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Antioxidant Activities of *Hibiscus furcatus* Roxb. ex DC. Extracts

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Abstract: The antioxidative potential of different solvent extracts of *Hibiscus furcatus* flowers were evaluated using 1,1-Diphenyl-2-Picrylhydrazyl (DPPH), 2, 2'-Azino-Bis(3-ethylbenzthiazoline-6-Sulphonic acid) (ABTS), superoxide radical, hydroxyl radical, nitric oxide radical scavenging activities and lipid peroxidation inhibition assay. Among those solvent extracts, ethyl acetate extract of *H. furcatus* exhibited highest level of antioxidant activities. The ethyl acetate extract also inhibited H_2O_2 mediated haemolysis and lipid peroxidation in human RBC.

Key words: *H. hisspidissimus, H. furcatus*, gossypin, DPPH, superoxide, hydroxyl, nitric oxide and lipid peroxidation

INTRODUCTION

Free radicals are constantly generated *in vivo* as a by-product of cellular metabolism. The predominant cellular free radicals are the superoxide (O_2) and hydroxyl (OH°) species and other molecules, such as Hydrogen Peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), although not themselves free radicals can lead to the generation of free radicals through various chemical reactions. These molecules are collectively called as Reactive Oxygen Species (ROS) and have the ability to cause oxidative changes within the cell. ROS can oxidise critical cellular components such as membrane lipids, proteins and DNA. These oxidative damages has been associated with diverse patho-physiological events, including cancer, atherosclerosis, diabetics, renal disease and neuro-degeneration (Seitz and Stickel, 2006; Simonian and Coyle, 1996; Halliwell, 2001).

Erythrocytes (RBC) have been extensively used to study oxidative damages. The Red Blood Cell (RBC) is unique among cells in that it combines very large concentrations of both iron (hemoglobin) and oxygen. This potentially dangerous combination of oxygen and iron within the RBC makes it a powerful promoters of oxidative processes are extremely susceptible to oxidative damages to poly unsaturated fatty acids of their membranes (Clemens *et al*., 1987; Scott *et al*., 1993). Many defense mechanisms have developed in living organisms to limit the levels of ROS and the damages they cause. Included among them are endogenous antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. Apart from these many naturally occurring substances in plants have antioxidant activities. *Hibiscus furcatus* DC. (now known as *Hibiscus hisspidissimus* Griffith) is a member of Malvaceae family growing throughout India.

The leaves of these plants are used to improve digestion, for eye diseases and are considered antihelminthic, the root bark is given as a remedy for poisons, swellings and for cleaning kidneys (Bindu *et al*., 1997). The flowers were reported for the presence of gossypin, gossypitrin and hibiscatin (Nair *et al*., 1981). The aim of the present study is to evaluate the *in vitro* antioxidant activities and inhibition of oxidative hemolysis and lipid peroxidation in human RBC induced by H_2O_2 of *H. furcatus* extract.

MATERIALS AND METHODS

Chemicals: 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) and 2, 2'-Azino-Bis (3-ethylbenzthiazoline-6-Sulphonic acid) (ABTS) and gossypin were purchased from Sigma-Aldrich (India). Nitroblue Tetrazolium (NBT), naphthylethylene diamine dihydrochloride, Thiobarbituric Acid (TBA) and potassium persulfate from SRL (India). HPLC grade acetonitrile and water were purchased from Merck (India). All other reagents were used of analytical quality.

Collection and preparation of extracts: *H. furcatus* flowers were collected from the outskirts of Amala campus and Thrissur. The flowers were separately dried in hot air oven (40°C) and powdered. The powdered flowers (100 g) were then extracted firstly with petroleum ether then with

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chloroform, ethyl acetate, ethanol and water using a soxhlet apparatus. The extracts were concentrated and evaporated under vacuum. The extracts thus obtained were used to check the antioxidant activities.

***In vitro* antioxidant assays**

Sample preparation: The extracts of *H. furcatus* in petroleum ether, chloroform, ethyl acetate were dissolved in minimum volume of DMSO and made to desired concentration with distilled water. Double distilled water is used for dissolving ethanol and water extracts. But for the DPPH assay all the extracts were dissolved in methanol.

1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging activity: DPPH scavenging activity was determined by the method proposed by Coruh *et al.* (2007). DPPH dissolved in methanol (0.05 mg mL^{-1} and a series of extract solutions with varying concentrations were prepared by dissolving the dried extracts in methanol and 0.1 mL of solutions from each extract was added to 1.4 mL of DPPH solution. The absorbance at 517 nm was recorded after 5 min of incubation at room temperature. Radical scavenging capacity of each extract has been calculated as the percentage of inhibition = ((absorbance of control - absorbance of extract)/absorbance of control) x 100.

2, 2'-Azino-Bis(3-ethylbenzthiazoline-6-Sulphonic acid) (ABTS) radical scavenging activity: The assay was carried out by interacting the extract with a model stable free radical derived from 2, 2'-Azino-Bis(3-ethylbenzthiazoline-6-Sulphonic acid) (ABTS). The production of radical cation was accomplished as described by Long and Halliwell (2001) with some modifications. Briefly a stock solution of ABTS (7 mM) was prepared in water.

To this solution ammonium persulphate (2.45 mM final conc) was added and the solutions were allowed to react leading to an incomplete oxidation of ABTS to generate ABTS radical.

The ABTS radical solution was diluted to an absorbance of 0.75 at 734 nm in phosphate buffer saline (PBS, pH 7.4) and 10 μL of different concentrations of the extract were added to 1 mL of ABTS radical solution. Absorbance was measured spectrophotometrically at 6 min after initial mixing using PBS as reference. Percentage of inhibition was calculated using the equation ((absorbance of control-absorbance of extract/ absorbance of control) x 100.

Superoxide radical scavenging activity: The reaction mixture contained 3 mg NaCl dissolved in EDTA (6 μM), riboflavin (2 μM) NBT (50 μM) and various concentrations (10 - 1000 μg mL^{-1}) of the extract and phosphate buffer (pH 7.8) in a final volume of 3 mL. The tubes containing the reaction mixture were uniformly illuminated with an incandescent lamp for 15 min and the absorbances were measured at 530 nm before and after the illumination (McCord and Fridovich, 1969). Percentage inhibition of superoxide radical was calculated using the equation ((absorbance of control-absorbance of extract)/ absorbance of control) × 100.

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging activity of the extract was measured by studying the competition between deoxyribose and test compounds for the hydroxyl radicals generated from Fe3+/ascorbate/EDTA/H_2O_2 system (Fenton reaction). The hydroxyl radicals attack deoxyribose which eventually results in the formation of thiobarbituric acid reacting substances (Kunchandy and Rao, 1990).

The reaction mixture contained deoxyribose (2.8 mM), ferric chloride (0.1 mM) EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM), KH_2PO_4- KOH (20 mM, pH 7.4) and various concentrations of the extracts were incubated for 1 h at 37°C. Deoxyribose degradation was measured as thiobarbituric acid reactive substrate by the method of Ohkawa *et al.* (1979). The inhibition produced by different concentrations of the extracts were calculated. Percentage inhibition of hydroxyl radical was calculated using the equation ((absorbance of control - absorbance of extract)/ absorbance of control) x 100.

Nitric oxide radical scavenging activity: Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were by Griess reaction (Green *et al.*, 1982). The reaction mixture (3 mL) containing sodium nitroprusside (10 mM) in Phosphate Buffered Saline (PBS) and the *H. furcatus* extracts (from 1 μg to 1000 μg mL^{-1}) was incubated at 25°C for 150 min. After incubation, 0.5 mL of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbence of the chromophore formed was measured at 546 nm. Percentage of inhibition = ((absorbance of control - absorbance of extract)/ absorbance of control) x 100.

Lipid peroxidation assay: The level of lipid peroxidation was measured by the method of Ohkawa *et al.* (1979). Different concentrations of extract (10-1000 μg mL^{-1}) was incubated with 0.1 mL rat liver homogenate (25%)

containing 30 mM KCl, Tris-HCl buffer (0.04 M, pH 7.0), ascorbic acid (0.06 mM) and ferrous ion (0.16 mM) in a total volume 0.5 mL for 1 h. After incubation, 0.4 mL of reaction mixture was treated with 0.2 mL of SDS (8.1%), 1.5 mL of TBA (0.8%) and 1.5 mL of acetic acid (20%, pH 3.5) were incubated for 1 h in a boiling water bath at 100°C. After 1 h, the reaction mixture was removed from the water bath, cooled and added 5 mL of pyridine: butanol (15:1), mixed thoroughly and centrifuged at 3000 rpm for 10 min.

Absorbance of the clear supernatant was measured at 532 nm against pyridine : butanol. Percentage inhibition of lipid peroxidation was calculated using the equation ((absorbance of control - absorbance of extract)/ absorbance of control) x 100.

Protective effect of *H. furcatus* extract against H_2O_2 induced haemolysis and lipid peroxidation

Determination of inhibition of haemolysis: The inhibition of human erythrocyte hemolysis by crude methanol exract of *H. furcatus* was evaluated according to the procedure described by Tedesco *et al.* (2000) with slight modifications.

Human erythrocyte hemolysis was performed by with H_2O_2 as free radical initiator. To 100 µL of 5% (v/v) suspension of erythrocyte in PBS (pH 7.4), added 50 µL of extract with different concentrations (10-25 µg in PBS, pH 7.4). To this, 100 µL of 100 µM H_2O_2 (in PBS, pH 7.4) was added. The reaction mixtures were incubated at 37°C for 3 h. The reaction mixture was diluted with 3 mL of PBS and centrifuged at 2000 rpm for 10 min. The absorbance of the resulting supernatant was measured at 540 nm by spectrophotometer to determine the hemolysis. Likewise, the erythrocyte was treated with 100 µm H_2O_2 without addition of extract to obtain a complete hemolysis. The absorbance of the supernatant was measured at the same condition. Percentage of hemolysis was calculated by taking hemolysis caused by 100 µm H_2O_2 as 100%. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

Lipid peroxidation of RBC: Lipid peroxidation was measured by the method of Stocks and Dormandy (1971). Erythrocytes were mixed with 20% Trichloroacetic Acid (TCA) (1:1). After 1 h incubation at 4°C, samples were centrifuged (1500 rpm for 20 min at 20°C). Thiobarbituric Acid (TBA) was added to supernatant and samples were heated at 100°C for 15 min. The supernatant was collected and were measured spectrophotometrically at 532 nm. Results were presented as percent of lipid peroxidation in control.

HPLC analysis of *H. furcatus* extract for gossypin: Determination of gosyypin was perfomed by using a Shimadzu SPD-10 AVP HPLC system equipped with a multi solvent delivery system and an UV-VIS detector. The column was a Purospher star column rp-18, end capped, 5 µm, 250×4.60 mm (Merck, Germany). The mobile phase was composed of Acetonitrile and water (60:40) with isocratic elution. The flow rate was 1 mL min^{-1} with UV absorbance detection at 272 nm and sample injection volume was 20 µL. The column temperature was kept at 25°C. The extract was dissolved in Acetonitrile and water (1:1) and centrifuged for 15 min at 3000 rpm. The supernatant was filtered using 0.45 µm membrane filter. The gossypin in samples were identified by comparing the retention time (±5) and quantified by integrating peak areas with standard gossypin (Sigma).

Statistical analysis: All the experiments were done in triplicate and the data's subjected to statistical analysis using standard deviation of the mean.

RESULTS AND DISCUSSION

***In vitro* antioxidant assays:** *In vitro* antioxidant methods used for the evaluation of antioxidant activities of *H. furcatus* extracts includes, DPPH assay, superoxide radical, ABTS radical, hydroxyl radical, nitric oxide radical scavenging assay and lipid peroxidation assays. The results were expressed in the IC_{50} values i.e., the quantity of the extract needed to scavenge 50% of the radical produced in the reaction mixture. In addition, extracts having low IC_{50} values is considered to posses strong antioxidant property.

Table 1 shows the IC_{50} values of extracts to scavenge the DPPH and ABTS radical. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.

The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Of the ethyl acetate extract showed stronger DPPH scavenging activity rather than other extracts. *H. furcatus* extracts showed ABTS radical scavenging ability of this ethyl acetate extract produced least IC_{50} value.

Table 2 shows the superoxide radical, hydroxyl radical, nitric oxide radical scavenging and inhibition of lipid peroxidation of *H. furcatus* extracts. Superoxide radicals derived from riboflavin reaction reduces NBT.

Table 1: DPPH and ABTS radical scavenging activity of *H. furcatus* extracts

	IC_{50} values* (μg mL^{-1})	
Extracts	DPPH	ABTS
Petroleum ether	LA	LA
Chloroform	96.0±3.60	LA
Ethyl acetate	1.33±0.57	51.66±3.51
Ethanol	28.0±2.00	96.33±4.04
Water	LA	LA

*IC_{50} value is the amount of extract needed for scavenging 50% of the radical produced in the reaction mixture. LA-extract which has percentage inhibition <50% upto 1 mg mL^{-1}

Table 2: Superoxide radical, hydroxyl radical, nitric oxide radical scavenging activities and inhibition of lipid peroxidation by *H. furcatus* extracts

	IC_{50} values* (μg mL^{-1})			
Plant part	Superoxide radical scavenging activity	Hydroxyl radical scavenging activity	Nitric oxide scavenging activity	Lipid peroxidation assay
Petroleum ether	LA	LA	LA	LA
Chloroform	LA	LA	LA	LA
Ethyl acetate	22±2.64	20.33±3.78	30 .0±3.25	25.0±1.24
Ethanol	472.66±6.42	387.0±9.53	420.0±10.00	400.0±5.55
Water	LA	LA	LA	687.0±10.25

*IC_{50} value is the amount of extract needed for scavenging 50% of the radical produced in the reaction mixture. LA: extract which has percentage inhibition <50% upto 1 mg mL^{-1}

The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. With regards to their IC_{50} values, the ethyl acetate extract was considerably more effective superoxide radical scavenger to other extracts.

The hydroxyl radical scavenging ability of *H. furcatus* ethyl acetate extract with an IC_{50} value of 91 μg mL^{-1} was found to be more effective in quenching the hydroxyl radical produced in the reaction mixture. Nitric oxide radical generated from sodium nitroprusside at physiological pH was inhibited by *H. furcatus* extracts. Among them the ethyl acetate extract showed more inhibition of nitric oxide radical with low IC_{50} values.

The capacity of *H. furcatus* extracts to prevent lipid peroxidation was assayed using malondialdehyde formation as an index of oxidative breakdown of membrane lipids, following incubation of rat liver homogenates with the oxidant chemical species Fe^{2+}. The ethyl acetate extract had the greatest activity in reducing lipid peroxidation, reflected by its low IC_{50} value when compared to other extracts.

Protective effect of *H. furcatus* against H_2O_2 induced haemolysis and lipid peroxidation: *H. furcatus* ethyl acetate extract is used for this experiment since it posses high antioxidant activity. Table 3 shows the inhibitory effect of different concentrations of extract (1-25 μg) on H_2O_2 induced haemolysis and lipid peroxidation in human RBC. The *H. furcatus* ethyl acetate extract showed a dose depended inhibition of haemolysis and showed 50% inhibition (IC_{50}) at a concentration of extract 14.75±1.98 μg mL^{-1}. The effect of different concentrations of *H. furcatus* ethyl acetate extract on H_2O_2 induced lipid peroxidation in human RBC showed a dose dependent inhibition of lipid peroxidation. The amount of the extract needed for 50% inhibition (IC_{50}) of peroxidation was found to be 21.50±1.63 μg mL^{-1}.

Table 3: Effect of *H. furcatus* ethyl acetate in inhibiting H_2O_2 induced haemolysis and lipid peroxidation

Plant part	IC_{50} value* (μg mL^{-1})
Haemolysis assay	17.75±1.98
Lipid peroxidation assay	21.50±1.63

*IC_{50} value is the amount of extract needed for scavenging 50% of the radical produced in the reaction mixture

HPLC analysis of *H. furcatus* ethyl acetate extract: The HPLC analysis of *H. furcatus* ethyl acetate extract produced chromatogram peaks at a retention time of 2.06 min with both extract and standard gossypin. No other interfering peaks were observed at around 2.06 min Fig. 1.

The role of ROS as the final common mediators of tissue damage in diseases of diverse etiologies emphasizes the wide range of therapeutic applications of antioxidants. The results demonstrated that this plant exhibits an interesting antioxidant activity. It was able to quench the synthetic DPPH radical, ABTS radicals and scavenged superoxide, hydroxyl, nitric oxide radicals and inhibited tissue lipid peroxidation.

Superoxide radical are generated during the normal physiological process mainly in mitochondria. Despite its involvement in many pathological processes, superoxide by itself is a weak oxidant. But it can give rise to the more toxic hydroxyl radicals and singlet oxygen, damaging bio-macromolecules directly or indirectly with severe consequences (Ames *et al*., 1993). The hydroxyl radicals being the most reactive and predominant radical generated endogenously, capable of causing lipid peroxidation process (Kappus, 1991).

In addition, the toxic byproducts of lipid peroxidation can damage lipids, proteins and DNA which contributes to carcinogenesis, mutagenesis and cell toxicity (Aruoma *et al*., 1989). The physiological importance of nitric oxide scavenging is because the excessive production of nitric oxide resulting from inducible nitric oxide synthases induction is cytotoxic and is implicated in many pathologic and physiological disorders, including vasodilatation, inhibition of platelet aggregation, neurotransmission and immunomodulation and neurodegenerative diseases (Bolanos *et al*., 1997; Law *et al*., 2001).

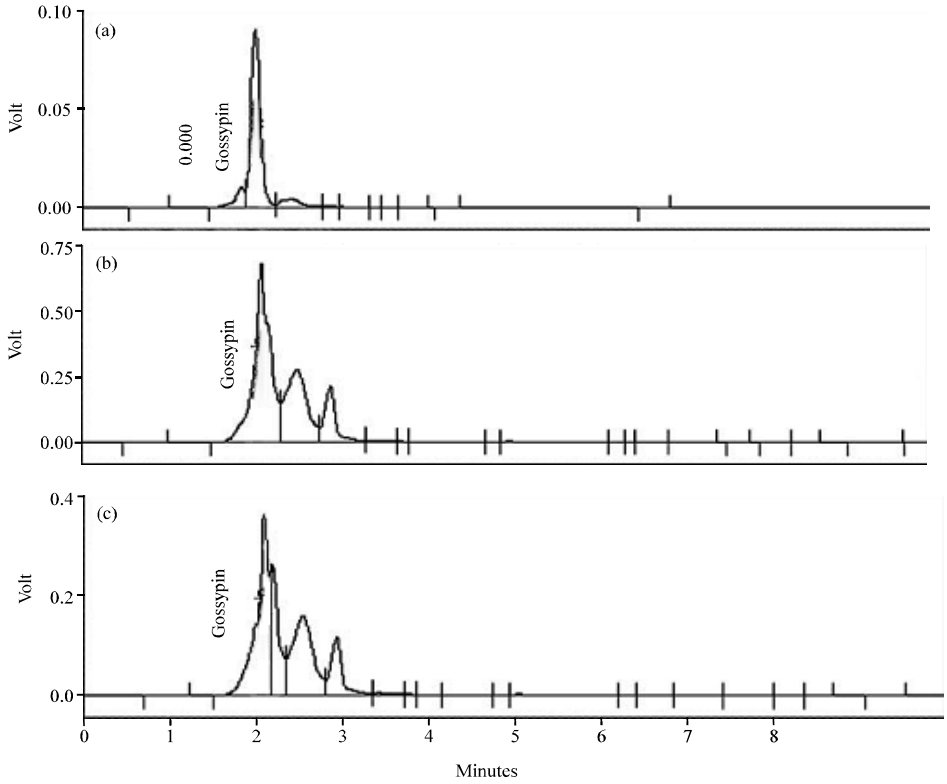

Fig. 1: a) Chromatogram of standard gossypin (Sigma); b) Chromatogram of *H. Furcatus* et extract; c) Chromatogram of *H. Furcatus* ethylacetate extract together with gosspin

Therefore, the radical scavenging by the *H. furcatus* extract has got medicinal value. Erythrocytes are critical targets for natural products and plants as well as many other drugs. Moreover, human erythrocytes are excellent subjects for studies of biological effects of free radicals, since they are both structurally simple, easily obtained and are continually exposed to high oxygen tensions they are unable to replace damaged components, the membrane lipids are composed partly of polyunsaturated fatty acid side chains which are vulnerable to peroxidation and they have antioxidant enzyme systems (Bukowska, 2003).

H. furcatus extract were also found protective on haemolysis and lipid peroxidation of erythrocytes against H_2O_2 induced oxidative damage. The products of lipid peroxidaion has been shown to cross-link erythrocyte phospholipids and proteins to impair a variety of the membrane-related functions and ultimately leading to diminished erythrocytes survival (Chiu *et al*., 1989; Sugihara *et al*., 1991; Ault and Lawrence, 2003). The *H. furcatus* ethyl acetate extract effectively reduced the haemolysis and lipid peroxidation in human erythrocytes. There are several proteins and biomolecules in the living organism which act as free radical scavengers. More over, several dietary supplements containing vitamins, polyphenols, especially flavonoids also play a significant role in this matter (Feher *et al*., 1987; Teixeira *et al*., 2005). The *H. furcatus* flowers were reported for the presence of gossypin, gossypitrin and hibiscatin (Nair *et al*., 1981). The antioxidant activity of gossypin has also been reported. The HPLC analysis of the *H. furcatus* extract also showed the presence of gossypin, so scavenging of reactive species by *H. furcatus* may be due to the presence of these phytochemicals.

CONCLUSION

On the basis of the results of this study, it is clear that the *H. furcatus* extracts have powerful antioxidant activity against various free radicals.

This may be a reason for the protective effect *of H. furcatus* on RBC systems *in vitro*; moreover, the ethyl acetate extract can be used as easily accessible source of natural antioxidants.

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