ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF FRUITS OF *FICUS BENGALENSIS LINN.*

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Summary

Antioxidant activity of defatted ethanolic extract of fruits of *Ficus bengalensis* was studied for its free radical scavenging property in different *in vitro* models as 1, 1-Diphenyl-2-picryl hydrazyl (DPPH), nitric oxide, superoxide, hydroxyl radical and lipid peroxide radical model. The extract showed significant dose-dependent free radical scavenging property in all the models except in hydroxyl radical inhibition assay. IC_{50} values were found to be 8.16, 14.81, 25.66 and 16.32 µg/ml respectively in DPPH, nitric oxide, superoxide and lipid peroxidation inhibition assays. However in hydroxyl radical inhibition assay 1000 µg/ml extract showed only 11% inhibition with respect to the control. Measurement of total phenolic compounds by Folin-Ciocalteu's phenol reagent indicated that 1mg of the extract contained 128.55µg equivalent of pyrocatechol, indicating the possibility of its having antioxidant property apparently due to its high phenolic content.

Key Words: *Ficus bengalensis* Antioxidant, 1, 1-Diphenyl-2-picryl hydrazyl (DPPH), phenolic content. ,

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Introduction

Oxygen is essential for the survival for all living things and during the process of its utilization in normal physiological and metabolic processes, approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals **(1, 2)**. All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the cells of human body rendering each cell to face about 10000 oxidative hits per second **(3).** When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to a number of physiological disorders **(4-6).** Free radicals are involved in the development of degenerative diseases **(6)**. They have also been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, neurological disorders, and in the process of aging **(7).** Many plants often contain substantial amounts of antioxidants including vitamin C and E, carotenoids, flavonoids and tannins etc., and thus can be utilized to scavenge the excess free radicals from human body **(8).**

Ficus bengalensis Linn. (Moraceae) commonly known, as "Bargad" or "Banyan" tree is widely grown along roadsides and also in lawns and gardens. It is reported to contain ketones, sterols, ficusin, bergaptin, bengalenoside and the flavonoid glycosides, leucocyanidin, leucopelargonidin, lupeol, β-sitosterol, rhamnoglycoside and Quercetin-3-galactoside **(9,10).** All parts of the plant are used both in Unani and Ayurvedic systems of medicine as astringent, anodyne vulnerary, anti-inflammatory, anti-arthritic, diaphoretic, antidiarrhoel, antiemetic, tonic and in ophthalmic conditions. The aerial roots are useful in the treatment of obstinate vomiting and leucorrhoea and are said to be used in the cure of osteomalacia **(11).** As its hepatoprotective activity was observed to be significant **(12),** it became imperative to investigate its total antioxidant activity.

Material and methods

All chemicals used were of analytical grade. 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. Sodium nitroprusside, sulphanilamide, O-phosphoric acid, napthyl ethylene diamine dihydrochloride, nitroblue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), 2-Deoxy-D-ribose, hydrogen peroxide, ascorbic acid, ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$), trichloroacetic acid (TCA), thiobarbituric acid (TBA), potassium chloride(KCl), potassium ferricyanide ($K_3Fe[CN]_6$), ethylene diamine tetra acetic acid (EDTA), tris hydrochloride buffer, Folin-Ciocalteu's phenol reagent (FCR) and all solvents were obtained from SISCO Research Laboratories Pvt. Ltd, Mumbai, India.

Plant material and preparation of ethanolic extract: Fresh mature fruits of *Ficus bengalensis* were collected from Bhopal and authenticated by the taxonomic division, National Herbarium of Cultivated Plants, National Bureau of Plant Genetic and Resources, New Delhi and the specimen voucher No. **NHCP/NBPGR/2006/93/307** was preserved in the department. The dried fruits in moderately coarse powder were defatted with petroleum ether ($60\text{-}80^0$) and then extracted thoroughly with ethyl alcohol (95%).

The alcohol from the extract was removed under reduced pressure and finally dried in a desiccator (Yield 10.4%). Its ethanolic extract was used for *in- vitro* antioxidant activity.

Animals: The Institutional Animal Ethical Committee approved the use of animals for the lipid peroxidation assay **(Ethical clearance number: 711/02/a/CPCSEA).** Swiss albino mice of both sex (20±2g) and Wistar albino male rats (130–170g) were used for the present studies. They were housed in clean polypropylene cages (38X23X10 cm) with not more than six animals per cage and maintained standard laboratory condition (temperature25±2°C) with dark and light cycle (12/12 h). They were allowed free access to standard pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*.

DPPH radical scavenging activity: DPPH radical scavenging activity was measured according to the method of Cotelle *et al* 1996 with some modification **(13).** In brief, 3 ml reaction mixture containing 200µl of DPPH (100 µM in methanol) and 2.8 ml of ethanolic extract of *F. bengalensis* (at various concentrations; 3-110µg/ml) in methanol was incubated at 37°C for 30 min and absorbance of the test mixture was read at 517 nm using Shimadzu model DU–40 spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the formula **(14)**:-

$$\text{Percentage inhibition} = \frac{\text{(Absorbance of control – Absorbance of test)}}{\text{Absorbance of control}} \times 100 \ldots (1)$$

Nitric oxide radical scavenging activity: Nitric oxide (NO) radicals were generated from sodium nitroprusside solution at physiological pH. Sodium nitroprusside (1ml of 10 mM) was mixed with 1 ml of ethanolic extract of *F. bengalensis* in different concentrations (3-110µg/ml) in phosphate buffer (pH 7.4). The mixture was incubated at 25°C for 150 min. To 1ml of the incubated solution, 1ml of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride) was added **(15,16).** Absorbance was read at 546nm and percentage inhibition was calculated using the formula (1).

Superoxide radical scavenging activity: Superoxide anion scavenging activity of ethanolic extract of *F. bengalensis* was measured according to the method of Robak *et al* 1998 with some modification **(17).** All the solutions were prepared in 100 mM phosphate buffer (pH 7.4). 1ml of NBT (156 µM), 1ml of NADH (468 µM) and 3ml of ethanolic extract of *F. bengalensis* (to produce final concentrations of 3-110µg/ml) were mixed. The reaction was started by adding 100 µl of phenazine methosulphate (PMS) (60µM) and the mixture then incubated at 25°C for 5 min followed by measurement of absorbance at 560nm. The percentage inhibition was calculated from the formula (1).

Inhibition of hydroxyl radical: Hydroxyl radical scavenging activity was measured according to the method of Kunchandy and Rao 1990, by studying the competition between deoxyribose and test extract for the hydroxyl radical generated by Fenton's reaction **(18).** 1ml of reaction mixture containing 500µl of ethanolic extract of *F. bengalensis* solution of different concentrations (3-1000µg/ml), and 100 µl of each of 2-Deoxy-D-ribose (28 mM), EDTA (1.04 mM), $FeCl_3$ (0.2 mM) and ascorbic acid (1.0 mM) were incubated at 37°C for 1hr. The damage imposed on deoxyribose due to the free radicals was determined colorimetrically by measuring the thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa *et al* 1979, **(19).** Percentage inhibition was calculated using the formula (1).

Inhibition of lipid peroxidation: Rat liver homogenate was used as the source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. Liver was collected immediately after the sacrifice of the animals by cervical dislocation under ether anesthesia. The liver was homogenized with 40mM tris – HCl buffer (pH 7) and centrifuged at 3000 rpm for 10 min to get a clear supernatant. Reaction mixture (4ml) containing 0.5 ml of supernatant, ethanolic extract of *F. bengalensis* solution of different concentrations (3-110µg/ml) and 100µl of each of 0.15 M KCl, 15 mM $FeSO_4$ and 6mM ascorbic acid was incubated at 37°C for 1 hr. TCA (1 ml; 10%) was added to the mixture and the samples centrifuged at 3000 rpm for 20 min at 4°C to remove insoluble proteins. Supernatant was removed and 1ml TBA (0.8%) was added to this fraction followed by heating at 90°C for 20 min in a water bath. After cooling, the coloured TBA- MDA complex was extracted with organic solvent (2ml butanol) and absorbance was measured at 532 nm **(20).** Percentage inhibition was calculated by formula (1).

Reductive ability: Reducing power of ethanolic extract of *F. bengalensis* was determined based on the ability of antioxidants to form colored complex with potassium ferricyanide, TCA and $FeCl_3$. 1ml of different concentrations of ethanolic extract of *F. bengalensis* (to produce final concentration 100 – 600µg/ml) was mixed with 2.5 ml potassium ferricyanide (1%) and 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 min after which 2.5 ml TCA (10%) was added to it and centrifuged at 3000 rpm for 10 minute. To the supernatant (2.5 ml) was added an equal amount of water and 0.5 ml of $FeCl_3$ (0.1%) were added to it. Absorbance was measured at 700 nm **(21).**

Determination of total phenolic compounds: The content of total phenolic compounds in ethanolic extract of *F. bengalensis* was determined by using Folin-Ciocalteu's phenol reagent (FCR) and determining absorbance at 760 nm according to the method of Slinkard and Singleton **(22).** The content was expressed as equivalent of pyrocatechol (µg) by using the following equation, which was obtained from a standard pyrocatechol graph **(23).**

$$\text{Absorbance} = 0.001 \times \text{pyrocatechol (µg)} + 0.0033$$

Statistical analysis: Experimental results are expressed as *mean* ± *SD*. All measurements were replicated three times. Linear regression analysis was used to calculate IC_{50} values.

Results

The extract showed significant antioxidant activity *in vitro* free radical scavenging models except in hydroxyl radical inhibition assay (Fig. 1a and b) where only 11% inhibition was noted with 1000 µg/ml of ethanolic extract of *F. bengalensis*. Thus IC_{50} value could not be determined for hydroxyl radical inhibition assay. In all other models, ethanolic extract of *F. bengalensis* showed dose dependent results. The percentage inhibition in various models *viz.* DPPH, nitric oxide, super oxide radical and lipid peroxidation is shown in Fig. 1(a). IC_{50} values were found to be 8.16, 14.81, 25.66 and 16.32 µg/ml respectively. The reducing power of ethanolic extract of *F. bengalensis* was observed to be dose dependent as shown in Table-1. Determination of total phenolic compounds showed that 1mg of ethanolic extract of *F. bengalensis* contains 128.55µg equivalent of pyrocatechol.

Table 1: Reductive ability of ethanolic extract of fruits of *Ficus bengalensis* Linn.

Concentration of ethanolic extract of *F. bengalensis* (µg/ml)	**Absorbance at 700 nm**
100	0.037 ± 0.007
200	0.188 ± 0.021
300	0.302 ± 0.036
400	0.437 ± 0.044
500	0.566 ± 0.060
600	0.675 ±0.068

[Values are mean ± S.E.M of 3 replicates.]

Discussion

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radicals can bring about many adverse reactions leading to extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals **(4, 5).** Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation.

DPPH is a relatively stable free radical and the assay determines the ability of ethanolic extract of *F. bengalensis* to reduce DPPH radical to the corresponding hydrazine by converting the unpaired electrons to paired ones, which infect is the action of the antioxidants. The dose dependent inhibition of DPPH radical (Fig 1a) indicates that ethanolic extract of *F. bengalensis* causes reduction of DPPH radical in a stoichometric manner **(24, 26).**

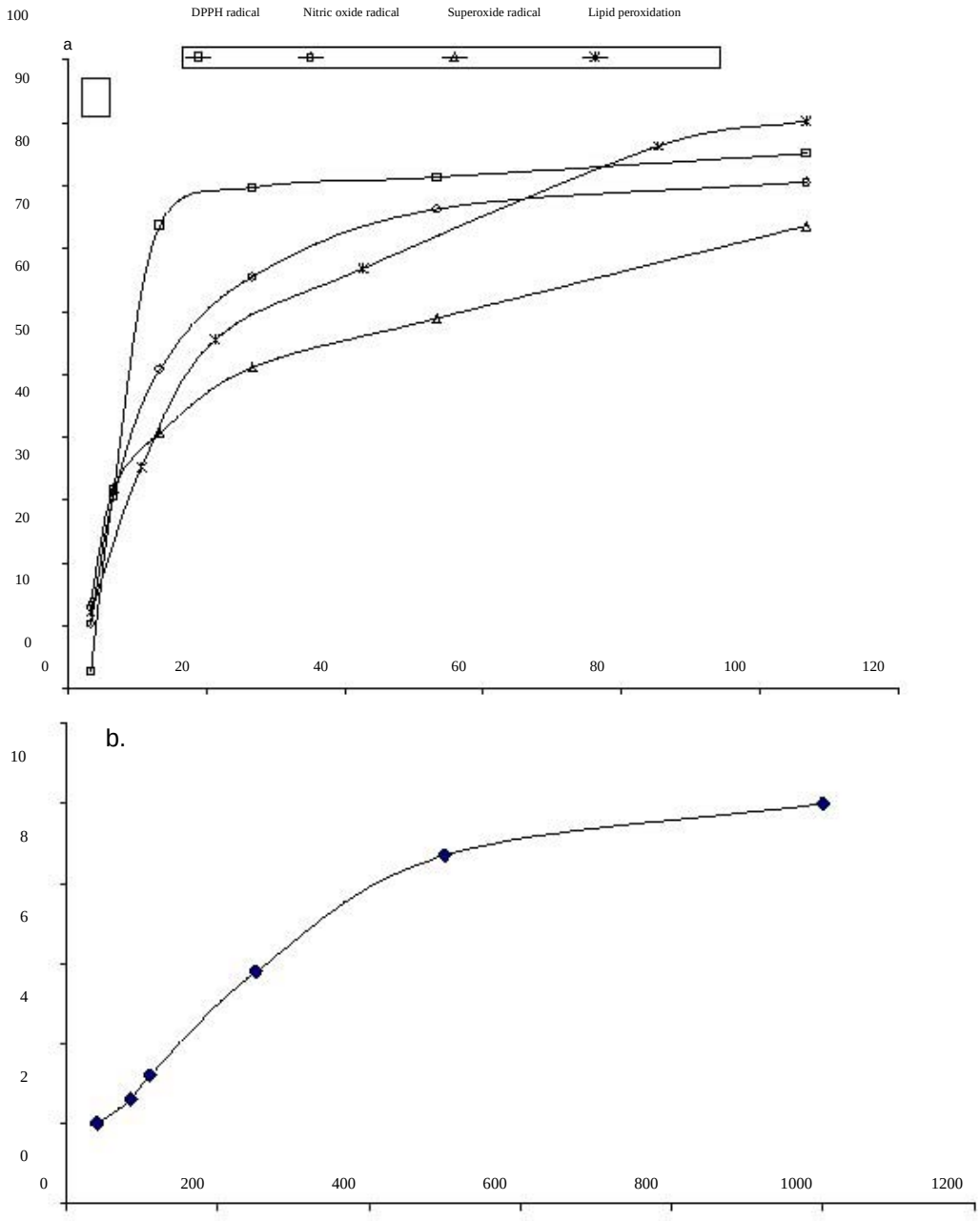
Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons etc and is involved in the regulation of various physiological processes **(3)**. Excess concentration of NO is associated with several diseases **(27, 28).** Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals **(4, 29)** .In the present study the extract competes with oxygen to react with nitric oxide and thus inhibits the generation of the anions.

Superoxides are produced from molecular oxygen due to oxidative enzymes **(29)** of body as well as *via* non-enzymatic reaction such as autoxidation by catecholamine **(30).** In the present study superoxide radical reduces NBT to a blue colored formazan that is measured at 560nm **(31).** The effect of ethanolic extract of *F. bengalensis* in this regard is shown in Fig 1a. The probable mechanism of scavenging the super oxide anions may be due to the inhibitory effect of MEDM towards generation of superoxides in the *in vitro* reaction mixture.

Ferrous salts can react with hydrogen peroxide and form hydroxyl radical *via* Fenton's reaction. The iron required for this reaction is obtained either from the pool of iron or the heme containing- proteins **(4).** The hydroxyl radical (OH*) thus produced may attack the sugar of DNA base causing sugar fragmentation, base loss and DNA strand breakage **(32).** Although ethanolic extract of *F. bengalensis* in concentration range of 3-110µg/ml is found to be sufficient to calculate IC_{50} values for different free radical inhibition models (Fig.1a), it has not been observed for hydroxyl radical model in which ease the concentration is increased up to 1000µg/ml **(14, 33)** which has not shown any promising effect. These differences can be explained by understanding the nature and generation of radicals as well as studying the differences in physical and chemical properties of the naturally occurring antioxidants **(34, 36).** The stable radicals like DPPH react stoichometrically with antioxidants which are hydrogen donors. But antioxidants which are effective chelators of transition metal ions may contribute differently to the antioxidant response in hydroxyl radical inhibition assay compared to the assays involving stable radicals, as Fe^{2+}/ Fe^{3+} is the active redox couple in Fenton reaction **(34).** The differences in antioxidant activity using different stable radicals are related to different redox potentials and steric properties of the radicals **(36).**

Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like brain and liver **(37).** In this study *in vitro* lipid peroxidation was induced to rat liver by using $FeSO_4$ and ascorbic acid. Lipid peroxidation occurs either through ferryl-perferryl complex or through OH^* radical by Fenton's reaction **(38).** Ethanolic extract of *F. bengalensis* has shown dose dependent prevention towards generation of lipid peroxides (Fig 1a).

Fig. 1 Effect of ethanolic extract of *F. bengalensis* on (a) different In vitro free radical models, (b) hydroxyl radical model



A substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atom **(21, 31).** The reducing property of ethanolic extract of *F. bengalensis* (Table 1) implies that it is capable of donating hydrogen atom in a dose dependent manner. The high content of phenolic compounds in the extract may be a contributing factor towards antioxidant activity because the phenolic compounds are known to have direct antioxidant property due to the presence of hydroxyl groups, which can function as hydrogen donor.

Preliminary phytochemical analysis shows the presence of tannins and flavonoids in ethanolic extract of *F. bengalensis* which are well known **(8, 39).** Thus the antioxidant potential of ethanolic extract of *F. bengalensis* fruits could be attributed due to the presence of polyphenolic compounds.

Acknowledgement

The authors are thankful to Dr. Anjula Pandey, Senior Scientist, Taxonomic Division, National Herbarium of Cultivated Plants, National Bureau of Plant Genetic and Resources, New Delhi for plants identification and its authentication. The author is also grateful for the Department of Pharmacy Barkatullah University, Bhopal for providing the necessary facilities.

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