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Evaluation of phytochemical and *in-vitro* antioxidant activity of *Filicium decipiens*

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Abstract

In the present study, preliminary phytochemical analysis and *in vitro* antioxidant activities of the ethanolic extracts of *Filicium decipiens* (FD) stem and bark was performed. The extract was screened for possible antioxidant activities by free radical scavenging activity (DPPH), xanthine oxidase inhibition (XOI), hydrogen peroxide scavenging activity (HPSA) and ferric-reducing antioxidant power (FRAP) assays. The FD stem and bark extracts exhibits antioxidant activity of 24.89% and 37.25% with an IC50 value of 2.3mg/ml and 1.8mg/ml in the DPPH radical scavenging method, 52.18 and 59.97% in the XOI assay, 43.5% and 47.64% by the HPSA method, and 0.675 mmol/Fe(II)g and 0.541 mmol/Fe(II)g in the FRAP method at the concentration tested. The FD stem and bark extract was found to contain total phenolics and flavonoid and their contents was 70.83mg and 76.27mg gallic acid equivalent (GAE) and 2.5 ± 0.15 mg and 5.4± 0.31mg of catechin equivalent per gram of dry extract, respectively. The results indicate that these extracts have both high free radical scavenging and xanthine oxidase inhibition activity.

Keywords: *Filicium decipiens*; antioxidant activity; phytochemical analysis.

Introduction

Filicium decipiens is a medium to large evergreen tree native to India and found abundantly in Nallamalai forest and available throughout the peninsular India. The leaves are compound and very large. Each leaf consists of 12 to 16 leaflets. Each leaflet is 4 to 6 inches in length and relatively narrows. The flowers are very small and white in colour, the tree produces both male and female flowers. The fruits are olive size, drupe, and dark blue in colour and form in clusters. The stem barks are black in colour [1]. Phytochemical investigations led to the identification of kaempferol, quercetin, 3',4'-di-0-methylquercetin, procyanidin, P-hydroxybenzoic acid, vanillic acid and melilotic acid, of tannin and of toxic saponins. Four new saponins have been isolated from the stem bark [2, 3].

The role of free radicals in many ailments has been well established. Several biochemical

reactions in our body system generate reactive oxygen species, which, if not effectively scavenged by cellular constituents, may lead to various disease conditions [4,5]. Much research into free radicals has confirmed that foods or plants rich in antioxidants play an essential role in the prevention of free radical related diseases [6, 7]. A wide range of antioxidants of synthetic origin such as butylated hydroxytoluene (BHT) has been proposed for use in the treatment of various free radicals related diseases [8,9], but it has been proven that these compounds also show toxic effects like liver damage and mutagenesis [10]. Hence, nowadays the search for natural antioxidants source is gaining much importance. The high antioxidant potential observed in many tropical plants is obviously part of their natural defense mechanism against noxious events causing oxidant damage, e.g. microbial infections. Although, *Filicium decipiens* is reported for different folk medical use, present work was carried out to explore the *in vitro* antioxidant potential of this plant.

Materials and Methods

Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ferric chloride ($FeCl_3$), TPTZ, xanthine, xanthine oxidase, Folin-Ciocalteu reagent, aluminium chloride ($AlCl_3$), allopurinol, gallic acid, ascorbic acid, butylated hydroxytoluene (BHT) and catechin standards were obtained from Sigma-Aldrich. HPLC grade methanol, dichloromethane, ethyl acetate, hydrogen peroxide, 95 % ethanol, hydrochloric acid (HCl), potassium ferricyaniade [$K_3Fe(CN)_6$] and trichloroacetic acid (TCA) were obtained from Merck. Ferric sulphate [$Fe(SO_4)_2$], sodium nitrate ($NaNO_2$), and sodium hydroxide (NaOH), were purchased from Fluka.

Plant Materials

The *Filicium decipiens* plant was collected from Nallamalai hills near Tirupati, Andhra Pradesh, India. The plant has been authentified by botanist Madhava Chetty S.V.University, Tirupati. The stems and barks were washed and cut into small pieces and dried for a week at 40 °C to remove the moisture content. The stems and barks were powdered.

Sample Preparation

The powdered stem and bark (250 g) were extracted with ethanol (1000 ml) by the maceration method. The extract was filtered through filter paper (Whatman No.1), collected and concentrated in a rotary evaporator (RII0 Buchi,) in vacuum at 50°C. The concentrated extract was dried to a consistent weight in an oven at 50 °C for a week.

Xanthine Oxidase Inhibition Assay

Xanthine oxidase activity was determined by measuring the formation of uric acid from xanthine. FD stems and bark extracts of 1 mg/ml was prepared in 50 mM phosphate buffer solution (pH 7.0). Two milliliters of the sample was mixed with solution containing xanthine oxidase (2 ml, 0.4 U/ ml) and xanthine (100 µM). After incubating at room temperature (24^oC) for 3 minutes, uric acid production was determined by measuring the absorbance at 295 nm. The blank used was buffer and the control was a solution containing xanthine and xanthine oxidase. The inhibition percentage of xanthine oxidase activity was calculated according to the formula = [(Abs. of control –Abs. of sample) / Abs. of control] x 100% [10].

Determination of Scavenging Activity against Hydrogen Peroxide

The FD stems and bark extracts radical scavenging activity against hydrogen peroxide was determined using the method of Ruch *et al.* [13]. Samples with different concentration were added to 0.1 M phosphate buffer solution (pH 7.4, 3.4 ml), respectively, and mixed with 43 mM

hydrogen peroxide solution (0.6 ml). After 10 min, the reaction mixture absorbance was determined at 230 nm. The reaction mixture without sample was used as the blank. Ascorbic acid was used as a reference compound [9]. The percentage inhibition activity was calculated as: [(Abs. of control – Abs. of sample)/Abs. of control] × 100%.

Ferric-Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was carried out according to the procedure of Benzene and Strain [10,11]. The FRAP reagent was prepared by mixing acetate buffer (25 ml, 300 mmol/l, pH 3.6), 10 mmol/l TPTZ solution (2.5 ml) in 40 mmol/l HCl and 20 mmol/l $FeCl_3$ solution (2.5 ml) in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh and warmed to 37^oC in a water bath prior to use. One hundred and fifty microliters of the sample was added to the FRAP reagent (4.5 ml). The absorbance of the reaction mixture was then recorded at 593 nm after 4 min; the assay was carried out in triplicates. The standard curve was constructed using $FeSO_4$ solution (0.5-10 mg/ml). The results were expressed as μmol Fe (II)/g dry weight of plant material. L-ascorbic acid was also used as a comparative model for this assay.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The free radical scavenging activity was measured by using DPPH assay. The quantitative estimation of radical scavenging activity was determined according to the methods described by Blois (1958) [19]. Five milliliters of 0.04% DPPH radical solution was added to FD stem and bark extracts solutions ranging from 0.031 to 2 mg/ml respectively. The mixtures were vortex-mixed and kept under darkroom conditions for 30 min. The optical density (OD) was measured at 517 nm. Methanol was used as baseline control. Ascorbic acid, BHT and vitamin E were used as positive controls. The DPPH radical concentration was calculated using the following equation: Scavenging effect (%):

[(Abs. of control –Abs. of sample) / Abs. of control] x 100%]

The IC50 (concentration providing 50% inhibition) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs. the corresponding scavenging effect.

Determination of Phenolic and Flavonoids Content

The total phenolic content in the methanol extracts was measured using Folin-Ciocalteu reagent method [20]. The FD stems and barks extracts ethanolic solution (0.4 ml, 1mg/ml) was transferred into a test tube. To this solution, distilled water (1.0 ml) and Folin-Ciocalteu reagent (1.0 ml) were added, and the tubes shaken thoroughly. After 1 min, sodium carbonate solution (Na_2CO_3, 1.6 ml, 7.5%) was added and the mixture was allowed to stand for 30 min with intermittent shaking. A linear dose response regression curve was generated using absorbance reading of gallic acid at the wavelength of 765 nm using UV / Vis - Double beam spectrophotometer (Elico SL 196). The total phenolic compounds concentration in the extract was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of extract. The content of phenolic compounds in the plant extracts was calculated using this formula: C = A/B; where C is expressed as mg GAE/g dry weight of the extract; A is the equivalent concentration of gallic acid established from calibration curve (mg); and B is the dry weight of the extract (g)

The total flavonoid content of the extract was determined according to colorimetric method as described in [21]. In brief, the sample solution (0.5 ml) was mixed with distilled water (2 ml) and subsequently with 5% $NaNO_2$ solution (0.15 ml). After 6 min of incubation, 10% $AlCl_3$ solution

(0.15 ml) was added and then allowed to stand for 6 min, followed by additon of 4% NaOH solution (2 ml) to the mixture. Consequently, water was added to the sample to bring the final volume to 5 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. The mixture's absorbance was determined at 510 nm. The total flavonoid content was expressed in mg of catechin per gram of extract.

Statistical Analysis

The data was presented as mean ± standard deviation (SD) for the three determinations.

Results and Discussion

Inhibition of Xanthine Oxidase

Xanthine oxidase is a flavoprotein that catalyses the oxidation of hypoxanthine to xanthine and generates superoxide and uric acid [10]. It has been shown that xanthine oxidase inhibitors may be useful for the treatment of hepatic disease and gout, which is caused by the generation of uric acid and superoxide anion radical [11]. *Filicium decipiens* (FD) stem and bark ethanolic extracts exhibits anti-superoxide activity. Ethanolic extract was used in this study due to its polarity and because it was anticipated that it would more effectively extract the polyphenols which possessed antioxidant activity from plant samples. At concentrations of 1 mg/ml the FD stem and bark extracts inhibited superoxide formation with a value of 52.18± 0.008 and 59.97± 0.006% respectively. The FD stems and bark extracts showed a lesser antioxidant activity compare to allopurinol (87.51 ± 0.001%). The preventive antioxidant activities of FD stem and bark extracts largely comes from their ability to inhibit various oxidative enzymes. However, their role as antioxidants in the human body when orally ingested is unknown, but there are several possibilities. The relevance of the *in vitro* experiments in simplified systems to *in vivo* protection from oxidative damage should be carefully considered. The results obtained from this study indicate that the further *in vivo* evaluation is needed.

Table 1. The xanthine oxidase inhibitory activity, hydrogen peroxide scavenging and ferric-reducing antioxidant power (FRAP) activity assays for *Felicium decipiens* stem and bark *ex*tracts

Assays	% Inhibition
Inhibition of xanthine oxidase	
FD stem extract (1mg / ml)	52.18 ± 0.025
FD bark extract (1mg / ml)	59.97 ± 0.053
Allopurinol (1mg / ml)	87.51 ± 0.001
Hydrogen peroxide scavenging activity	
FD stem extract (1mg / ml)	43.50 ± 0.029
FD bark extract (1mg / ml)	47.64 ± 0.072
Ascorbic acid (1mg / ml)	51.13 ± 0.010
Ferric-reducing antioxidant power	
FD stem extract	0.675 ± 0.058 mmol/Fe (II)/g
FD bark extract	0.728 ± 0.031 mmol Fe (II)/g
Ascorbic acid	0.405 ± 0.048 mmol Fe (II)/g

The results are expressed as mean ± SD

Determination of Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging activity of *Filicium decipiens* (FD) stem and bark ethanolic extracts is given in Table 1. The scavenging ability of both these extracts is comparable to that of

ascorbic acid (stem and bark extract: 43.5 ± 0.029% and 47.64 ± 0.072% and ascorbic acid: 51.1 ± 0.01%). Addition of H_2O_2 to cells in cultures can lead to transition metal ion dependent OH mediated oxidative DNA damage. Levels of H_2O_2 at or below about 20–50 mg seem to have limited cytotoxicity to many cell types [12, 13]. Since phenolic compounds present in the plant extract are good electron donors, they may accelerate the conversion of H_2O_2 to H_2O [13], hence, FD stem and bark extracts might also help to accelerate the conversion of H_2O_2 to H_2O.

Ferric-Reducing Antioxidant Power Assay (FRAP)

The FRAP assay is versatile and can be readily applied to ethanolic extracts of different plants. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron. The standard curve was generated in the range of 0.5 to 10 mg/ml of ferrous sulphate and the results were expressed as mmol ferrous ion equivalent per gram of sample dry weight for stem and bark extracts ($y = 0.0029x + 0.0769$, $y = 0.0087x + 0.0884$, $r2 = 0.973$, 0.988) respectively. Table 1 shows the results for the ferric-reducing antioxidant power assay. *Filicium decipiens* (FD) bark ethanolic extracts (0.728 ± 0.031 mmol Fe (II)/g) shows approximately two-fold higher ferric reducing capacity compared to the standard reference ascorbic acid (0.405 ± 0.048 mmol Fe(II)/g). Our results also show that the *Filicium decipiens* (FD) *stem and bark* ethanolic extract has better ferric reducing power than date palm (soft dates, sahroon: 26.93 ± 1.96 and dry dates, Kharak: 387.34 ± 1.94 μmol Fe (II)/g) from Iran [14].

2, 2-Diphenyl-2-picrylhydrazyl (DPPH) Assay

The scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples, including plant extracts [15]. The measured DPPH radical scavenging activity is shown in Table 2. The *Filicium decipiens* (FD) stem and bark ethanolic extracts scavenging antioxidant activity was 24.89 ± 0.04% and 37.25 ± 0.27% at 1 mg/ml. The scavenging effect on the DPPH radical decreased in the order: ascorbic acid > vitamin E > BHT > FD stems and bark extracts. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability [16]. Although the DPPH radical scavenging abilities of the extracts were significantly lower than those of ascorbic acid, vitamin E and BHT, it was evident that the extracts did show some proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. The quality of the antioxidants in the extracts was determined by the IC_{50} values shown in Table 2. A low IC_{50} value indicates strong antioxidant activity in a sample. The IC_{50} values of FD stem and bark extract was 2.64 mg/ml. Although the IC_{50} values of FD stem and bark extracts were greater than the reference antioxidant, it was comparable to other tropical fruits which are considered to have a good antioxidant powers (guava IC50: 2.1 ± 0.63; star fruit IC50: 3.8 ± 2.1, papaya IC50: 3.5 ± 0.9 mg/ml and pumpkin stem and bark IC50: 5.13 mg/ml) [17,18].

Table 2. DPPH antioxidant activity of *Felicium decipiens* stem and bark extract.

Plant Extract (1 mg/ml)	**% (DPPH) radical scavenging Activity**
FD stem extract	24.89 ± 0.04%
FD bark extract	37.25 ± 0.27%
Butylated hydroxytoluene	82.54 ± 0.19 %
Ascorbic acid	86.84 ± 0.24 %
Vitamin E	82.41 ± 0.26 %
IC50 of FD stem extract	2.3 mg/ml

The results are expressed as mean ± SD

Determination of Phenolic and Flavonoids Contents

The total phenolic and flavonoids contents are represented in Table 3. Total phenol compounds, as determined by the Folin Ciocalteu method, is reported as gallic acid equivalents by reference to standard curve (y = 9.892x + 0.092, and r^2 = 0.997,). The total phenolic content of FD stem and bark extract was 23 ± 0.46 and 28 ± 0.26 mg GAE/g respectively. The total flavonoid content of stem and bark extract was 3.42 ± 0.19 and 2.52 ± 0.43 mg of catechin equivalent per gram of sample, respectively, by reference to standard curve (y = 0.0038x + 0.0272 and r^2 = 0.9985). The FD stems and bark extract had good total phenolic content that may be responsible for the antioxidative activities of this extract. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (20)

Table 3. Total phenolic and flavonoid content of Filicium decipiens (FD) stem and bark ethanolic extract

Assays	FD stem extract (1 mg/ml)	FD bark extract
Total phenolic content	$23 \pm 0.46_a$	$28 \pm 0.26_a$
Total flavonoid content	$3.42 \pm 0.19_b$	$2.52 \pm 0.43_b$

$_a$ Gallic acid equivalents mg/g; $_b$ Catechin equivalents mg/g

Conclusion

FD stems and barks ethanolic extracts antioxidant capacity is comparable to that of other tropical plants. Various assays were used to prove the antioxidant activity of the extract in this study. This is important since some plant extract with good antioxidant activity don't necessarily show good antioxidant activity with any one particular one assay. Hence, more assays are needed to conclusively prove antioxidant activity. The presence on antioxidant bioactive compound(s) like phenolic compounds, flavonoids in the FD stem and bark extract could possibly contribute to the antioxidant activity.

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