

Evaluation of Antioxidant Properties of *Ficus bengalensis* Bark.

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ABSTRACT

Antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of the *Ficus bengalensis* bark have been tested using various antioxidant model systems viz, DPPH, hydroxyl, superoxide and ABTS. Methanol extract of *Ficus bengalensis* bark is found to possess higher DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activities. Like the antioxidant activity, reducing power of the extract increases with increase in concentration. The results suggest that phenolics and flavonoids in the bark provide considerable antioxidant activity. The results also suggest the potential of *Ficus bengalensis* bark as a medicine against free radical associated oxidative damage.

Keywords: Antioxidant activity, Solvent extraction, Phenolics, ABTS.

INTRODUCTION

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease¹⁻³. The potentially imprudent derivatives of oxygen, endorsed as ROS (Reactive Oxygen Species) such as O₂, H₂O₂ and OH radical are incessantly generated within the human body, if ROS overproduction or derisory antioxidant argument, this equilibrium is hindered favoring the ROS gain that culminates in oxidative hassle. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA. This oxidative damage produces a lot of chronic human diseases like diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases etc; nowadays more interest has been shown in the field of free radical biology, because the reason is to avoid the causes of chronic human diseases⁴⁻⁶.

Epidemiological studies have brought into being that the intake of antioxidants such as Vitamin-C (ascorbic acid) reduces the risk of coronary heart disease and cancer. The use of synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, tert-butylhydroquinone and propylgallate has been negatively perceived by consumers due to safety and health effects⁷. Hence, there is an increasing interest in the search of natural antioxidants from plant sources. It is well known that many botanicals possess natural antioxidants with high antioxidant activity⁸⁻¹⁰ and investigations on these were initiated based on their uses in traditional folkloric medicines.

The genus *Ficus* includes some 750 species of plants occurring in most tropical and subtropical forests throughout the world. The genus is remarkable for the large variation in the habits of its species¹¹. The different parts of the *Ficus bengalensis* tree have been found to

possess medicinal properties; leaves are good for ulcers, aerial roots are useful in gonorrhoea, seeds and fruits are used as coolant and tonic. The roots of *Ficus bengalensis* are given for obstinate vomiting and infusion of its bark is considered as a tonic and astringent and is also used in diarrhea, dysentery and diabetes. Ayurvedic practitioners in India are using the milky juice (latex) of stem bark of *Ficus bengalensis* for the treatment of rheumatism and other inflammatory diseases. The bark of the plant is used in Ayurvedic medicine for the treatment of diabetes. So our present study is focused on *Ficus bengalensis* to determine their free radical scavenging properties. The literature survey showed very little information on this plant and this prompted us to analyze this plant.

MATERIALS AND METHODS

The bark of *Ficus bengalensis* L. was collected from Courtallam, Tirunelveli District, Tamil Nadu. The plant was identified with help of local flora and authenticated in Botanical Survey of India, Southern circle, Coimbatore, Tamil Nadu.

Preparation of extract for antioxidant activity:

The bark of *F. bengalensis* was dried in shade, and then coarsely powdered separately in a willy mill. The coarse powder (100g) was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250 ml in a Soxhlet apparatus for 24 hrs. All the extracts were filtered through Whatman No.41 filter paper. All the extracts were concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity. The methanol extract was used for the estimation of total phenolics and flavonoids.

Estimation of Total Phenolics:

Total phenolic content was estimated using Folin-Ciocalteu reagent based assay as previously described¹² with little modification. To 1mL of each extract (100µg/mL) in methanol, 5mL of Folin-Ciocalteu reagent

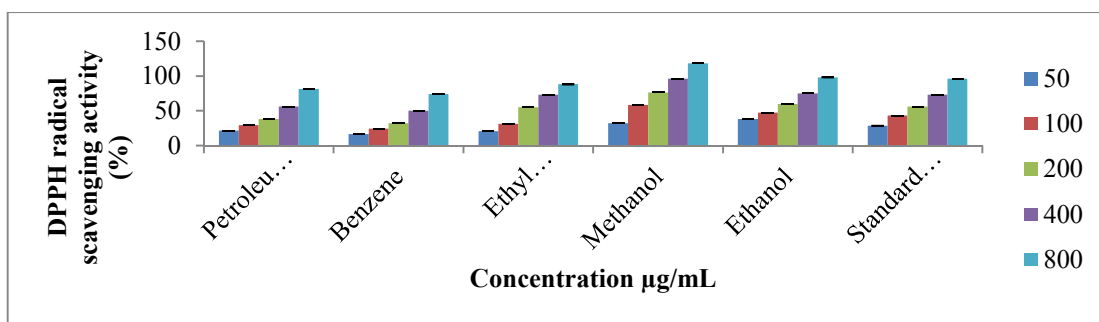


Figure 1: DPPH radical scavenging activity of different extracts of *F. bengalensis* bark

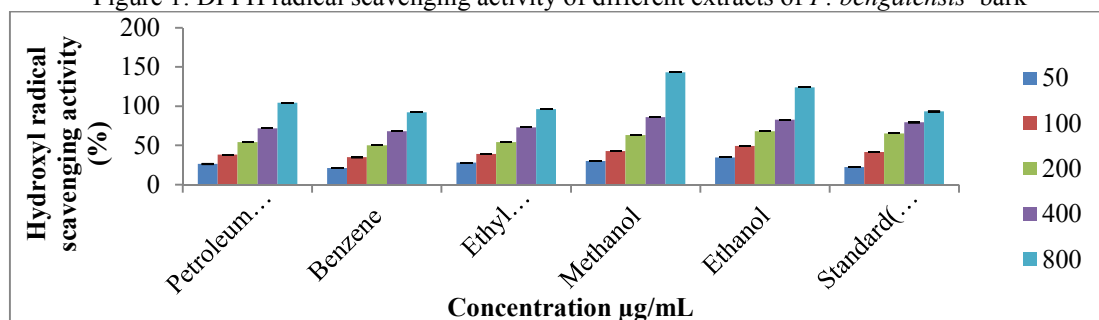


Figure 2: Hydroxyl radical scavenging activity of different extracts of *F. bengalensis* bark

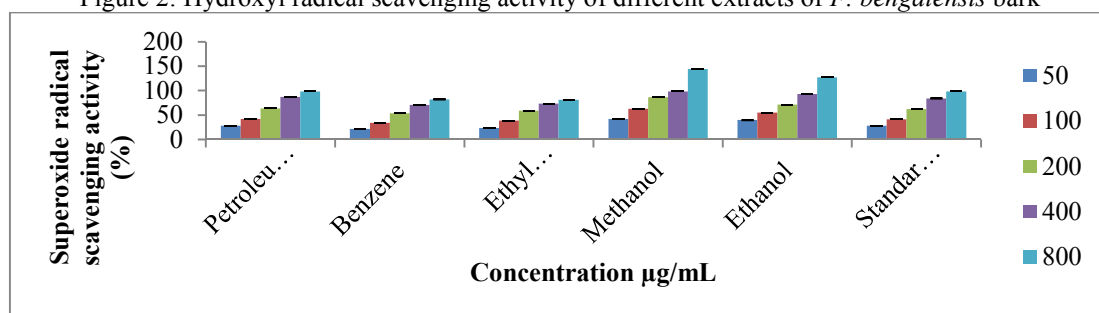


Figure 3: Superoxide radical scavenging activity of different extracts of *F. bengalensis* bark

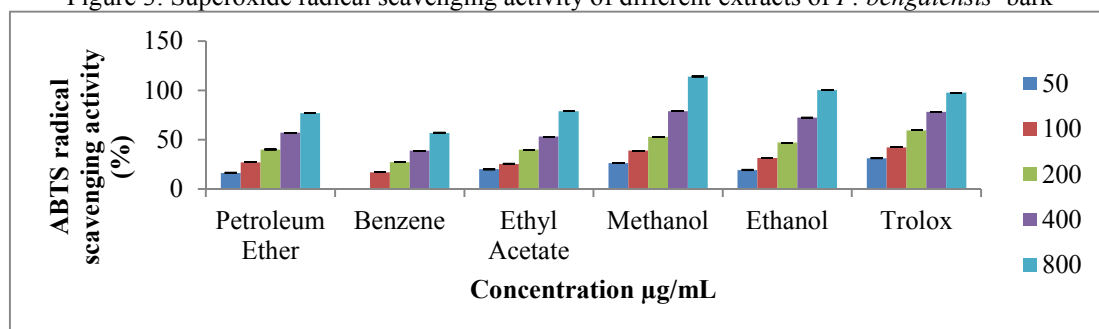


Figure 4: ABTS radical scavenging activity of different extracts of *F. bengalensis* bark

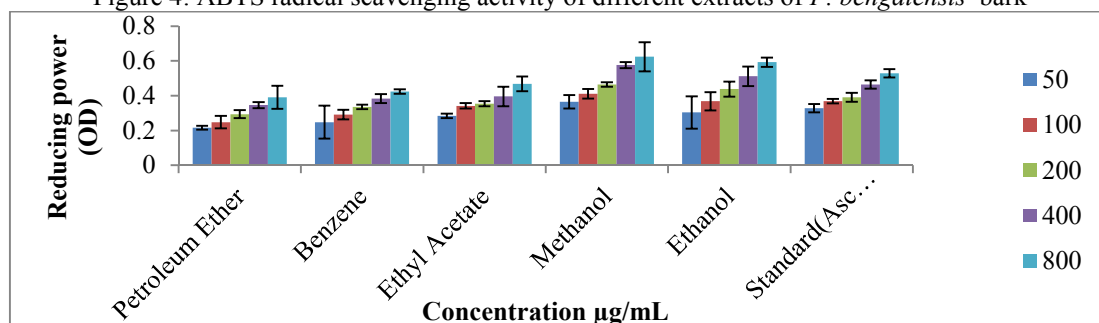


Figure 5: Reducing power ability of different extracts of *F. bengalensis* bark

(diluted ten-fold) and 4mL (75g/L) of Na₂CO₃ were added. The mixture was allowed to stand at 20°C for 30min and the absorbance of the developed colour was recorded at 765nm using UV-VIS spectrophotometer. 1mL aliquots of 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100g dry weight of extract).

Estimation of Flavonoids:

The total flavonoid content was determined according to Eom *et al*¹³. An aliquot of 0.5 mL of sample (1mg/mL) was mixed with 0.1mL of 10% aluminium chloride and 0.1mL of potassium acetate (1M). In this mixture, 4.3mL of 80% methanol was added to make 5mL volume. The mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H¹⁴.

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method¹⁴. Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 ml of the solution of all extracts in methanol at different concentration (50,100,200,400 & 800µg/mL). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0\} * 100$

Where, A₀ is the absorbance of the control reaction, and A₁ is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged

Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell¹⁵. Stock solutions of EDTA (1mM), FeCl₃ (10mM), Ascorbic Acid (1mM), H₂O₂ (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1mL EDTA , 0.01mL of FeCl₃, 0.1mL H₂O₂, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration (50,100,200,400 & 800µg/mL) dissolved in distilled water, 0.33mL of phosphate buffer (50mM , pH 7.9), 0.1mL of ascorbic acid in sequence . The mixture was then

incubated at 37°C for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan *et al*¹⁶. The superoxide anion radicals were generated in 3.0mL of Tris – HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 ml NADH (0.936mM) solution, 1.0 mL extract of different concentration (50,100,200,400 & 800µg/mL), and 0.5 mL Tris – HCl buffer (16mM, pH8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25°C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula

Antioxidant activity by radical cation (ABTS. +)

ABTS assay was based on the slightly modified method of Huang *et al*¹⁷. ABTS radical cation (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Reducing power

The reducing power of the extract was determined by the method of Kumar and Hemalatha¹⁸. 1.0 mL of solution containing 50,100,200,400 & 800µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6) and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50°C for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5°C) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

Statistical analysis

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

RESULT

Table 1: IC₅₀ values of different solvent extracts of bark of *F. bengalensis*^a

| Solvent | IC ₅₀ (µg/mL) | | | |
|-----------------|--------------------------|------------------|------------|-------|
| | DPPH | Hydroxyl radical | Superoxide | ABTS |
| Petroleum ether | 29.65 | 30.39 | 36.18 | 30.28 |
| Benzene | 28.13 | 29.15 | 32.33 | 21.35 |
| Ethyl acetate | 29.18 | 32.15 | 29.68 | 26.42 |
| Methanol | 43.81 | 36.29 | 48.13 | 43.83 |
| Ethanol | 33.40 | 31.63 | 46.91 | 39.27 |
| Ascorbic acid | 31.75 | 31.22 | 34.84 | - |
| Trolox | - | - | - | 37.84 |

All the values are mean by triplicate determines*

Total phenolics and total flavonoid content

The total phenolic content and total flavonoid content of the methanol extract of *F. bengalensis* bark were found to be 1.28g100g⁻¹ and 1.14g100g⁻¹ respectively.

DPPH radical scavenging activity

DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *F. bengalensis* bark was shown in Fig 1. The scavenging effect increases with the concentration of standard and extracts. Among the solvent tested, methanol extract of *F. bengalensis* bark exhibited highest DPPH radical scavenging activity. At 800µg/mL concentration, methanol extract of *F. bengalensis* possessed 118.16% scavenging activity on DPPH. The concentration of methanol extract of *F. bengalensis* needed for 50% inhibition (IC₅₀) was found to be 43.81µg/mL; whereas 31.75µg/mL was needed for ascorbic acid.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *F. bengalensis* bark was shown in Fig 2. Methanol extract showed very potent hydroxyl radical scavenging activity. At 800µg/mL concentration, methanol extract of *F. bengalensis* possessed 143.16% scavenging activity on hydroxyl radical. The quantity of *F. bengalensis* methanol extract required to produce 50% inhibition of hydroxyl radical was 36.29µg/mL; whereas 31.22µg/mL was needed for ascorbic acid (Table 1).

Superoxide radical scavenging activity

The different solvent extracts of *F. bengalensis* bark were subjected to be superoxide radical scavenging assay and the results were shown in Fig 3. It indicates that methanol extract of *F. bengalensis* bark (800µg/mL) exhibited the maximum superoxide scavenging activity of 143.91% which is higher than the standard ascorbic acid whose scavenging activity is 98.22%. The IC₅₀ values of methanol extract of *F. bengalensis* on hydroxyl radical were found to be 48.13µg/mL and 34.84µg/mL for ascorbic acid, respectively (Table 1).

ABTS radical cation scavenging activity

The different solvent extracts of *F. bengalensis* bark were subjected to the ABTS radical cation scavenging activity and the results were shown in Fig 4. The methanol extract exhibited potent ABTS radical cation scavenging activity in concentration dependent manner. At 800µg/mL concentration, *F. bengalensis* bark possessed 113.95% scavenging activity on ABTS which is higher than the standard trolox whose scavenging activity is 97.63%. The

concentration of methanol extract of *F. bengalensis* needed for 50% inhibition (IC₅₀) was found to be 43.83µg/mL; whereas 37.84µg/mL was needed for trolox (Table 1).

Reducing Power

Figure 5 showed the reducing ability of different solvent extracts of *F. bengalensis* bark compared to ascorbic acid. Absorbance of the solution was increased when the concentration increased. A higher absorbance indicates a higher reducing power. Among the solvent tested, methanol extract exhibited higher reducing ability.

DISCUSSION

Phenolics are ever-present secondary metabolites in plants and possess a wide range of therapeutic uses. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. Flavonoids are a group of polyphenolic compounds, which exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, anti allergic, antiviral, anticancer activities. They also inhibit enzymes such as aldose reductase and xanthine oxidase. They are able of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups and are potent antioxidants^{19,20}. The presence of high phenolic and flavonoid content in the bark extracts has contributed directly to the antioxidant activity by neutralizing the free radicals.

In vitro antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *F. bengalensis* bark were investigated in the present study by DPPH, hydroxyl, superoxide, ABTS radical cation scavenging activities. These methods have proven the effectiveness of the extracts in comparison to that of the reference standard antioxidants, ascorbic acid and trolox. DPPH radical scavenging activity is a very useful method as it is highly sensitive and rapid assay. This assay is independent on substrate polarity where DPPH can accept an electron or hydrogen radical to become a stable diamagnetic molecule. When an antioxidant scavenges the free radical by hydrogen donation, the purple color of DPPH in assay solution turns to yellow, which can be monitored spectrophotometrically at 517nm²¹. The results of this study indicate that all the solvent tested have noticeable effect on DPPH radical. Among the solvent tested, methanol extract of *F. bengalensis* bark exhibited more DPPH radical scavenging activity.

The hydroxyl radical is the most reactive oxygen species that induces severe damage in biomolecules. The hydroxyl scavenging ability of different solvent extracts of *F.*

bengalensis bark was estimated by generating hydroxyl radicals using deoxyribose method. The hydroxyl radical generated through Fenton reaction which degraded deoxyribose using Fe^{2+} as an important catalytic component. The potential of bark extract to inhibit hydroxyl radical-mediated deoxyribose damage was determined by means of iron (II) dependent DNA damage assay. In this assay, the test and standard compound colour changed to various shades of pink. Antioxidant efficiency of bark extracts compared to the standard control ascorbic acid was determined as the ability to scavenge the free radical generated. When *F. bengalensis* bark extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. Among the solvent tested, methanol extract possessed more hydroxyl radical scavenging activity when compared with standard ascorbic acid. The ability of the extracts to quench hydroxyl radicals can be related to the prevention of lipid peroxidation. Moreover, it seemed to be a good scavenger of active oxygen species, thus reducing the rate of chain reaction.

Superoxide anions damage biomolecules directly or indirectly by forming H_2O_2 , OH, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation²². The superoxide scavenging activity of the plant extract was increased markedly with the increase in concentrations. Methanol extract showed potent superoxide radical scavenging activity. Thus, higher inhibitory effects of methanol extract of *F. bengalensis* bark on superoxide anion formation showed here in possibly renders them as promising antioxidants.

ABTS radical cation scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS for the estimation of antioxidant activity²³. The present study, methanol extract of bark of *F. bengalensis* was fast effective scavengers of ABTS radical and this activity was higher than that of trolox standard. Proton radical scavenging is an important attribute of antioxidants. ABTS a protonated radical has characteristic absorbance maxima at 734nm which decreases with the scavenging of the proton radical²⁴.

In reducing power, the presence of antioxidants in the samples would result in reducing Fe^{3+} to Fe^{2+} by donating an electron by the extracts. The extracts with reducing power reveal that they are electron donors, reduce the oxidized intermediates and act as primary antioxidant substances²⁵. Increasing absorbance at 700nm indicated an increase in reducing ability. The high absorbance of extracts may be due to its strong reducing potential. The reducing power of the extracts may be caused by the bioactive compounds in the extracts which possess potent donating abilities.

The result of the present study showed that the methanol extract of *F. bengalensis* bark, which contains phenolic and flavonoid compounds, exhibited the great antioxidant activity. This study also demonstrated that the methanol is the best solvent to obtain the main antioxidant constituents. All these findings indicate that *F. bengalensis* bark extracts possess antioxidant activity. By these findings and purification of the active substance(s) present in the extracts of *F. bengalensis* bark, it will be possible to discover new natural drugs serving as antioxidant agents for application in the nutritional or pharmaceutical fields, in the prevention of free radical mediated diseases. Further studies are needed to explore the potential phenolic and flavonoid compounds from *F. bengalensis* bark and *in vivo* studies are needed for better understanding their mechanism of action.

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