


***In vitro* Antioxidant and Cytotoxic Activities of Methanolic Leaf Extract of *Ficus hispida* Linn.**

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ABSTRACT

This study was aimed to investigate *in vitro* antioxidant and cytotoxic activities of methanolic leaf extract of *Ficus hispida* Linn. Antioxidant potential of the extract was evaluated by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, total antioxidant capacity and reducing power methods. Cytotoxic activity was evaluated using brine Shrimp lethality bioassay. The extract showed good antioxidant activities in all antioxidant assays compared to the reference antioxidant ascorbic acid in a dose dependent manner. In DPPH scavenging assay, the IC₅₀ value of the extract was 58.14µg/ml while the IC₅₀ value of ascorbic acid was 48.78µg/ml. Total antioxidant activity also increased in a dose dependent manner. Moreover, the extract showed strong reducing power. In Brine Shrimp Lethality Bioassay, the LC₅₀ value of the extract was 7.94 µg/ml while LC₅₀ of Vincristine sulfate (reference standard) was 0.078 µg/ml. These results suggest that leaves of *Ficus hispida* Linn. has *in vitro* antioxidant and cytotoxic activities.

Key words: *Ficus hispida* Linn. DPPH free radical scavenging, antioxidant, cytotoxicity.

INTRODUCTION

Free radicals or reactive oxygen species (ROS) derived during metabolism and other activities are thought to play a major role in the etiology of a wide variety of diseases. (Cross et al.1994; Mikulikova and Popes, 2001). The most common free radicals or reactive oxygen species (ROS) include superoxide (O₂⁻) anion, hydrogen peroxide (H₂O₂), peroxy (ROO[·]) radicals, and reactive hydroxyl (OH[·]) radicals. Mammalian body possesses antioxidant defense mechanisms such as catalase, superoxide dismutase, glutathione peroxidase enzymes and antioxidant nutrients i.e. vitamin E, ascorbic acid which arrest the damaging properties of ROS (Halliwell et al., 1995). But continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage (Tseng et al., 1997). It results in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury, cardiovascular diseases, diabetes and neurodegenerative diseases (Halliwell and Gutteridge, 1999; Liao and Yin, 2000, Sies and Sian et al. 2003). In treatment of these diseases, antioxidant therapy has gained an immense importance. Currently, restriction is being imposed on the use of synthetic antioxidants because of their carcinogenicity and the need for natural antioxidants therefore become imperative and desirable (Grice, 1986; Wichi, 1988; Behera et al., 2006). Thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (Jayaprakash and Rao, 2000). The naturally occurring antioxidants like vitamins are a balanced mixture of redox with reduced and oxidized form while the synthetic antioxidants are unbalanced in this respect and they themselves produce harmful free radicals in some cases emphasizing the importance of the naturally occurring antioxidants over the synthetic ones (Blot et al., 1993). In this respect, flavonoids and other polyphenolic compounds have received the

greatest attention. Polyhydroxy flavones, flavanones, flavanols, isoflavones, chalcones and many members of these groups of natural substances have a high degree of antioxidant activity and they are found to be wide spread in plant material (Hudson, 1991).

Ficus hispida Linn. (Bengali name: Dumur) belongs to the family Moraceae, is a shrub or moderate sized tree and grows in almost all districts of Bangladesh (Ghani, 2003). It is also widely distributed throughout India, Sri Lanka, Myanmar, southern regions of the China, New Guinea and Queensland in Australia. Almost all parts of this plant can be used in traditional medicine for the treatment of various ailments (Shanmugarajan et al, 2008, Pratumvinit et al, 2009). This plant has anti-diarrheal (Mandal and Kumar 2002), hypoglycemic (Ghosh, et al., 2004), antineoplastic (Pratumvinit et al., 2009), hepatoprotective (Mandal et al., 2000), emetic, astringent, antitussive, antipyretic and anti-inflammatory activity (Mandal and Kumar, 2002; Peraza-Sanchez et al., 2002). As a part of our ongoing investigations on local medicinal plants of Bangladesh (Saha et al., 2008), in this article, we have reported a study on the *in vitro* antioxidant and cytotoxic activities of methanolic leaf extract of *Ficus hispida* Linn. The antioxidant power was evaluated by *in vitro* DPPH free radical scavenging, reducing power and total antioxidant capacity assays and *in vitro* cytotoxic activity was investigated with brine Shrimp lethality bioassay.

MATERIALS AND METHODS

Chemicals

DPPH (1, 1-diphenyl, 2-picrylhydrazyl), TCA (trichloroacetic acid) and ferric chloride were obtained from Sigma Chemical Company, USA. Ascorbic acid was obtained from SD Fine Chem. Ltd., Biosar, India. Ammonium molybdate was purchased from Merck, Germany.

Plant material

Leaves of *Ficus hispida* Linn. were collected from Dhaka, Bangladesh and identified by the expert of the National Herbarium of Bangladesh. The accession number of the plant was 32539; a voucher specimen for this collection has been retained in the National Herbarium, Dhaka, Bangladesh.

Extraction

About 100 gm of powdered material was taken in a clean, flat-bottomed glass container and soaked in 500 ml of 80% methanol. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper (Bibby RE200, Sterilin Ltd., UK). The filtrate (methanolic extract) obtained was completely evaporated until dried. It rendered a gummy concentrate of greenish color designated as crude extract of methanol.

Phytochemical screening

The freshly prepared extract was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorff's reagent, Mayer's reagent and Fehling's Solutions, flavonoids with the use of Mg and HCl, tannins with ferric chloride and potassium dichromate solutions, steroids with sulfuric acid and saponins with ability to produce suds. Gum was tested using Molish reagents and concentrated sulfuric acid. These were identified by characteristic color changes using standard procedures (Trease et al., 1983).

In vitro Antioxidant tests

DPPH free radical scavenging activity

The free radical scavenging capacity of the extracts was determined using DPPH (Braca et al., 2001). DPPH solution (0.004% w/v) was prepared in 95% methanol. Methanolic leaf extract was mixed with 95 % methanol to prepare the stock solution (5 mg/ml). Freshly prepared DPPH

solution (0.004% w/v) was taken in test tubes and the extracts was added followed by serial dilutions (10 µg to 500 µg) to every test tube so that the final volume was 3 mL and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV-Visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (5 mg/ml). Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95 % methanol was served as blank. The percent (%) scavenging of DPPH free radical was measured by using the equation $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/ standard. The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition \pm standard deviation. IC_{50} values was also determined.

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure describe by Prieto et al. (1999). The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer) after cooling to room temperature. Methanol (0.3 ml) in the place of extract was used as the blank. The antioxidant activity was expressed as the number of equivalents of ascorbic acid.

Reducing power

Different concentrations of crude extract (100 µg-800 µg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution (Oyaizu, 1986). The absorbance of the final reaction mixture of two parallel experiments was taken and was expressed as mean \pm standard deviation.

Cytotoxicity study

Brine shrimp lethality bioassay (Meyer et al., 1982) technique was applied for the determination of cytotoxic property of methanolic leaf extract of *Ficus hispida* Linn. For brine shrimp lethality bioassay the eggs of Brine Shrimp (*Artemia salina*, Red Top®, Ocean Star International, USA) were hatched in a tank at a temperature around 37°C equipped with constant oxygen supply for 24 hours. Stock solutions of both the samples were prepared by dissolving required amount of extract in specific volume of pure dimethyl sulfoxide (DMSO). Fifty four clean vials were taken; eighteen of these were for the extract in nine concentrations (two vials for each concentration) and the rest thirty six vials for negative and positive control test.

Preparation of test samples

4ml of seawater was given to each of the vials. Then with the help of micropipette specific volumes of samples were transferred from the stock solutions to the vials to get final sample concentrations of 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320µg/ml. The concentration of DMSO in these vials should not exceed 40µl per 4 ml of Brine Shrimp nauplii because above this concentration DMSO may become toxic to the nauplii (Meyer et al., 1982).

Preparation of positive control group

Vincristine sulphate was used as the positive control. Measured amount of vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions were made using DMSO to get 10 µg/ml, 5 µg/ml, 2.5µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml and 0.0390 µg/ml. Then the positive control solutions were added

to the premarked vials containing ten living brine shrimp nauplii in 4 ml simulated sea water to get the positive control groups.

Preparation of negative control group

30 μ l of DMSO was added to each of premarked glass vials containing 4 ml of simulated seawater and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test was considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted.

RESULTS

Phytochemical Screening

The Results of phytochemical screening are summarized in Table 1.

Table 1: Result of chemical group test of methanolic leaf extract of *Ficus hispida* Linn.

Extract	Steroid	Alkaloid	Tannin	Gum	Flavonoid	Saponin
MEFH	-	+	+	+	+	-

MEFH: Methanolic extract of *Ficus hispida* Linn., (+): Present; (-): Absent

DPPH radical scavenging activity

Result of DPPH free radical scavenging assay is given in Figure 1. The IC_{50} values of the extract and Ascorbic acid were 58.14 μ g/ml and 48.78 μ g/ml respectively. % scavenging of DPPH radical was found to rise with increasing concentration of the crude extract.

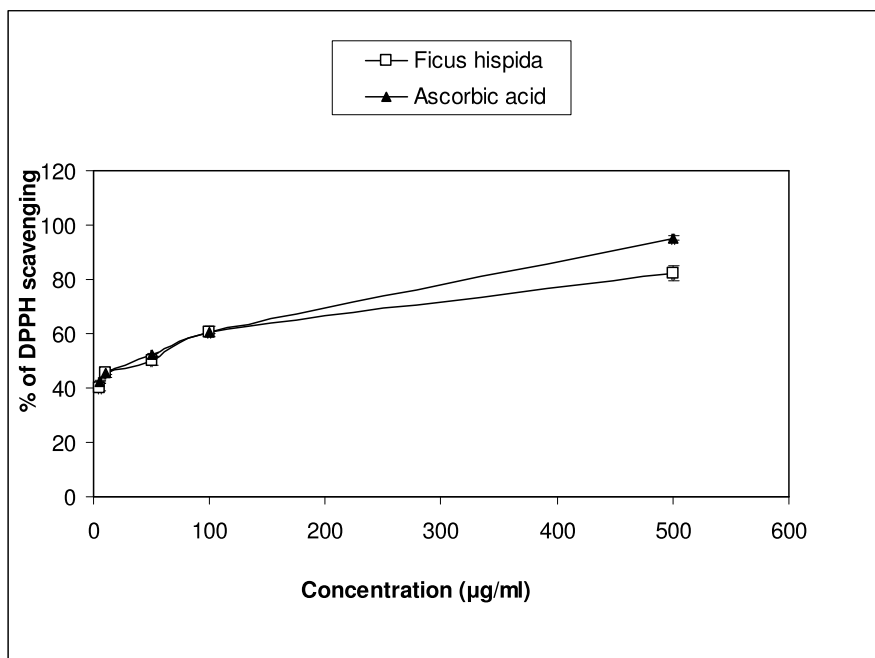


Figure 1: DPPH radical scavenging activity of the methanolic leaf extract of *Ficus hispida* Linn.

Total antioxidant capacity

The total antioxidant capacity of the plant extract is given in Table 1. Total antioxidant capacity is expressed as the number of equivalents of ascorbic acid (AAE).

Table 2: Total antioxidant capacity of methanol leaf extract of *Ficus hispida* Linn.

Materials	Concentration ($\mu\text{g/ml}$)	Equivalent to ascorbic acid ($\mu\text{g/mg}$)
Methanolic extract of <i>Ficus hispida</i> Linn.	100	1.00 ± 0.20
	200	1.80 ± 0.05
	400	2.50 ± 0.17
	600	3.25 ± 0.15
	800	4.68 ± 0.21

Reducing power

Figure 2 shows the reductive capabilities of the plant extract compared to ascorbic acid. An increase in absorbance in the reducing power method implies that extracts are capable of donating hydrogen atoms in a dose dependent manner.

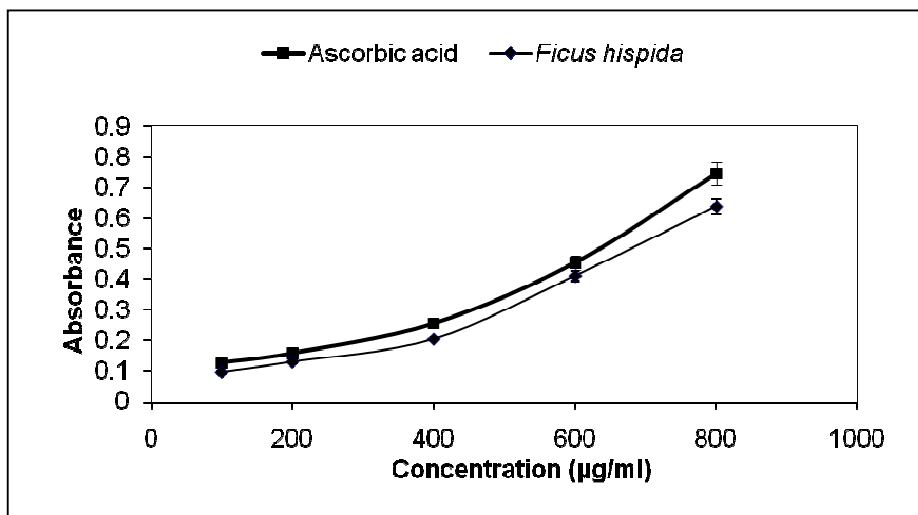


Figure 2: Reducing power of the crude methanolic leaf extract of *Ficus hispida* Linn.

Brine Shrimp lethality bioassay

The result of Brine Shrimp lethality bioassay is given in Table 3. *Ficus hispida* Linn. leaf extract displayed strong cytotoxic activity. LC_{50} value of the extract was $7.94 \mu\text{g/ml}$ whereas LC_{50} value of the extract was $0.078 \mu\text{g/ml}$.

Table 3: LC_{50} data of *Ficus hispida* L.in brine shrimp lethality bioassay

Sample	LC_{50} ($\mu\text{g/ml}$)
<i>Ficus hispida</i> Linn.	7.94
Vincristine sulphate	0.078

DISCUSSION

DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. On the other hand, the phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. Whereas for the measurements of the reductive ability, it has been investigated from the Fe^{3+} to Fe^{2+} transformation in the presence of extract samples using the method followed by Oyaizu (Oyaizu, 1986). An increase in absorbance in the reducing power method implies that extracts are capable of donating hydrogen atoms in a dose dependent manner. The reducing power of the extract was found remarkable and the reducing power of the extract was observed to rise as the concentration of the extract gradually increased.

In the past few years, there has been growing interest in the involvement of reactive oxygen species (ROS) in several pathological situations. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans et al., 1997; Jorgensen et al., 1999). Many plants contain substantial amounts of antioxidants including Vitamin C and E, carotenoids, flavonoids, tannins and thus can be utilized to scavenge the excess free radicals from the human body (Pratt, 1992).

Radical scavenging activity of the plant extract can be explained by the presence of flavonoids, or other chemoprotective agents present in the plant and preliminary phytochemical screening of leaf extract showed the presence of flavonoids. This *in vitro* antioxidant study also can be correlated with the previously reported antioxidant action of methanolic leaf extract of *Ficus hispida* Linn. against azathioprine induced oxidative stress and liver injury in male Wistar rats (Shanmugarajan et al, 2008)

In the current study brine Shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer activity of natural products. It is indicative of cytotoxicity and a wide range of pharmacological activity of the compounds (Meyer et al.1982). As compared to positive control vincristine sulphate, the plant extract exhibited considerable cytotoxic activity. As phytochemical tests showed the presence of alkaloids in methanolic extract, it might be responsible for cytotoxic effect. This finding can be correlated with utilization of stems of *Ficus hispida* Linn. for the treatment of malignancies in Thai traditional medicinal formulations. Another study reveals that methanolic extract of the stems exhibited antineoplastic activity against human breast cancer cell lines (Pratumvinit et al, 2009) and it proves that *Ficus hispida* Linn. has cytotoxic potential. This finding clearly indicates the presence of bioactive principles in this crude extract which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents.

CONCLUSION

It can be speculated that the methanolic leaf extract of *Ficus hispida* Linn. possesses both antioxidant and cytotoxic properties. Further investigation is required to isolate pure compounds for establishing its mechanism of action.

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