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In *vivo* and in *vitro* antioxidant potentials of aqueous and ethanol extracts of the leaves of *Bauhinia tomentosa* L.

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Abstract

Bauhinia tomentosa, belonged to the family Caesalpiniaceae, is a well known plant species for its various biological properties. Aqueous and ethanol extracts of *B. tomentosa* leaves were tested for their antioxidant potentials both *in vitro* and *in vivo.* The *in vitro* tests included DPPH assays, Ferric Reducing Power assay, Nitric oxide radical scavenging assay, Superoxide anion radical scavenging assay, ABTS radical scavenging assay and hydroxyl radical scavenging assay. The above test methods showed the antioxidant potentiality of the leaf extracts of *Bauhinia tomentosa* leaf extracts *in vitro*. The *in vivo* methods include SOD, GSH and LPO tests. All the tested methods showed the presence of antioxidant potentials of the *B. tomentosa in vivo.*

Key words: *Bauhinia tomentosa* L. - aqueous and ethanol extracts- *in vivo* and *in vitro* antioxidant activities.

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INTRODUCTION

Traditional system of medicine not only cures the diseases but also treats human mind. Better understanding is needed for the proper use of medicinal plants. Scientists should evaluate the pharmacological activity of medicinal plants by conducting active research.

The culminating research findings not only provide scientific validation for the traditional system of medicine but also help to formulate and develop new drugs. Besides patenting of such compounds helps the economy of the country.. Hence frequent attempts have been made find out alternate therapy using the plants and plant derived compounds, without side effects (Essawi and Srour, 2000).

The relatively large *Bauhinia* genus (Family: Caesalpiniaceae) includes trees, climbers and shrubs. The plants are distributed in a wide range of geographic locations. Certain

Bauhinia species have a long history of traditional medicinal applications (Valdir, 2009). *Bauhinia tomentosa* is a scrambling, many-stemmed shrub or small tree and the branches are often dropping with many slender twigs (Orwa *et al.,* 2010). It is called 'Adavimandaramu' in Telugu and 'Phalgu' in Sanskrit. It has been reported to contain amino acids, proteins, fatty acids, minerals, quercetin and isoquercetin (Row and Viswanadham, 1954).

The dried leaves and flower buds of *B. tomentosa* and a decoction of the root and bark area being used medicinally by the African doctors of South Africa. In Madura, the leaf is an ingredient in the preparation of a plaster applied to cure abscesses. In the traditional system of medicine *B. tomentosa* leaf is reported to have antidiarrhoeal, antihelmentic and antimicrobial properties (Agbede 2007). The dried leaves, buds and flowers are prescribed to control dysentery (Shivkumar, 2011). Hence leaves of these plants were selected and assessed for antioxidant potentials both *in vivo* and *in vitro.*

The various medicinal properties of *B. tomentosa* include digestive, antibacterial, antioxidant, antifungal, stomachic and antihelmintic. Modern scientific studies proved the medicinal potential of the plant by demonstrating antidiabetic, antihy perglycemic (Manivannan *et al.,* 2010), antioxidant (Mannangatti *et al.,* 2010), anti-inflammatory, immunomodulatory (Aderogba *et al.,* 2008) and antimicrobial (Mythreyi *et al.,* 2005) activities by using the different plant parts.

Oxidative stress is produced during normal metabolic process in the body as well as induced by a variety of environmental and chemical factors, which cause generation of various reactive free radicals and subsequent damage to the macromolecules like protein, DNA and lipids. This oxidative damage is a decisive etiological factor concerned in quite a lot of chronic human diseases such as ulcer, arthritis, cancer etc., (Ajay *et al.,* 2011) .

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Antioxidants are chemical substances that donate an electron to the free radical and convert it to a harmless molecule. In this way antioxidants intercept free radicals and protect cells from oxidative damage that leads to ageing and disease (Pallavi and Balaraman, 2004). All plants produce chemical compounds as a part of their normal metabolic activities. These are divided into primary metabolites and secondary metabolites. These compounds are responsible for all kinds of biological activities.

The antioxidants may reconcile their upshot by directing reaction with ROS, quenching them and / or chelating the catalytic metal ions. Several synthetic antioxidants like BHA and BHT are commercially accessible but are perilous and their toxicity is a problem of disquiet. Natural antioxidants are safe and also bioactive. Therefore, in the current years, substantial attention has been directed towards credentials of plants with antioxidant potentialities. Hence the present article deals with the, *in vitro* and *in vivo* antioxidant potentials of aqueous and ethanol leaf extracts of *Bauhinia tomentosa*.

MATERIALS AND METHODS

Preparation of the leaf extract

Fresh leaves of *Bauhinia tomentosa* were dried under shade and powdered using a mechanical grinder. The leaf powder (150 gm) was mixed with water (AEBT) and ethanol (EEBT) separately. The leaf powder mixed with water was boiled and filtered with a muslin cloth and it was condensed in hot air oven at 50°C. The leaf material was soaked in ethanol for 3 days, filtered and allowed to condense at 50°C. The aqueous and ethanol extracts were stored in a container and refrigerator for future use (Jonathan, 2009).

I) *In vitro* methods for the evaluation of antioxidant activity

A great number of *in vitro* methods have been developed to determine the efficiency of natural antioxidants either as pure compounds or as plant extracts. α, α - diphenyl-β-picryl-hydrazyl radical scavenging assay (DPPH), Ferric reducing antioxidant power (FRAP), Nitric oxide radical scavenging assay, Superoxide anion radical scavenging assay, ABTS radical scavenging assay and Hydroxyl radical scavenging assay are the *in vitro* antioxidant assay methods used to assess the antioxidant activity of the leaf extracts of *B.tomentosa*.

1. DPPH assay: (2, 2-diphenyl-1-picryl hydrazyl)

a) Assay by DPPH staining

An aliquot (3 μL) of the samples and standard (Quercetin and Ascorbic acid) were carefully loaded onto a 10 cm X 10 cm silica gel plate (silica gel 60 F254; Merck) and allowed to dry for 3 minutes. After 5 minutes, the TLC plate was sprayed with 0.2 % DPPH in methanol. Discolouration of DPPH indicated scavenging potential of the compound tested (Chang *et al.,* 2002 ; Mc Donald *et al.,* 2001).

b) DPPH assay by using TLC

This preliminary test was performed with a rapid TLC screening method using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) as a spray reagent (Chang *et al.,* 2002; McDonald *et al.,* 2001) Analytical TLC silica gel plate (10cm X10cm) was developed using chloroform: methanol: water (61:32:7) after application of 1μL, 2μL and 3μL of the test sample solution (1 mg / mL), dried and sprayed with DPPH solution (0.2 %, MeOH). After 5 minutes, the active compound appeared as yellow spots against a purple background. The purple stable free radical 2, 2-diphenyl-1-picrylhydrazyl was reduced to yellow diphenylpicryl hydrazine. Quercetin was used as a positive control.

c) DPPH Radical Scavenging Assay (Spectrophotometric assay)

The free radical scavenging capacity of aqueous and ethano extracts of *B. tomentosa* was determined using DPPH. DPPH solution (0.004 % w / v) was prepared in 95 % methanol. The leaf extracts of *B. tomentosa* leaves were mixed with 95 % methanol to prepare the stock solution (10 mg / 100 mL). The concentration of the extract solution was 10 mg / 100 mL or 100 mg / mL. From the stock solution 2 mL, 4 mL, 6 mL, 8 mL and 10 mL of the solution were taken in five test tubes and serially diluted. They were made up to final volume of each test tube to 10 mL whose concentrations were then 20 μg / mL, 40 μg / mL, 60 μg / mL, 80 μg / mL and 100 μg / mL respectively. Freshly prepared DPPH solution (0.004 % w / v) was added in each of these test tubes containing extracts and after 10 minutes, the absorbance was taken at 517 nm using a spectrophotometer (Systronics UV - Visible Spectrophotometer 119, INDIA). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10 mg / 100 mL or 100 μg / mL) of the extracts. From the stock solution 1 mL, 2 mL, 3 mL, 4 mL and 5 mL of the solution were taken in five test tubes and serially diluted. They were made upto final volume of each test tube to 10 mL whose concentrations were then 10 μg / mL, 20 μg / mL, 30 μg / mL, 40 μg / mL and 50 μg / mL respectively. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank (Soler-Evans *et al.,* 1997).

2. Ferric reducing power assay

This experiment was carried out as described previously (Cuendet *et al.,* 1997). 1mL of the plant

extract solution (final concentration 100-500 mg / L) was mixed with 2.5 mL phosphate buffer (0.2M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN_6)$] (10 g / L), then the mixture was incubated at 50°C for 20 minutes. To this 2.5 mL of trichloroacetic acid (100 g / L) was added, and centrifuged at 3000 rpm for 10 minutes. Finally, 2.5 mL of the supernatent solution was mixed with 2.5 mL of distilled water and 0.5 mL $FeCl_3$ (1 g / L) and the absorbance was measured at 700 nm with UV-Visible Spectrophotometer (Systronics UV-Visible Spectrophotometer 119, India). Ascorbic acid was used as standard and phosphate buffer as blank solution. The absorbance of the final reaction mixture of two parallel experiments was expressed as mean ± standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

$$\text{Percentage increase in reducing power} = \frac{A_{test}}{A_{blank}} 1 \times 100$$

A_{test} is the absorbance of test solution and A_{blank} is absorbance of blank. The antioxidant activity of the leaf extract was expressed as IC_{50} and compared with standard.

3. Nitric oxide scavenging assay

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of NO compete with oxygen leading to reduce production of NO. Nitric oxide was generated from sodium nitroprusside, which at physiological pH liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions (NO_2-) which diazotize with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink colour, which can be measured at 546 nm. Sodium nitroprusside (10 mM, 2 mL) in phosphate buffer saline was incubated with the test compounds in different concentrations (20-100 μg / mL) at room temperature for 30 minutes. After 30 minutes, 0.5 mL of the incubated solution was added with 1mL of Griess reagent and the absorbance was measured at 546 nm (Polshettiwar *et al.,* 2007).

4. Superoxide radical scavenging assay (PMS-NADH System)

Superoxide anions were generated using PMS / NADH system. The superoxide anions were subsequently made to reduce nitroblue tetrazolium, which yielded a chromogenic product, which was measured at 560 nm. Phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system was used for the generation of superoxide anion. It was assayed by the reduction of nitroblue tetrazolium (NBT). About 1mL of nitro blue tetrazolium (156 μM), 1 mL NADH (468 μM) in 100 mM phosphate buffer of pH 7.8 and 0.1 mL of sample solution of different concentrations (20-100μg / mL) were mixed. The reaction was started by adding 100μl PMS (60 μM). The reaction mixture was incubated at 25°C for 5 minutes and absorbance of the mixture was measured at 560 nm against blank samples. The percentage inhibition was determined by comparing the results of control and test samples (Kumarasamy *et al.,* 2007)

5. ABTS radical scavenging assay

ABTS (2, 2'-azinobis-3-ethylbenzothiozoline-6-sulphonic acid) assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process that is associated with a change in absorption. The relatively stable ABTS radical was green and it was quantified spectrophotometrically at 734 nm. ABTS radical cations were produced by the reaction of ABTS and APS. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and the percentage inhibition was calculated. The stock solutions included were 7 mM ABTS solution and 2.4 mM potassium per sulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowed them to react for 14 hrs at room temperature in dark. The solution was then diluted by mixing 1 mL ABTS solution with 60 mL methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Leaf extract (1 mL) was allowed to react with 1mL of the ABTS solution and the absorbance was taken at 734 nm after 7 minutes using a spectrophotometer. All the determinations were performed in triplicates (Blois *et al.,* 1958)

6. Hydrogen peroxide scavenging assay

H_2O_2 scavenging ability of aqueous and ethanol extracts of *B. tomentosa* leaves was determined according to the method of Ali *et al.* (2009). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). The aqueous and ethanolic extracts at 20-100 μg / mL concentrations in 3.4 mL phosphate buffer were added to H_2O_2 solution (0.6 mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing the phosphate buffer without H_2O_2.

Assessment of % inhibition and IC_{50}

Radical scavenging activity of the extract and standard were expressed in terms of % inhibition. It was calculated using the formula $[(A_{Control}-A_{Sample}) / A_{Control}] \times 100$. Where $A_{Control}$ is the absorbance of the control,

and A_{Sample} is the absorbance in the presence of the sample of aqueous and ethanol extracts. The IC_{50} value is defined as the concentration (in μg / mL) of extracts that produced 50% antioxidant effect.

IC_{50} = Concentration of extract / % inhibition X 50.

II) *In vivo* Antioxidant assays

The experimental protocols for all the *in vivo* assays employed in the present investigation were subjected to the scrutinization of the Institutional Animals Ethical Committee (Reg.No.Ph.D MB1 / 2010-11) and were cleared by the same. All the experiments were performed during morning according to CPCSEA guidelines for care of laboratory animals and the ethical guideline for investigations of experimental pain in conscious animals.

Experimental Design

The following method was adopted for studying the in vivo antioxidant activity (Verma *et al.,* 2010) . Swiss albino mice (20-35 g) of either sex obtained from TANUVAS, Madavaram, Chennai were used. They were divided into 9 groups comprising 6 animals in each group. They were housed in polypropylene cages in the departmental animal house at 26±2º C for one week before and during the experiments. Fresh dry husks were used as bed material. They were fed commercially with standard pellet diet and distilled water. Food was withdrawn 18-24 h before the experiment though *ad libitum* was allowed. Gastric ulcers were induced in rats by administrating absolute acid alcohol (0.3M Hydrochloric acid in 60% ethanol). The experimental designs given below were followed for the present study.

Group I - Normal control- receives saline
Group II - Ulcer induced disease control - ethanol-acid (25 mL / Kgof 0.3 M HCl in 60 % ethanol)
Group III - Ulcer induced + received ranitidine 32 mg / Kg
Group IV - Ulcer induced + received AEBT 200 mg / Kg
Group V - Ulcer induced + received AEBT 400 mg / Kg
Group VI - Ulcer induced + received AEBT 600 mg / Kg
Group VII - Ulcer induced + received EEBT 200 mg / Kg
Group VIII - Ulcer induced + received EEBT 400 mg / Kg
Group IX - Ulcer induced + received EEBT 600 mg / Kg

After the experimental period, the mice were sacrificed by cervical decapitation. Liver tissue was dissected out and washed in ice-cold saline. Stomach tissues were homogenized in 0.1M phosphate buffer (pH 7.4) and were subjected to *in vivo* antioxidant studies.

Estimation of Protein

0.2 ml of working standard solution was taken in 5 test tubes and made up to 1ml using distilled water. The test tube with 1 ml distilled water served as blank. 4.5 ml of Reagent I was added and incubated for 10 minutes. After incubation 0.5 ml of Reagent II was added and incubated for 30 minutes. Absorbance was recorded at 660 nm and a standard graph was plotted. The test protein sample was performed as that of the standard solution and the amount of protein present in the given sample was estimated (Lowry *et al.,* 1951).

Methods for the determination of *in vivo* antioxidant activity

Various *in vivo* methods were used to evaluate the ability of antioxidants to reduce or dispropionate the radical. Antioxidant activity can be measured using both serum sample as well as tissue homogenate. The objective of this study is to compile the different *in vivo* antioxidant screening methods in the literature such as methods for superoxide dismutase (SOD), reduced glutathione (GSH) and lipid peroxidation (LPO).

1. Superoxide Dismutase (SOD)

The assay of SOD was carried out using the method of (Misra and Fridovich ,1972). Reagents required include Carbonate and bicarbonate buffer – 0.1 M pH 10.2; EDTA solution-0.6 mM; Epinephrine – 1.8 mM, absolute alcohol and chloroform. 0.1 ml of tissue homogenate was added to tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chill) and centrifuged. To 0.5 ml of supernatent 0.5 ml of EDTA solution and 1ml buffer were added. The reaction was initiated by the addition of 0.5 ml epinephrine and the increase in absorbance was measured at 480 nm. The enzyme activity is expressed as 50 % inhibition of epinephrine auto oxidation.

2. Reduced Glutathione (GSH)

To measure the GSH level, the tissue homogenate (in 0.1 M phosphate buffer pH 7.4) was taken. The procedure was followed initially as described by (Ellman and Fiches, 1959). The homogenate was added with equal volume of 20 % trichloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. The supernatent (200 µl) was then transferred to a new set of test tubes and added with 1.8 ml of the Ellman's reagent

(5, 5'-dithiobis-2-nitrobenzoic acid) (0.1 mM) which was prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes were made upto the volume of 2ml. After completion of the total reaction, solutions were measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from known GSH.

3. Lipid Peroxidation (LPO)

The TBARs levels were estimated as per the spectrophotometric method described by Ohkawa *et al.,* (1979). Briefly, to each test tube, 0.5 ml of tissue homogenate, 0.5 ml of normal saline, 1 ml of 20 % trichloroacetic acid (TCA) and 0.25 ml of TBA reagent (200 mg of thiobarbituric acid in 30 ml distilled water and 30 ml of acetic acid) were added. The test tubes were kept for boiling at 95ºC for one hour. To each of the test tubes, 3 ml of n-butanol was added and mixed well. The tubes were centrifuged at 3000 rpm for 10 minutes. The separated butanol layer was collected and read in a spectrophotometer against the reagent blank at 535 nm. Thiobarbituric reactive substances concentration was expressed in terms of nmol of malondialdehyde per milliliter of tissue homogenate.

Statistical analysis

All the values were expressed as mean SD (Standard deviation). Statistical analysis was carried out by using Origin software package (version 6.0). Statistical significance of differences between the control and experimental groups was assessed by One-way ANOVA. The value of probability less than 5% ($P < 0.05$) was considered statistically significant (Panse and Sukhatme, 1978)

RESULTS AND DISCUSSION

I) In vitro methods

The antioxidant activity of the leaf extracts of *B. tomentosa* were assessed using DPPH method, Ferric reducing power assay method, Nitric oxide radical scavenging method, Superoxide radical scavenging method, ABTS radical scavenging method and H_2O_2 scavenging methods.

The DPPH radical scavenging activity was assessed using three different methods. All the methods confirmed the radical scavenging activity of the plant extracts. 1µl, 2µl and 3µl concentration of the leaf extracts and standard were spotted on TLC plate. Results revealed that discolouration was recorded in all the spots except distilled water spot and the size of the spot was increased with increased concentration. When the extracts and standard (Ascorbic acid and Quercetin) were chromatographed using suitable mobile phase, at particular spots both standard and extracts showed DPPH colour discolouration. The DPPH assay has been largely used as a quick, reliable and reproducible parameter to search the *in vitro* general antioxidant activity of pure compounds as well as plant extracts (Koleva *et al.,* 2002 and Goncalves *et al.,* 2005)

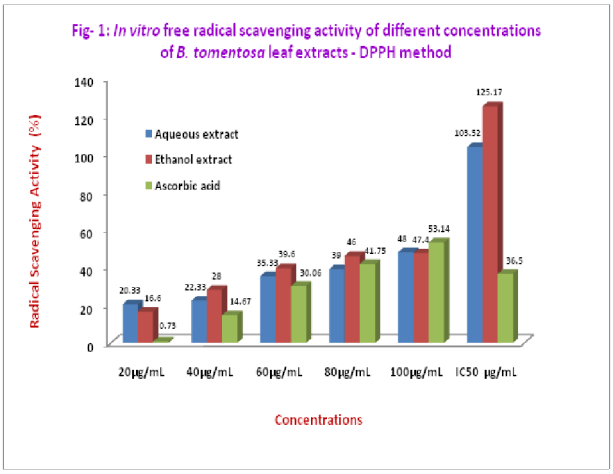
Spectrophotometric assay of DPPH mediated free radical scavenging activities of aqueous and ethanol extracts of *Bauhinia tomentosa* leaf and standard are presented in Table-1 and Fig. 48±3 % (significant at $p<0.001$ level) free radical scavenging power was exhibited by aqueous extract at 100µg/mL concentration. Ethanol extract exhibited 47.4±6.8 % (significant at $p<0.05$ level) of free radical scavenging activity. IC_{50} value of the aqueous and ethanol extracts and standard were recorded as 103.52±0.75, 125.17±1.09 and 36.5±1.15 µg/mL respectively. The decrease in absorbance by the DPPH radical with increase in concentration of the extract manifested in the rapid discolouration of the purple DPPH, suggests that AEBT and EEBT have antioxidant activity due to their proton donating ability (Adesegun *et al*., 2007).

The ferric reducing power of *Bauhinia tomentosa* leaf aqueous and ethanol extracts were recorded (Table-2

Table 1. *In vitro* free radical scavenging activity of different concentrations of *B. tomentosa* leaf extracts - DPPH method

Extracts	Percentage of Scavenging activity (mean±SD)					
	20µg/mL	40µg/mL	60µg/mL	80µg/mL	100µg/mL	IC_{50} µg/mL
Aqueous extract	20.33±2.08*	22.33±2.5*	35.33±2.45*	39±3.6*	48±3*	103.52±0.75
Ethanol extract	16.6±2.3*	28.0±5.0*	39.6±4.1***	46±7.2***	47.4±6.8***	125.17±1.09
Concentration	10µg/mL	20µg/mL	30µg/mL	40µg/mL	50µg/mL	
Ascorbic acid	0.73±0.080*	14.67±0.81*	30.06±1.37*	41.75±0.88*	53.14±0.76*	36.5±1.15

* Significant at $p<0.001$ level ** Significant at $p<0.01$ level *** Significant at $p<0.05$ level



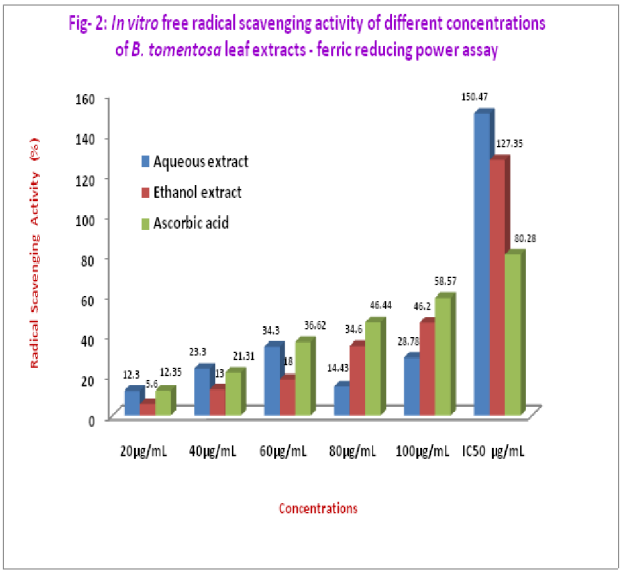
and Fig-2). Here yellow coloured test solution changed to various shades of green and blue, depends on the available reducing power components. Reducing components converts Fe^{3+} to Fe^{2+}, which was analysed spectrophotometrically. Ethanol extract (100 µg/mL) yielded better ferric reducing power (46.2±5.7 %) at a significant level of p<0.001, with an IC_{50} 127.35±1.71 µg/mL. Similarly aqueous extract (100 µg/mL) also produced good ferric reducing activity (28.78±0.67) at a significant level of p<0.001, with an IC_{50} of 150.47±0.98 µg/mL. IC_{50} for ascorbic acid was found to be 80.28±1.49 µg/mL (Table-9). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging (Hazra *et al.,* 2008). The reducing capacity of a compound could serve as an important indicator of its potential antioxidant activity (Meir *et al.,* 1995). The antioxidant activities of putative antioxidants have been attributed to various mechanisms, such as the prevention of chain initiation, transition metal ion catalyst binding, peroxides decomposition, prevention of continued proton abstraction, and radical scavenging (Diplock, 1997).

Significant nitric oxide scavenging activity was exhibited by aqueous and ethanol extracts of *tomentosa* leaves (Table-3, Fig-3). Ethanol extract showed 63.9±7.4 % (significant at p<0.001 level) nitric oxide scavenging activity at 100 µg/mL concentration. Similarly aqueous extract exhibited 64.9±3.15 % (significant at p<0.01 level) inhibition and 58.55±0.57 % (significant at p<0.01 level) inhibition was recorded for standard at 50 µg/

Table 2. *In vitro* free radical scavenging activity of different concentrations of *B. tomentosa* leaf extracts - ferric reducing power assay

Extracts	Percentage of Scavenging activity (mean±SD)					
	20µg/mL	40µg/mL	60µg/mL	80µg/mL	100µg/mL	IC_{50} µg/mL
Aqueous extract	12.3±3.6*	23.3±2.6*	34.3±3.3*	14.43±1.00*	28.78±0.67*	150.47±0.98
Ethanol extract	5.6±1.5*	13.0±4.5*	18±4*	34.6±4.1*	46.2±5.7*	127.35±1.71
Concentration	10µg/mL	20µg/mL	30µg/mL	40µg/mL	50µg/mL	
Ascorbic acid	12.35±0.47*	21.31±0.56*	36.62±0.94*	46.44±0.93**	58.57±0.55*	80.28±1.49

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level



mL concentration. Antioxidants compete with oxygen and reduce nitric oxide production which in turn reduces the burden of nitric oxide. The leaf extracts of *tomentosa* revealed significant antioxidant activity with increased concentration of extracts. It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (Tylor *et al.,* 1997). The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$) (Huie and Padmaja, 1993). Reactive oxygen species, nitric oxide has been implicated in inflammation, cancer and other pathological conditions (Moncada *et al.,* 1991).

Table 3. *In vitro* free radical scavenging activity of different concentrations of *B. tomentosa* leaf extracts - nitric oxide scavenging assay

Extracts	Percentage of Scavenging activity (mean±SD)					
	20µg/mL	40µg/mL	60µg/mL	80µg/mL	100µg/mL	IC_{50} µg/mL
Aqueous extract	18.5±3.5**	25.0±7.9*	31.2±6.9**	35.9±7.2*	64.9±3.15**	36.21±1.19
Ethanol extract	11.94±1.5**	19.9±1.5*	24.8±4.6*	54.8±4.6*	63.9±7.4*	40.56±0.64
Concentration	10µg/mL	20µg/mL	30µg/mL	40µg/mL	50µg/mL	
Ascorbic acid	12.31±0.47**	21.32±0.43**	36.3±0.74**	46.11±0.25*	58.55±0.57**	80.162±0.97

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

Table 4. *In vitro* free radical scavenging activity of different concentrations of *B. tomentosa* leaf extracts - superoxide radical scavenging assay method

Extracts	Percentage of Scavenging activity (mean±SD)					
	20µg/mL	40µg/mL	60µg/mL	80µg/mL	100µg/mL	IC_{50} µg/mL
Aqueous extract	14.6±1.0**	21.3±2.5*	27.3±1.1**	43.4±5.7*	58.6±5.6***	31.38±6.72
Ethanol extract	11.5±1.9***	16.6±1.9*	21.7±2.28*	29.7±5.6**	35.46±8.7**	33.43±1.69
Concentration	10µg/mL	20µg/mL	30µg/mL	40µg/mL	50µg/mL	
Ascorbic acid	66.36±0.80***	66.58±1.01**	68.12±0.80**	71±0.61*	72±0.63*	43.05±0.94

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

Table 5. *In vitro* free radical scavenging activity of different concentrations of *B. tomentosa* leaf extracts - ABTS radical scavenging assay method

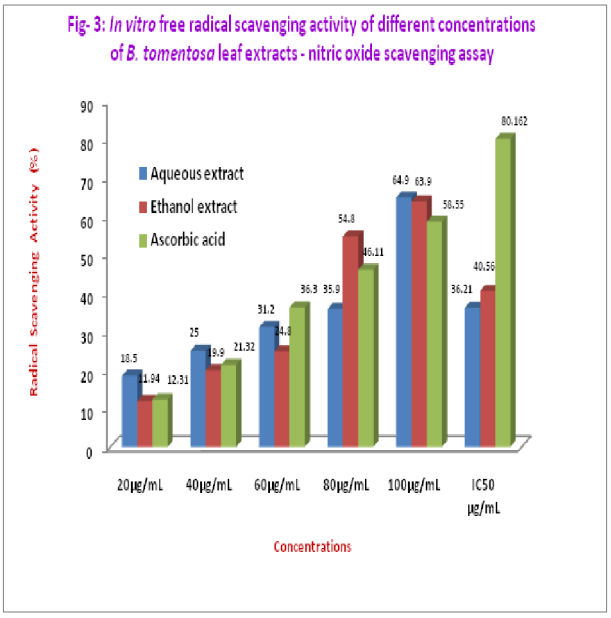
Extracts	Percentage of Scavenging activity (mean±SD)					
	20µg/mL	40µg/mL	60µg/mL	80µg/mL	100µg/mL	IC_{50} µg/mL
Aqueous extract	39.6±4.8**	55.07±5.6*	65.9±2.3*	71.9±2.0*	87.4±2.0*	30.65±1.75
Ethanol extract	35.6±2.8**	45.5±5.2**	57.8±5.5*	62.3±7.1 *	76.3±7.3*	30.71±3.35
Concentration	10µg/mL	20µg/mL	30µg/mL	40µg/mL	50µg/mL	
Ascorbic acid	10.34±1.16*	28.93±0.55*	32.27±1.25*	41.86±1.21*	51.18±1.24*	33.2±5.12

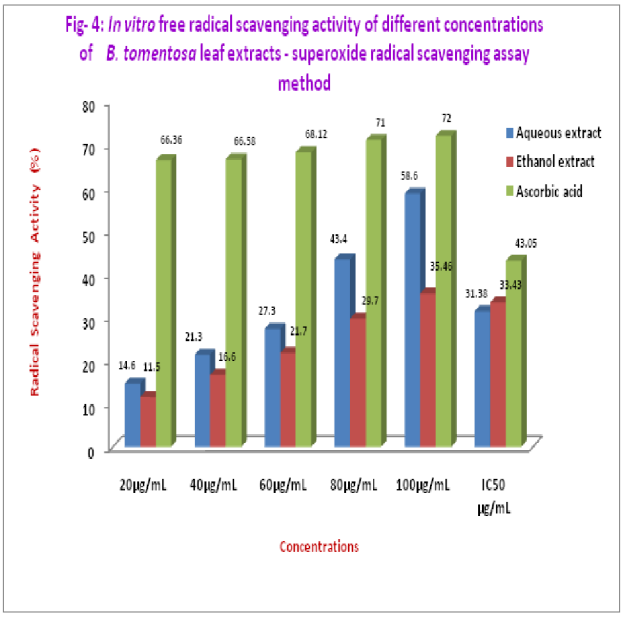
* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level

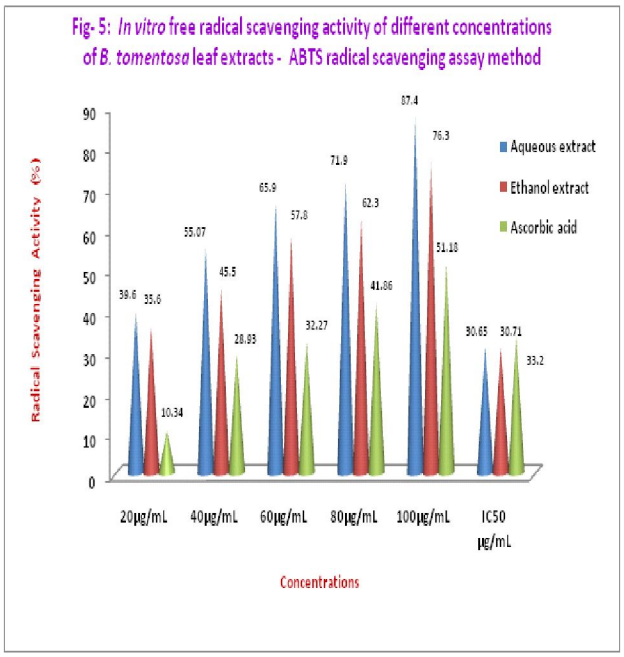
Table 6. *In vitro* free radical scavenging activity of different concentrations of *B. tomentosa* leaf extracts - H_2O_2 scavenging method

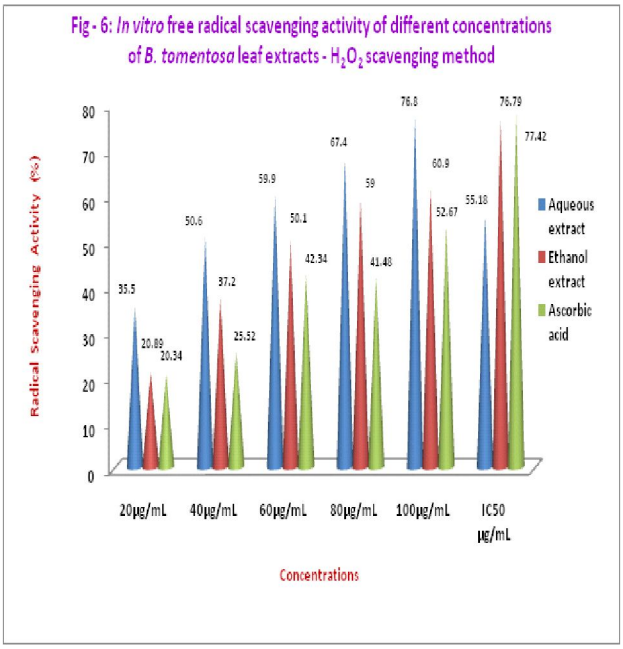
Extracts	Percentage of Scavenging activity (mean±SD)					
	20µg/mL	40µg/mL	60µg/mL	80µg/mL	100µg/mL	IC_{50} µg/mL
Aqueous extract	35.5±2.0**	50.6±1.7**	59.9±6.1*	67.4±4.5*	76.8±3.3 *	55.18±0.90
Ethanol extract	20.89±1.1**	37.2±3.1***	50.1±4.6**	59.0±0.7 *	60.9 ±1.5*	76.79±1.35
Concentration	10µg/mL	20µg/mL	30µg/mL	40µg/mL	50µg/mL	
Ascorbic acid	20.34±0.68**	25.52±0.52*	42.34±0.81*	41.48±0.82*	52.67±0.84*	77.42±0.95

* Significant at p<0.001 level ** Significant at p<0.01 level *** Significant at p<0.05 level









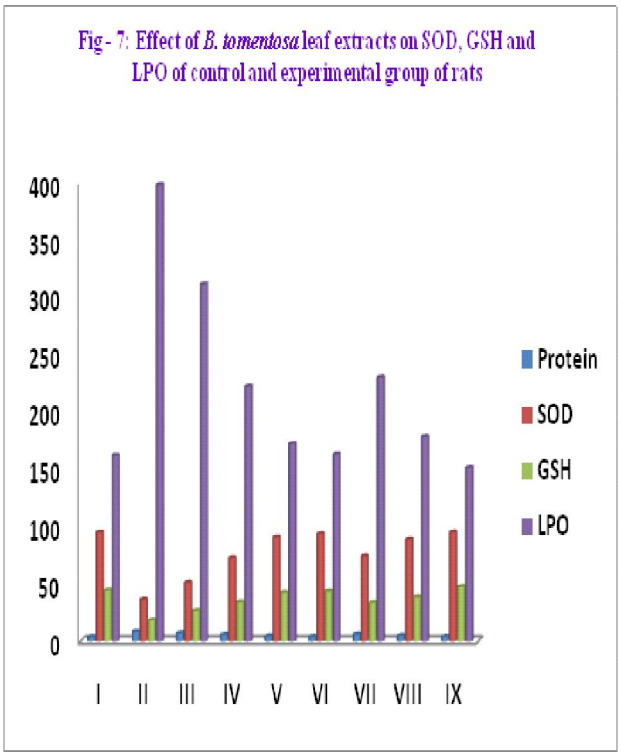
Effective superoxide radical scavenging activity of extracts of *Bauhinia tomentosa* leaves was noted in Table-4 and Fig-4. Aqueous extract (100 µg/mL) produced 58.6±5.6 % (significant at $p<0.05$ level) superoxide radical scavenging activity followed by 35.46±8.7 % (significant at $p<0.01$ level) for ethanol extract (100 µg/mL) and 72±0.63 % (significant at $p<0.001$ level) for standard (at 50 µg/mL) with 33.43±1.69, 31.38±6.72 and 43.05±0.94 µg/mL IC_{50} respectively. Superoxide anions are highly toxic to cellular components. Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Okhawa *et al.,* 1979). The flavonoids are effective antioxidants mainly because they scavenge superoxide anions (Hazra *et al.,* 2008).

Leaf extracts of *Bauhinia tomentosa* showed better ABTS radical scavenging activity (Table-5, Fig-5). Aqueous extract (100 µg/mL) produced 87.4±2.0 % (significant at $p<0.001$ level) ABTS scavenging power with 30.65±1.75 µg/ml IC_{50} value followed by ethanol extract (100 µg/mL) 76.3±7.3 % (significant at $p<0.001$ level) with 30.71±3.35 IC_{50} value. IC_{50} value for ascorbic acid was found to be 33.2±5.12 µg/ml. Chromatophore $ABTS^{+}$ was formed by the reaction between ABTS and potassium persulphate and reduced to ABTS by the action of antioxidants available in the extracts. High molecular weight phenolics (tannins) have more abilities to quench free radicals (ABTS) and their effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups (Hagerman et al., 1998).

Table 7. Effect of *B. tomentosa* leaf extracts on SOD, GSH and LPO of control and experimental group of rats

Groups	PROTEIN (gm/dl)	SOD (U/mg protein)	GSH (nM of glutathione oxidized/ mg protein)	LPO (nM of MDA/mg protein)
Group I	4.142±0.0402	95.06±0.847	44.44±0.3049	162.2±0.8366
Group II	8.428±0.0132	36.72±0.593	18.44±0.3847	398.2±1.303
Group III	7.144±0.0270*	51.46±0.456*	26.52±0.5385**	311.4±0.9617**
Group IV	5.89±0.010**	72.66±0.427**	34.34±0.3633*	222.5±1.118**
Group V	4.634±0.0194*	90.58±0.3288**	42.46±0.4375**	172.3±1.036*
Group VI	4.184±0.0110*	93.56±0.5176**	43.54±0.4159*	163.4±0.894**
Group VII	6.2±0.1225**	74.2±0.4472*	33.8±1.303**	230.2±1.789*
Group VIII	5.06±0.1673*	88.8±1.095*	38.6±0.8944**	178.6±0.8944*
Group IX	4.184±0.0110*	95.22±0.76**	47.54±0.95**	151.4±0.21**

Values are given as mean ± standard deviation of six animals each.
* Values are statistically significant at p<0.01 level
**Values are statistically significant at p<0.05 level.



Good H_2O_2 conversion of *tomentosa* leaf extracts and standard are indicated in Table-6 and Fig-6. Among the extracts, aqueous extract (100 µg / mL) yielded good H_2O_2 scavenging power (76.8±3.3) at a significant level of p<0.001, with an IC_{50} value of 55.18±0.90 µg / mL. Obviously standard showed good antioxidant power in all methods tested. Ethanol extract at 100 µg / mL exhibited 60.9±1.5 % (significant at p<0.001 level) radical scavenging activity with an IC_{50} of 76.79±1.35 µg / mL. Results of antioxidant nature of both extracts and standard were significantly different among various concentrations (p<0.05). Hydrogen peroxide inactivates a few enzymes directly, usually by oxidation of essential thiol group (-SH). It can cross membranes and reacts with Fe^{2+} and Cu^{2+} ions to form hydroxy radical and are responsible for various toxic effects and this may be the origin of many of its toxic effect (Miller *et al.,* 1993). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate (Miller, 1996). Antioxidant compounds react with H_2O_2 and convert into H_2O, which is involved in normal metabolic activities of the cell (Hazra *et al.,* 2008). Quantitative phytochemical analysis indicated that the plant contains significant amounts of phenolic compounds such as total phenolic acids, tannins and flavonoids. These classes of compounds were responsible for antioxidant and free radical scavenging effect of the plant material (Noda *et al.,* 2002).

Biological and chemical research in life science proved that free radicals and reactive oxygen species could be involved in a high number of diseases (Jain and Agarwal, 2008). Numerous physiological and

biochemical processes in the human body may produce oxygen centered free radical and other reactive oxygen species and byproducts. Over production of such free radicals cause oxidative damage to biomolecules leading to many chronic diseases (Halliwell, 1994). Plants are the important source for free radical scavenging molecules. Intake of natural antioxidants has been associated with reduced risk of cancer; cardiovascular diseases, diabetes and other diseases associated with ageing.

Antioxidant is one of the most essential ingredient of today's menu / therapy because the antioxidative system protects the animal against reactive oxygen species (H_2O_2, n the use, but they are suspected to be carcinogenic (Singh *et al.,* 2002). Natural antioxidants, therefore, have gained importance. Aqueous and ethanol extracts of *tomentosa* fruit rind has been studied for its antioxidant properties using different *in vitro* antioxidant methods. Flavonoids, phenolic acids, tannins and steroids were found in the lead extracts of *tomentosa.* The leaf extracts of *tomentosa* showed good antioxidant activity, which could be due to the available phytoconstituents. In this respect, polyphenolic compounds commonly found in plants have been reported to have multiple biological effects like anticancer (Seeram *et al.,* 2005), wound healing (Nasr *et al.,* 1996) and antibacterial (Das *et al.,* 1999) activities including antioxidant activity (Gil *et al.,* 2000).

Potent antioxidant activity of the leaf extracts of *tomentosa* was analyzed by making use of 6 different methods. However, the efficiency of each extract differed against various free radicals depending on the specific assay methodology, which reflects the complexity of the mechanisms and diversity of the chemical nature of the plant material. This is the first of this kind of work to assay the antioxidant potentiality of the leaf of *tomentosa*. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating activity (Baumann *et al.,* 1979). Hydrogen donor in the extract could be responsible for DPPH radical scavenging power. Radical scavenging power of the extracts increased with the increased concentration of the extract (Lim *et al.,* 2007). Aqueous extracts indicated better antioxidant or free radical scavenging power than ethanol extracts.

II) *In vivo* antioxidant assays

In vivo antioxidant activity of the leaf extracts of *tomentosa* is presented in Table-7 and Fig-7. SOD, GSH and LPO levels were significantly regained in leaf extract treated animal groups. Glutathione is one of the best antioxidants. Higher level of glutathione indicates that the cells are in good condition. Lipid peroxidation and lipid peroxidation product which are high during diseased condition. SOD levels were assessed in terms of U / mg protein whereas GSH and SOD were assessed in terms of nM of the respective compound oxidized / mg of protein in tissues. Reduced level of SOD (36.72±0.593) and GSH (18.44±0.3847) were noted in group II animals. Very high level of LPO was noted in tissues of group II animals (398.2±1.303). Leaf extract treated groups regained their level of antioxidant enzymes in a dose dependent manner (observed from group IV to VI for AEBT and VII to IX for EEBT).

Leaf extract treated groups effectively regained their antioxidant levels as in group I animals. 151.4±0.21 and 163.4±0.894 (both the values are significant at $p<0.05$ level) nM of MDA / mg protein of LPO was noted in leaf extract treated animal groups received 600 mg / Kg of AEBT (Gp VI) and EEBT (Gp IX) extracts. EEBT 600 mg / Kg treated animal group showed good regaining power of LPO, SOD and GSH than AEBT. Leaf extract treated animal groups regained best SOD and LPO levels than ranitidine treated groups (Group III - 51.46±0.456 (significant at $p<0.01$ level) and 311.4±0.9617 (significant at $p<0.05$ level) respectively). All the results were significantly different at $p<0.01$ and 0.05 levels. SOD involves in scavenging superoxide ions and catalase is a heme enzyme which removes hydrogen peroxide (Lee *et al.,* 2004). Level of LPO was high among ulcer induced animal tissues as well as in the samples of ulcer induced disease control group and decreased level of SOD was noted in ulcer induced mice. Both the extracts of *tomentosa* effectively showed their antioxidant powers.

MDA is one of the end product in the lipid peroxidation process (Valenzuela, 1989). GSH is a naturally occurring substance that is abundant in many living creatures. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and injury. GSH is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. It protect cells against free radicals, peroxides and other toxic compounds. The concept of a glutathione- SH threshold for drug detoxification was discussed by Jollow (1980). Glutathione was also implicated in diseases like cataract and aminoaciduria. SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense by lowering the steady-state level of O_2. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage.

Antioxidants ensure limited survival of reactive oxygen species such as superoxide and H_2O_2 in the extracellular fluid by binding the metals and thus

diminishing their ability to accelerate lipid peroxidation (Gutteridge, 1995). Secondary metabolites from medicinal plants function as small molecular weight antioxidants through direct antiradical, chain-breaking of the free radical propagation and interaction with transition metals. Other mechanisms include the inhibition of ROS-generating enzymes such as Xanthine oxidase, inducing nitric oxide synthase, and improving the endogenous cellular antioxidant mechanisms such as the up-regulation of the activity of SOD (Halliwell and Whiteman, 2004).

Furthermore, phenolic compounds function as high-level antioxidants because they possess the ability to adsorb and neutralize free radicals as well as quench reactive oxygen species. Flavonoids, as one of the most diverse and widespread groups of natural compounds, are also probably the most natural phenolics capable of exhibiting *in vitro* and *in vivo* antioxidant activities. In addition, plant flavonoids which show an antioxidant activity *in vitro* also function as antioxidants *in vivo* (Dorman *et al.,* 2003).

The screening studies for antioxidant properties of medicinal and food plants have been performed increasingly for last few decades with the hope of finding an effective remedy for several present-day diseases and means to delay aging symptoms (Jimoh *et al.,* 2009). The disorders related to the excessive oxidation of cellular substrates (oxidative stress) include type II diabetes, neurodegenerative diseases, and some types of cancer (Karuna *et al.*, 2009). Furthermore, there is also a huge demand for natural antioxidants in food and related industries, for replacing the synthetic preservatives (Dorman *et al.,* 2003).

CONCLUSION

The antioxidant activities of medicinal plants may be due to the presence of phenolic compounds, containing the hydroxyl groups that confer the hydrogen donating ability and the flavonoids which are involved in free radical scavenging activity. The findings of this study supports the view, that the aqueous and ethanol extracts of the leaves of *Bauhinia tomentosa* are promising sources of potential antioxidants and may be efficient as preventive agents in some diseases and can be considered as a natural herbal source in pharmaceutical industry. Further detailed studies on isolation of phytoconstituents of the plant extracts are essential to characterize them as biological antioxidants. The medicinal plants which possess good antioxidant potential are the best supplements for the diseases associated with oxidative stress.

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