In Vitro Antioxidant Studies and Total Phenolic Content of *Ficus Religiosa* Fruits Extract

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Summary

The aim of this study was to assess the *in vitro* potential of methanolic extract of *Ficus religiosa* fruits as a natural antioxidant. The DPPH activity of the *Ficus religiosa* fruit extract (FRFE) (10, 20, 40, 80, and 160 µg/ml) was increased in a dose dependent manner, which was found in the range of 43.5 - 73.92% as compared to ascorbic acid 55.25 - 85.79%. There is a significant increase in reducing power of FRFE with increasing the concentration. FRFE demonstrated the ability to inhibit the formation of TBARS in a concentration dependent manner. Measurement of total phenolic content of the methanolic extract of *Ficus religiosa* fruit was achieved using Folin-Ciocalteau reagent containing 0.2%w/w of phenolic content, which was found significant. The results obtained in this study clearly indicate that *Ficus religiosa* fruits have a significant potential to use as a natural antioxidant agent. The overall results of this study indicates that the various extract conc. from *Ficus religiosa* fruits have interesting antioxidative properties and represent a potential source of medicine for the treatment of inflammatory diseases.

Keywords: *Ficus religiosa* fruits; Antioxidant activity; DPPH; Reducing power assay; TBE assay; Total phenolic content

Introduction

Several physiological functions require free radicals for their normal modulation and these free radicals are generated in different forms at a low level in cells. There is a balance between the generation and quenching of these free radicals in the body. Imbalance between the generation and quenching system results in the excess amount of free radicals in the body which may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease like aging, arthritis, asthma, carcinogenesis, diabetes, rheumatism and various neurodegenerative diseases(1). Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases. But there is a need to replenish these antioxidant compounds because these are used up in the process of neutralizing free radicals (2). The traditional medicine all over the world is nowadays

revalued by an extensive activity of research on different plant species and their therapeutic principles.

As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity. Fruits and vegetables contain different antioxidant compounds, such as ascorbic acid, tocopherol, glutathione and carotenoids, which may contribute to protection against oxidative damage (3). Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors and from the ability of the polyphenol-derived radicals to stabilize and delocalize the unpaired electron, and from their ability to chelate transition metal ions (termination of the Fenton reaction) (4). Thus, polyphenols may play a role in the antioxidant ability related to fruit browning.

Ficus Linn is the largest genus of the family Moraceae comprising of about 755 fig tree species worldwide (5). It is retained as a single, large genus because it is well defined by its unique reproductive system, involving Syconia fig and specialized pollinator wasps (6) *Ficus religiosa* commonly known as Bodhi tree is one of the most important species of this genus. Traditionally almost every part of this plant is used for treatment of various diseases. Leaf juice along with honey is used for treatment of asthma, cough, sexual disorders, diarrhoea, haematuria, earache and toothache, migraine, eye troubles, gastric problems (7) and scabies. Fruits are used for the treatment of asthma and respiratory disorders. Fruit paste is taken to cure scabies. Stem bark is used in the treatment of gonorrhoea, bleeding, cuts, wounds, paralysis, diabetes, diarrhea, bone fracture and used as antiseptic, astringent and antidote. Bark paste along with honey is used to treat cough and cold as well as accompanying mild fever. Aerial root juice is used for treatment of menustral problems (8).

The objective of the current research was to evaluate the antioxidant properties of polyphenols present in *Ficus religiosa* fruits.

Material and Methods

Collection of plant material:

The fruits of *Ficus religiosa* were collected in the month of Nov.-Dec, 2008 from Chandigarh and were authenticated from Dept. of Botany Punjab University Chandigarh. The *Ficus religiosa* fruits were subjected to shed drying and further crushed to powder, and then the powder was passed through the mesh 40.

Preparation of extract:

250 g of coarse powder of drug was first defatted with petroleum ether and then extracted in Soxhlet apparatus with methanol for 12 hours and filtered to yield the extract. The extract was then concentrated and finally dried to a constant weight. The dried extract was used for the evaluation of antioxidant activity through various *in vitro* models. Preliminary phytochemical analysis showed the presence of steroids, amino acids, phenolic compounds and carbohydrates in the drug extract.

Determination of total phenolic content using Folin- Ciocalteu phenolic reagent: (9)

Preparation of calibration curve using gallic acid as standard:

Weigh 10mg of standard gallic acid and dissolve in 100mL distilled water in a volumetric flask (100µg/mL of stock solution). From the above stock solution pipette out aliquots of 0.5 to 2.5mL into 25mL volumetric flasks. Add 10mL of distilled water and1.5 mL of Folin-Ciocalteu reagent, diluted according to the label specification to each of the above volumetric flasks. After 5 minutes add 4mL of 20% sodium carbonate solution, make up the volume to 25mL with distilled water. After 30 minutes the absorbance was recorded at 765 nm and a calibration curve of absorbance *vs* concentration was plotted. The results were expressed as

gallic acid equivalents (GAE), milligrammes per 100 g of dry weight (dw). All determinations were performed in triplicate (n = 3).

Preparation of test solution:

1g of accurately weighed powdered drug was extracted with (3 × 15mL) 50% aqueous methanol by cold maceration for 2 hours with intermittent shaking. Filtered and the final volume of the combined methanolic extract was made up to 50mL. From this test solution 1mL was pipette out into 25mL volumetric flask and same procedure was followed for color development using Folin- Ciocalteu reagent. The amount of total phenolics was calculated using the standard curve of gallic acid.

***In-vitro* assays:**

Determination of DPPH radical scavenging activity:

Scavenging activity of *Ficus religiosa* fruits extract against DPPH radicals was assessed according to the method of Blois (10) and Lia (11). A rapid, simple and inexpensive method to measure antioxidant capacity involves the use of the free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. 0.1 mM solution of DPPH in methanol was prepared and 1.0 mL of this solution was added to 3.0 ml of extract solution in water at different concentrations (8-250 µg/mL). It was incubated at room temperature for 45 minutes and the absorbance was measured at 517 nm against the corresponding blank solution. The assay was performed in triplicates. Ascorbic acid was taken as reference. Percentage inhibition of DPPH free radical was calculated based on the control reading, which contain DPPH and distilled water without extract using the following equation:

$$\text{DPPH Scavenged (\%)} = \frac{(\text{A cont} - \text{A test}) \times 100}{\text{A cont}}$$

Where A cont is the absorbance of the control reaction and A test is the absorbance in the presence of the sample of the extracts.

Reducing power assay: (12-13)

For the measurement of the reductive ability, we investigated the Fe^{3+}- Fe^{2+} transformation in the presence of methanolic extract of fruits of *Ficus religiosa*. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (14). The reducing power was determined according to the method of Oyaizu (1986). The different concentration of the extracts (100-1000 µg/mL) in 1 mL of deionized water were mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6) and 1% potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL). The mixture was incubated at 50°C for 20 minutes. The reaction was stopped by adding trichloroacetic acid (2.5 mL, 10%) to the mixture, which was then centrifuged at 1000 x g for 10 min. The upper layer of 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. Ascorbic acid was taken as a reference.

Lipid peroxidation by thiobarbituric acid assay (TBA):

During lipid peroxidation low-density lipoprotein (LDL) breaks down into TBARS and the amount of TBARS can be used as an index of lipid peroxidation (15). As phenolic antioxidants are suggested to act as inhibitors of LDL oxidation by means of free radical scavenging, so it is expected that the presence of these compounds in these extract might be responsible for inhibiting the LDL oxidation by donating the hydrogen atom (16). The main interest of the recent research suggests that LDL oxidation may play an important role in the

pathogenesis of atherosclerotic complications, including coronary heart disease (CHD). The radical-mediated oxidative chain reaction is a possible mechanism involved in LDL oxidation. LDL oxidation is believed to be a complex and multistep process involving both lipid and protein fractions through different mechanisms (17). Antioxidants, including vitamins C and E, flavonoids and other plant phenolics, have been shown to suppress LDL oxidation and delay the development of heart diseases (18).

TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (19). Normal male rats (250 g) were used for the preparation of liver homogenate. The perfused liver was isolated and 10% (w/v) homogenate was prepared with homogenizer at 0-4°C with 0.15 M KCl. The homogenate was centrifuged at 8,000 x g for 15 minutes and clear cell-free supernatant was used for the *in vitro* lipid peroxidation assay with. Different concentrations (50-700 µg/ml) of extract/fractions were dissolved in methanol in test tubes and then 1 ml of 0.15 M KCl and 0.5 mL of rat liver homogenates were added to the test tubes. Peroxidation was initiated by adding 100 µL of 0.2 mM ferric chloride. After incubation at 37 °C for 30 minutes, the reaction was stopped by adding 2 mL of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid (TCA), 0.38% TBA, and 0.5% butylated hydroxytoluene. The reaction mixtures were heated at 80°C for 60 minutes. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm. The percentage inhibition of lipid peroxidation is calculated by the formula: Inhibition of lipid peroxidation (%) = 1 – (sample OD/blank OD) X 100

Statistics:

All analyzed were run in triplicate. The average value and standard deviation were calculated using Excel. Analysis of variance (ANOVA) was used to evaluate the significant difference among various treatments with the criterion of P = 0.05 (29).

Results and Discussion

DPPH radical scavenging activity:

DPPH is a stable free radical at room temperature which when accepts an electron or hydrogen radical becomes a stable diamagnetic molecule (20).The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 517 nm, induced by antioxidants. The absorption maximum of a stable DPPH radical in methanol was at 517 nm. On reaction with antioxidant or free radical there is decrease in absorbance of DPPH radical because of scavenging of the radical by hydrogen donation. There is change in color from purple to yellow which is visually noticeable. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity (21). Data showed the DPPH radical scavenging activity of FRFE and the standard ascorbic acid. The FRFE significantly inhibited the activity of DPPH radicals in a dose-dependent manner. FRFE at different concentration of 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml and 160µg/ml showed the percentage inhibition were 43.5%, 55.64%, 69.64%, 72.37% and 73.92% respectively and the percentage inhibition showed by the ascorbic acid at same conc. was 55.25%, 64.47%, 71.53%, 79.37%, 85.79% respectively, It appears that FRFE have a strong hydrogen-donating capacity and can efficiently scavenge DPPH radicals. The results also indicate that the methanolic extract of FRF reduces the DPPH radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles (22). On reaction of DPPH free radicals with suitable reducing agents, the free electrons become paired off and the color of solution is lost stoichiometrically depending on the number of electron taken up (11). Figure 1 illustrates the percentage inhibition by extract in comparison to the standard.

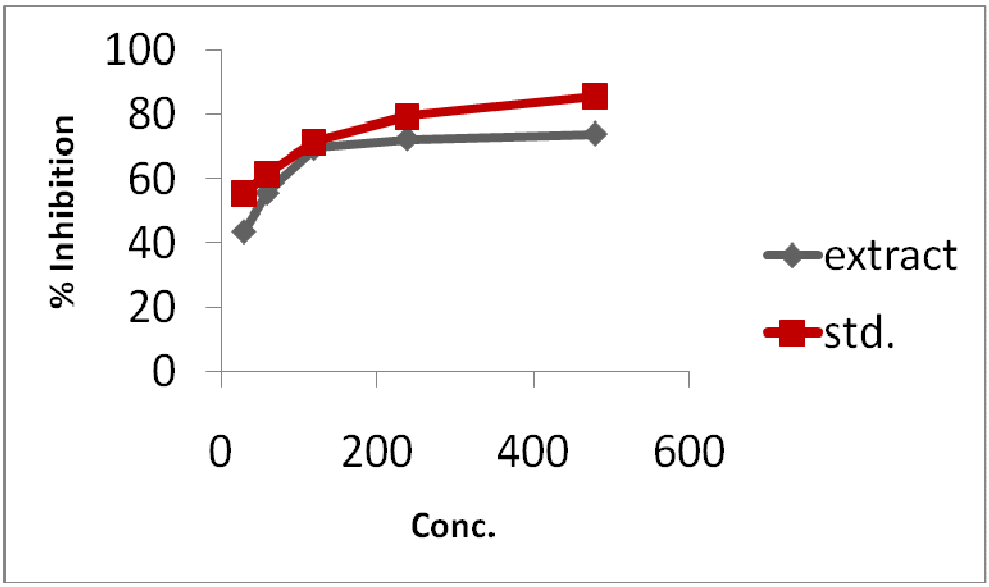

Figure 1: Effect of methanolic extract of *Ficus religiosa* fruits on DPPH scavenging activity

Reducing power assay:

It has been reported that reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (23). As shown in data FRFE exhibited a comparable reducing power than ascorbic acid, suggesting that the polyphenols had strong electron-donating capacity. Like the antioxidant activity, the reducing power of the different concentration of FRFE increased with increasing the concentration. The reducing power showed by the FRFE is statistically significant ($p<0.01$).The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging (24). Figure 2 shows the reductive capability of the methanolic extract of FRF to ascorbic acid (standard).

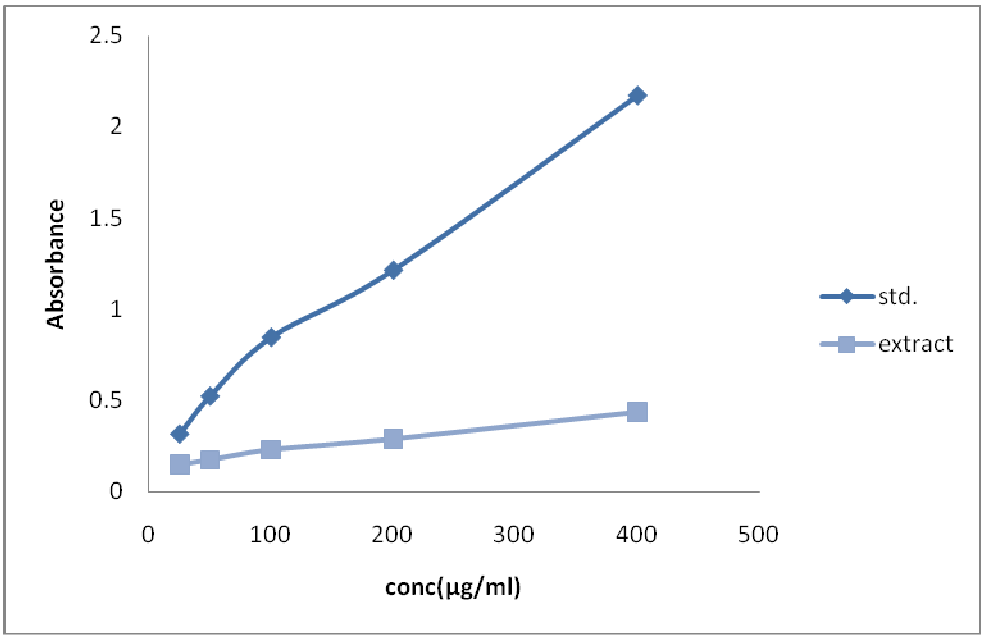

Figure 2: The reductive capability of the methanolic extract of fruits of *Ficus religiosa* to ascorbic acid (standard)

Lipid peroxidation by thiobarbituric acid assay:
The total antioxidant activity, which reflected the ability of the extracts to inhibit the $FeCl_3$/ascorbic acid induced phosphatidylcholine liposome oxidation, was measured and compared with that of a control which contained no antioxidant component. The inhibitory effects of FRFE at different concentrations on lipid peroxidation in liposomes are shown in Figure 3. FRFE demonstrated the ability to inhibit the formation of TBARS in a concentration dependent manner. The FRFE exhibited almost comparable antioxidant activity with reference to the standard. FRFE at various concentrations of 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml, 800µg/ml and 1000µg/ml showed percentage inhibition of 47.65%, 50.33%, 52.34%, 56.37%, 59.73% and 61.74% respectively. Ascorbic acid showed comparable percentage inhibition at the same concentrations. The inhibitory effects of Ascorbic acid on TBARS formation were 57.7%, 85.23%, 86.57%, 88.59%, 89.93%, and 92.63% respectively at the same concentrations as that of FRFE. There was no significant difference ($p > 0.05$) in antioxidant activities between the various conc. of FRFE and ascorbic acid at 50µg/ml.

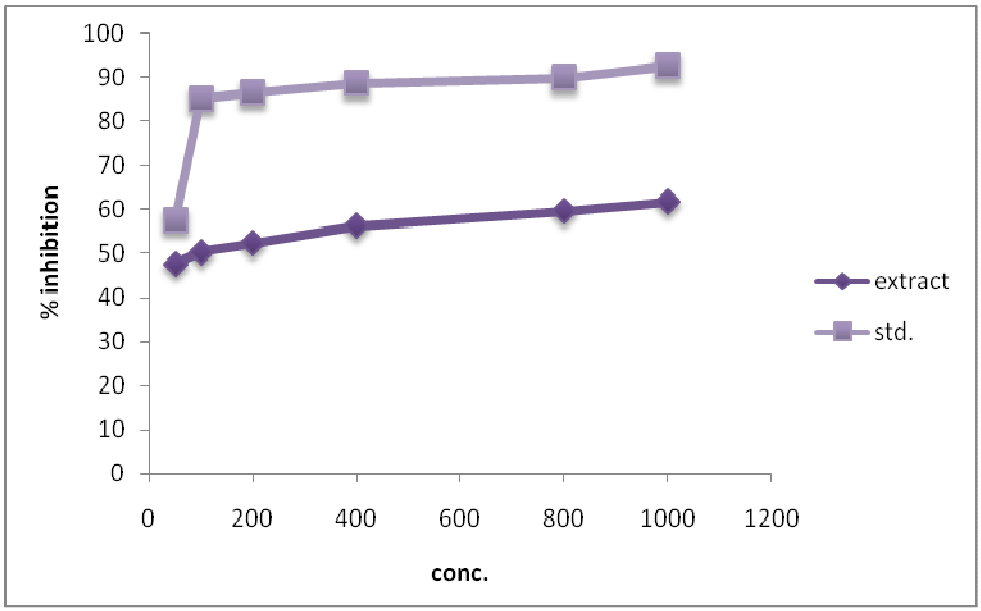

Figure 3: Effect of methanolic extract of *Ficus religiosa* fruits extract on lipid peroxidation

Determination of total phenolic content using Folin- Ciocalteu phenolic reagent:
Phenolic constituents are very important in plants because of their scavenging ability due to their hydroxyl group. Phenolic constituents are very important in plants because of their scavenging ability due to their hydroxyl groups (25). Percentage of total Phenolics present in the fruits of *Ficus religiosa* is 0.2%w/w of drug powder. Phenolic compounds are famous powerful chain breaking antioxidants (26).It has been suggested that up to 1.0 g polyphenolic compounds (from diet rich fruits or vegetables) ingested daily have remarkable inhibitory effects on mutagenesis and carcinogenesis in humans (27). In addition, it has been reported that phenolic compounds are associated with antioxidant activity and play a crucial role in stabilizing lipid peroxidation (28).

Conclusions

The results of the present study showed that the methanolic extract of *Ficus religiosa* Linn. fruits possess optimum antioxidant activity through the DPPH radical scavenging activity, reducing power assay and Lipid peroxidation by thiobarbituric acid assay. Preliminary phytochemical analysis indicates the presence of phenolic compounds which might be responsible for the antioxidant activity of the methanolic extract of fruits of *Ficus religiosa*.

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