liver homogenate (EC_{50} = 291.6100 μg/ml). The free radical scavenging property may be one of the mechanisms by which this drug is effective in several free radical mediated disease conditions.

KEY WORDS: *Hibiscus syriacus,* DPPH, Superoxide, Reducing power, Lipid peroxidation, TLC finger printing.

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Introduction

Flavonoids are diphenylpropanes that commonly occur in plants (more than 4000 flavonoids have been isolated) and are frequently components of the human diet (the daily human intake in the Western countries was recently reestimated to be about 23 mg/d). The immediate family members of flavonoids include flavones, isoflavones and the 2,3-dihydroderivatives of flavone, namely flavanones, which are interconvertible with the isomeric chalcones. Epidemiological evidence suggests an inverse relationship between dietary intake of flavonoids and the risk of coronary heart disease. It has been suggested that oxidative modification of LDL plays an important role in the development of human atherosclerosis. Thus, protecting LDL from oxidation by such compounds as flavonoids, may be an effective strategy to delay or prevent the progression of the disease (1, 2). The leaves are diuretic, expectorant and stomachic (3). A decoction of the flowers is diuretic, ophthalmic and stomachic. It is also used in the treatment of itch and other skin diseases (4). Report provided reliable information about its antitussive activity (5). The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial bio-molecules. If they are not effectively scavenged by cellular constituents, they lead to disease conditions 13. In recent years one of the areas which attracted a great deal of attention. The present study was undertaken to evaluate the potential antioxidant activity in the control of degenerative diseases in which oxidative damage has been implicated. Several plant extracts and different classes of phytochemicals have been shown to have antioxidant activity.

Materials and Methods

Plant material

Leaves of *Hibiscus syriacus* was collected from College campus Karnataka (India) in the month of May-June 2005 after these were identified by comparing the specimen and the herbarium is placed in the Pharmacognosy Department, Rural College of Pharmacy. The plant material was air dried, powdered and sieved through 40-mesh size and material was stored in well closed container.

Extract preparation and fractionation of extracts

Separate maceration of air-dried, powdered leaves of *Hibiscus syriacus* afforded methanol extract (ME) in 6.65% (w/w) yield. ME (40 g) was fractionated by loading on silica gel (100-200 mesh) and eluted with petroleum ether (5.23 g), benzene (5.76 g), chloroform (1.30 g) and methanol (CMF) (24.4 g).

Preliminary phytochemical screening and TLC finger print profile of *H. syriacus*.

The Methanolic extract and various chromatographic fractions were subjected to preliminary phytochemical screening (6) for detection of various phytoconstituents.

TLC finger printing profile was established for CMF using HPTLC. A stock solution (1 mg/ml) was prepared in methanol. Suitably diluted stock solution was spotted on pre-coated Silica gel G60 F254 TLC plates using CAMAG Linomat V Automatic Sample Spotter and the plates were developed in solvent systems of different polarities to resolve polar and non-polar components. The plates were scanned using TLC Scanner 3 (CAMAG) at 254 nm (absorbance/reflectance mode) and 366 nm (fluorescence/reflectance mode) and R_f values, spectra, λ max and peak areas of resolved bands were recorded. Relative percentage area of each of the bands was calculated from

peak areas. Developed chromatograms in different solvent systems were then sprayed with 5% methanolic ferric chloride to detect phenolic compounds and flavonoid-type compounds respectively.

In-vitro antioxidant activity:

Hydrogen Donating -Activity

Hydrogen donating activity was quantified in presence of stable DPPH radical on the basis of Blois method. (8). briefly, to a methanolic solution of DPPH (100 μ M, 2.95 ml), 0.05 ml of test compounds dissolved in methanol was added at different concentrations (2—10 mg/ml). Reaction mixture was shaken and absorbance was measured at 517 nm at regular intervals of 30 s for 5 min. Ascorbic acid was used as standard. The degree of discoloration indicates the scavenging efficacy of the extract. $EC_{50\ was}$ calculated as 50% reduction in absorbance brought about by sample compared with blank (8).

Assay for superoxide radical scavenging activity

Superoxide scavenging was carried out by using alkaline DMSO method (9). Solid potassium superoxide was allowed to stand in contact with dry DMSO for at least 24 h and the solution was filtered immediately before use. Filtrate (200 ml) was added to 2.8 ml of an aqueous solution containing nitroblue tetrazolium (56 m M), EDTA (10 m M) and potassium phophate buffer (10 m M, pH 7.4). Sample extract (1 ml) at various concentrations (30—1500m g/ml) in water was added and the absorbance was recorded at 560 nm against a control in which pure DMSO has been added instead of alkaline DMSO.

Determination of reducing power

The reducing power of ME and CMF was determined according to the method (10). Samples were mixed with 5 ml phosphate buffer (2M, pH 6.6) and 5 ml potassium ferricyanide (1%). The mixture was then incubated at 50^0 C for 20 minutes. 5 ml trichloroacetic acid (10%) was added and the mixture was centrifuged at 4000 rev./ min. The upper 5 ml solution was then mixed with 5 ml distilled water and 1 ml ferric chloride (0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid (0.3 mg) was used as standard.

Measurement of effect on lipid peroxidation on rat liver homogenate

Male Sprague-Drawley rats (160—180 g) were purchased from the animal house of the Gold Mohur Lipton India Ltd, India. Ethical clearance for the animal study was obtained from the institutional animal ethics committee. These were kept in the departmental animal house at 26 ± 2°C and relative humidity 44—55% light and dark cycles of 10 and 14 h respectively for one week before the experiment. Animals were provided with rodent diet (Amruth, India) and water ad libitum. Randomly selected male rats were fasted overnight and were sacrificed by cervical dislocation, dissected and abdominal cavity was perfused with 0.9% saline. Whole liver was taken out and visible clots were removed and weighed amount of liver was processed to get 10% homogenate in cold phosphate buffered saline, pH 7.4 using glass teflon homogeniser and filtered to get a clear homogenate.

Assay of Lipid Peroxidation

The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid-reactive substances (TBARS) by using the standard method (11). In brief, different concentration of extracts (200—1000 mg/ml) in water was added to the liver homogenate. Lipid peroxidation was initiated by adding 100 m l of 15 mM $FeSO_4$ solution to 3 ml of liver homogenate (final concentration was 0.5 mM). After 30 min, 100 ml of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA. After 10 min tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated in a hot water bath at 85 °C for 30 min to complete the reaction. The intensity of pink coloured complex formed was measured at 535 nm. The values of TBARS were calculated from a standard curve (absorption against concentration of Tetraethoxy propane) and expressed as nmol/mg of protein. The percentage inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls not treated with the extracts.

Results

The antioxidant activity of the CMF of *H. syriacus* leaves was evaluated in different *in-vitro* and *ex-vivo* models. Further, the same methanolic extract was subjected to preliminary phytochemical screening for the presence of different constituents **Table 1** and also characterized phytochemically by establishing its TLC fingerprint profile. Phenolics were found to be major groups present along with flavonoids in the methanolic extract. Compounds of different polarities were resolved in two different solvent systems and scanned. The TLC fingerprint profile comprises of the bands resolved, R_f values,

spectral details and λ max when scanned in UV 254 and 366 nm. Derivatization of the TLC plates with 5% methanolic ferric chloride revealed the presence of phenolic compounds. The details of the TLC finger print profile are given in **Table 2** and the chromatograms in **Figure 1**.

Table 1. Preliminary phytochemical screening of methanolic extract of *Hibiscus Syriacus* leaves

Tested for	**Presence/absence***	**Test performed**
Phenols	+++	5% Methanolic Ferric chloride solution
Tannins	-	Braemer's test
Steroids and Terpenoids	- Terpenoids	Liebermann Burchardt test
Alkaloids	-	Dragendorff's test
Anthraquinones	-	Bornträger test
Flavonoids	+++	Shinoda's test

* – absent; +++ Abundant

ANTIOXIDANT ACTIVITY

The antioxidant activity of the CMF of *H. syriacus* leaves was exhibited levels of antioxidant activity in all the models studied. It showed a concentration dependent hydrogen donating activity by inhibiting DPPH radical with EC_{50} = 248 µg /ml **Table 3**.

Table 2. TLC finger printing profile of bioactive fraction of *Hibiscus Syriacus* .

Scanned at	Solvent system 1			Solvent system 2			Solvent system 3		
	Rf	λ_{max}	Relative %	Rf	λ_{max}	Relative %	Rf	λ_{max}	Relative %
	0.11	319	3.68	0.31	330	64.30	0.23	200	0.98
	0.16	348	1.71	0.45	203	5.31	0.31	323	2.60
	0.20	312	5.90	0.74	200	30.39	0.44	318	8.13
254 nm	0.33	328	3.19				0.49	324	1.24
	0.62	580	0.74				0.60	328	12.31
	0.80	233	9.68				0.81	228	47.09
	0.90	332	75.10				0.91	327	27.63
	0.06	323	8.54	0.08	328	4.87	0.11	324	3.60
	0.13	313	5.96	0.37	329	92.34	0.14	323	1.90
	0.10	335	7.45	0.40	203	3.79	0.35	324	6.90
	0.23	309	23.36				0.38	317	1.21
366 nm	0.37	580	30.32				0.49	327	2.17
	0.47	613	24.37				0.56	327	11.11
	0.56	223	7.41				0.72	329	54.14
	0.92	332	20.15				0.78	200	4.35
							0.89	296	13.59
							0.95	228	1.03

Solvent system 1. Toluene/Ethyl acetate (7:3 v/v); Solvent system 2. Ethyl acetate/Methanol/Water (10:1.35:1.00 v/v); Solvent system 3. n-butanol/glacial acetic acid/water (6:2:2 v/v).

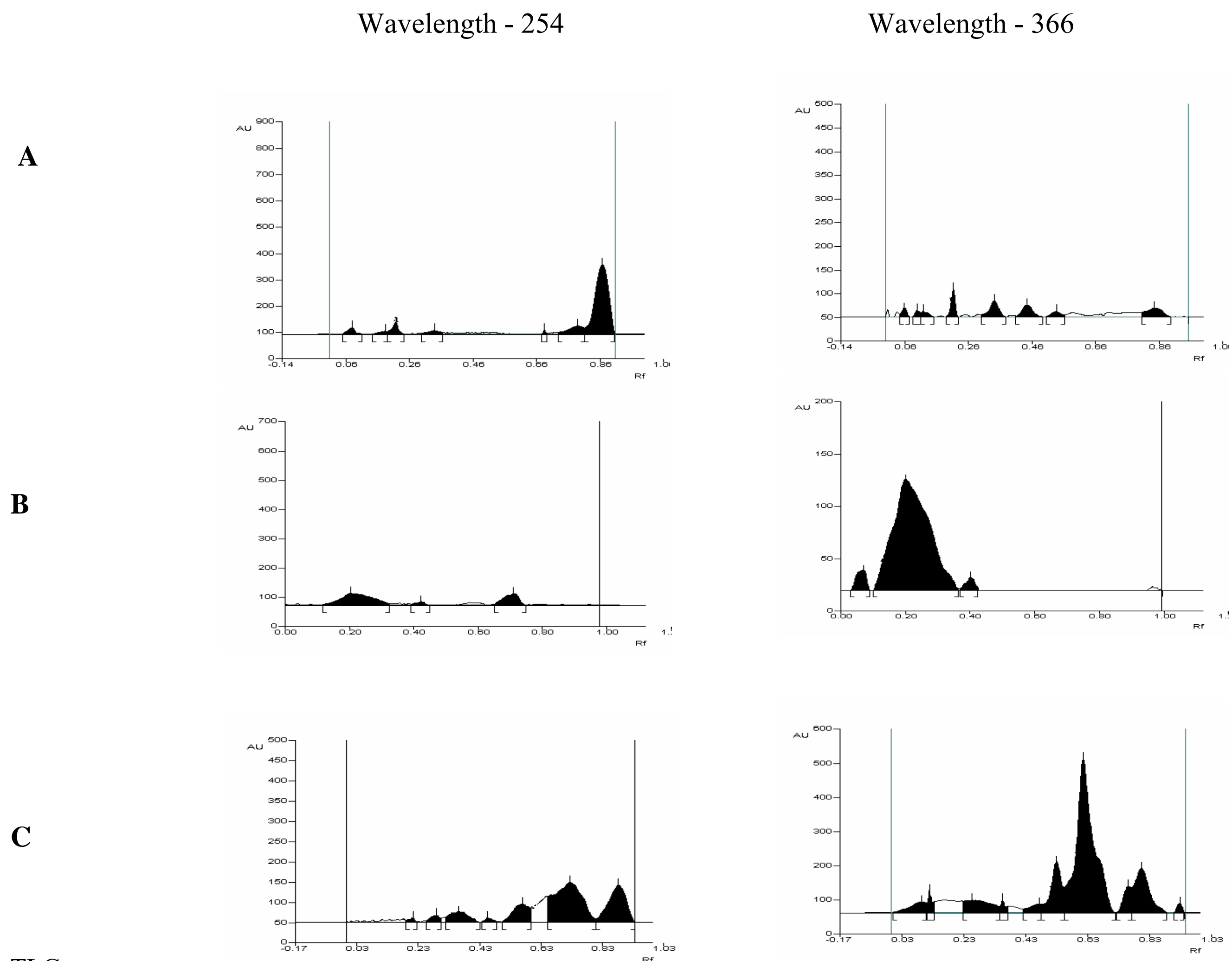

Figure 1. TLC chromatogram of bioactive fraction of *Hibiscus Syriacus* run in three different solvent system and scanned at 254 and 366 nm (solvent systems as in Table 3) **A-** Solvent system 1**,** B- Solvent system 2, **C-** Solvent system 3**.**

The extract scavenged superoxide radicals in a dose dependent manner with EC_{50} = 105 µg /ml, which is comparable to the activity of ascorbic acid (EC50 = 15.17 µg/ml) **Table 4**. The extract also showed a moderate reducing power activity between 5 to 15 mg/ml in a dose dependent manner **Table 5**. Protection from damage due to lipid peroxidation The extract protected hepatocytes from damage due to lipid peroxidation induced in rat liver homogenate by ferric-ADP and ascorbate in a dose dependent manner between 10–150 µg/ml, with an EC_{50} = 292.00 µg/ml **Table 6**.

Table 3. Antiradical activity of ME and CMF of *Hibiscus Syriacus* leaves observed with DPPH.

Samples	Concentration (µg/ml)	% inhibition	EC_{50} (µg/ml)
	250	18.81± 1.36	
ME	**500**	28.68 ± 1.82	798.0
	750	34.07 ± 0.81	
	1000	71.70 ± 1.69	
	100	23.67 ± 0.20	
CMF	**200**	40.18 ± 1.43	248.0
	300	54.21 ± 1.49	
	400	70.77 ± 1.40	

Values are mean ± S.E.M. of three replicate analyses.

Table 4. Superoxide anion scavenging activity of ME and CMF of *Hibiscus Syriacus* leaves

Samples	Concentration (µg/ml)	% inhibition	EC_{50} (µg/ml)
	250	25.76 ± 1.48	
ME	**500**	37.45 ± 2.09	678.0
	750	43.96 ± 1.64	
	1000	65.30 ± 3.76	
	50	32.14 ± 3.16	
CMF	**100**	53.20 ± 1.84	105.0
	150	65.73 ± 1.70	
	200	75.51 ± 2.22	
Ascorbic acid			24.0

Values are mean ± S.E.M. of three replicate analyses.

Table 5. Reducing power determination of different concentrations of ME and CMF of *Hibiscus Syriacus* .

Sample	Reducing powers of different concentrations (mg/ml).			
	0.0	5.0	10.0	15.0
ME	0.021 ± 0.01	0.100 ± 0.07	0.210 ± 0.12	0.323 ± 0.10
CMF	0.024 ± 0.01	0.129 ± 0.09	0.251 ± 0.16	0.373 ± 0.20

Values are mean ± S.E.M. of three replicate analyses.

Ascorbic acid (0.3 mg) was used as standard, giving a reading of 0.410 at 700 nm.

Table 6. Inhibition of lipid peroxidation induced in rat liver homogenate by ME and CMF of *Hibiscus Syriacus* leaves.

Samples	Concentration (µg/ml)	% inhibition	EC 50 (µg/ml)
ME	**200**	18.67 ± 0.98	525.06
	400	39.22 ± 1.26	
	600	65.07 ± 2.22	
	800	75.54 ± 0.99	
	1000	80.21 ± 2.56	
CMF	**100**	23.54 ± 0.87	291.61
	200	40.27 ± 0.91	
	300	52.10 ± 2.43	
	400	66.20 ± 3.05	
	500	73.30 ± 1.29	
Ascorbic acid			29.07

Values are mean ± S.E.M. of three replicate analyses.

Discussion

Free radicals have been implicated in many disease conditions, the important ones being superoxide radical, hydroxy radical, peroxyl radical and singlet oxygen. Herbal drugs containing radical scavengers are gaining importance in treating such diseases. Many plants exhibit efficient antioxidant properties owing to their phenolic constituents (12). In the present experiment, the preliminary phytochemical screening of the CMF of *H. syriacus* leaves showed the presence of phenolic compounds. TLC fingerprint profile

also suggests the presence of phenolic compounds. We investigated the antioxidant activity of the CMF of *H. syriacus* leaves and the possible mechanism involved, basing on the response obtained in the four different *in-vitro* and *ex-vivo* models covering major radicals' viz., superoxide, hydroxyl and reducing power activity.

DPPH is one of the free radicals generally used for testing preliminary radical scavenging activity of a compound or a plant extract. In the present study, CMF of *H. syriacus* leaves showed a good hydrogen donating activity by scavenging DPPH radical. The possible phenolic compounds, as observed on TLC plates produced blue colouration when derivatised with 5% methanolic ferricchloride solution. Furthermore, from the point of sample application to the last band (R_f = 0.33 and 0.60 in solvent system 1 and 3 respectively) that showed antiradical activity,

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species (13). The extract was found to be an efficient scavenger of superoxide radical generated in riboflavin-NBT-light system *in- vitro* and its activity was comparable to that of ascorbic acid. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (14). The extract showed a moderate nitric oxide scavenging activity. The lipid radicals thus generated would initiate chain reaction in the presence of oxygen, giving rise to lipid peroxide, which breaks down to aldehydes such as malondialdehyde, which are known to be mutagenic and carcinogenic (15). In the present study, the CMF of *H. syriacus* leaves showed potent inhibition of lipid peroxidation induced by TBARS in liver homogenate.

In conclusion, from the above investigation, using several *in- vitro* and *ex- vivo* models, CMF of *H. syriacus* leaves was found to scavenge superoxide radical, nitric oxide radical,

reducing power and inhibited the lipid peroxidation. The activity was due to phenolic components present therein as revealed by the quenching of the DPPH radical by the compounds separated on the TLC. The antioxidant activity of the extract can be attributed to the presence of phenolic compounds like Delphinidin, petunidin, malvidin, quercetin and cyaniding (16) which has already been shown to have antioxidant properties The free radical scavenging property may be one of the mechanisms by which this drug is effective in traditional medicine.

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