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Phytochemical analysis and free-radical scavenging activity of *Flemingia strobilifera* (Linn) R. Br.

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ABSTRACT

The present investigation is to evaluate the antioxidant activity of various extracts of root and leaf of *F. strobilifera* using different *in vitro* methods and its phytochemical analysis. The antioxidant activity was studied by DPPH radical scavenging method, nitric oxide radical inhibition assay and scavenging of hydroxyl radical by *p*-NDA method. Methanolic extract of root and leaf of *F. strobilifera* showed a very good DPPH radical scavenging activity with low IC_{50} values of 11.4 µg/ml and 38.0 µg/ml respectively. Butanolic extract of *F. strobilifera* root showed good nitric oxide radical inhibition activity with IC_{50} of 150.0 µg/ml in a dose-dependent manner. The methanolic extract of root showed hydroxyl radical scavenging by *p*-NDA method with IC_{50} value of 378.33 µg/ml. Subsequent quantification showed the presence of 13.75 and 8.84 % w/w phenolics (calculated as gallic acid), 2.14 and 3.26 % w/w of flavonol in methanol extract of root and leaf of *F. strobilifera* respectively. The high amount of flavonols and phenolics prompted us to evaluate its antioxidant activity. This study revealed that methanolic extract of *F. strobilifera* comprise effective potential source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

Keywords *Flemingia strobilifera*, DPPH, Nitric oxide radical inhibition assay, Scavenging of hydroxyl radical by p-NDA method

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INTRODUCTION

Flemingia strobilifera (Linn) R.Br. (Family Leguminosae), an important medicinal plant, commonly known as Kusrunt [1]. The plant is found in Sind, Rajputana, Bengal, South India and Andamans ranges of India [2]. The roots of this plant have been indigenously used in the treatment of epilepsy and hysteria whereas leaves are used as vermifuge [3]. Literature survey reveals the presence of various chalcones [3], flavonoid glycosides [4], aurone glycosides [5] and epoxy chromenes [6]. The plant was reported to show antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermis, Methicillin resistant Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* in our previous research studies [7].This paper reports the pharmacognostical investigation and the evaluation of free-radical scavenging properties of root and leaf of *F. strobilifera* in three *in-vitro* models. The study also includes determination of physico-chemical constants, preliminary phytochemical screenings of extracts of *F. strobilifera* root and leaf. To the best of our knowledge, there is no antioxidant activity and pharmacognostical investigation has been done on the parts of this plant.

MATERIAL AND METHODS

Collection and Identification of Plant material

Flemingia strobilifera roots and leaves were collected from forests of Shann Power House, Joginder Nagar, (Distt Mandi) Himachal Pradesh, India in October 2006. The identity of the plant material was verified by Dr. H.B Singh, Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi. A voucher specimen (NISCAIR/RHMD/Consult/06/757/74) is deposited in the herbarium of National Institute of Science Communication and Information Resources, New Delhi, India. The plant material was dried in a hot air oven (<50ºC), stored in airtight glass bottles and powdered to 40 mesh.

Physico-chemical analysis

Physico-chemical analysis i.e. percentage of ash values, loss on drying and extractive values were performed according to the official methods prescribed [8] and the WHO guidelines on quality control methods for medicinal plant materials [9]. Fluorescence analysis was carried out according to the method of Kokoski et al [10].

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out by using standard procedures described by Harborne [11]. Five-hundred milligrams (500 mg) of the dried methanolic extract of *F. strobilifera* root and leaf was reconstituted in 10 ml of methanol and used for preliminary phytochemical testing for the presence of different chemical groups of compounds

Chemicals

1, 1-Diphenyl-2-picryl hydrazyl (DPPH) were obtained from Sigma Aldrich Co., St. Louis, USA. Folin Ciocalteu's reagents and rutin were purchased from SD Fine chemicals, India. Naphthyl ethylene diamine dihydrochloride (NEDD) was obtained from Roch-Light Ltd., Suffolk, UK. *p*-nitroso dimethyl aniline (*p*-NDA) were obtained from Across Organics, New Jersey, USA. All chemicals used were of analytical grade.

Preparation of Extracts

The dried roots weigh about (30 gm) of *F. strobilifera* were powdered and extracted separately with DCM, BuOH and MeOH, for 24 h by maceration. The extracts were filtered, pooled and the solvent was removed under reduced pressure (yield was obtained 5, 6.4, 8.8 % w/w respectively), dried powdered leaves about (30 gm) were extracted only with MeOH (yield was obtained 10 % w/w).

Preparation of Test and Standard Solutions

The extracts and the standard antioxidants, ascorbic acid and rutin, were dissolved in distilled dimethyl sulphoxide (DMSO) separately and used for *in-vitro* antioxidant study. The stock solutions were serially diluted with DMSO to get required dilutions.

Estimation of total phenolics

The total phenol content of all the plant extracts was determined by using the standard Folin-Ciocalteu method [12]. Each extract solution (100 µl) was mixed with 2 ml of Folin-Ciocalteu reagent and 1.6 ml of sodium carbonate, shaken well and kept for 2 h. The absorbance was measured at 750 nm using Beckman (DU 640B) Spectrophotometer. Using gallic acid monohydrate as standard, Standard curve was prepared and linearity was obtained in the range of 2.5 to 25 µg/ml. The total phenol content of the extracts was obtained by using the standard curve. The total phenol content was expressed as gallic acid equivalent in % w/w of the extracts.

Estimation of total flavonols

The total flavonol content of the extracts was determined by aluminium chloride colorimetric method [13]. Each extract (0.5 ml) was mixed with 1.5 ml methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Beckman (DU 640B) Spectrophotometer. Using rutin as standard, Standard curve was prepared and linearity was obtained in the range of 1-10 µg/ml. Using the standard curve the total flavonol content was expressed as rutin equivalent in % w/w of the extracts.

DPPH radical- scavenging assay

The antioxidant activitiy [14] of the methanol extracts and standard compounds (rutin and ascorbic acid) were assessed on the basis of radical scavenging effect of the stable DPPH free radical. To 6ml of DPPH (20µg/ml) methanolic solution, 20 µl of DMSO solution of each extract was added separately, at room temperature. The mixture was shaken vigorously and kept aside for 5 min and absorbance was measured at about 517nm with Beckman (DU 640B) spectrophotometer against corresponding test blanks. All tests were run in triplicate and mean values were taken for calculation. IC_{50} value is the concentration of sample required to inhibit 50% of DPPH radical.

Nitric oxide radical inhibition assay

Nitric oxide is a free radical and scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide [15, 16]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite, which can be estimated by the use of Griess Illosvog reagent [17]. In the present investigation, Griess Illosvog reagent is modified by using naphthylethylene diamine dihydrochloride (0.1% w/v) instead of 1- naphthylamine (5%). The reaction mixture (1.5 ml) containing sodium nitroprusside (10 mM, 1 ml), phosphate buffer saline (0.25 ml) and extract solution (0.25 ml) was incubated at 25ºC for about 2 hr. After incubation, 0.5 ml of the reaction mixture containing nitrite ions was removed and added 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid), mixed well and allowed to stand for 5 min for completing diazotization, then 1 ml of naphthylethylene diamine dihydrochloride (0.1%) was added, mixed and allowed to stand for 30 min. A pink colored chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions with Beckman (DU 640B) spectrophotometer. IC_{50} value is the concentration of sample required to inhibit 50% of nitric oxide radical.

Scavenging of hydroxyl radical by *p*-NDA method

To a solution mixture containing ferric chloride (0.1 mM, 0.5 ml), EDTA (0.1 mM, 0.5 ml), ascorbic acid (0.1 mM, 0.5 ml), hydrogen peroxide (2mM, 0.5ml) and *p*-NDA (0.01 mM, 0.5 ml) in phosphate buffer (pH 7.4, 20 mM) was added various concentrations of extract or standard in distilled DMSO (0.5 ml) to produce a final volume of 3 ml. Absorbance was measured at 440 nm [18].

RESULTS AND DISCUSSION

Physico-chemical parameters i.e. ash value of a drug gives an idea of the earthy matter or the inorganic composition and other impurities present along with the drug. The ash values (Table 1) of the powdered *F. strobilifera* root and leaf revealed a high concentration of total ash. The total ash, water soluble ash and acid insoluble ash which are important parameter for detecting the presence of inorganic substances were found to 6.79, 1.77, 2.06 % w/w

respectively in root and 5.34, 2.47,0.86 % w/w respectively in leaves. Extractive values are primarily useful for the determination of exhausted or adulterated drugs. The water, ethanol, butanol, methanol and dichloromethane soluble extractives, which are indicator of total solvent soluble component, are 14.6, 10.4, 6.4, 8.8, 5.0 % w/w respectively (Table 2). Loss on drying of the powdered *F. strobilifera* root and leaf revealed the presence of 6.68 % and 8.76 % of moisture in a drug respectively. The results of fluorescence analysis of the powdered root and leaf are presented in Table 3 & 4 respectively, which helps preliminary for the presence of phenolic compounds, flavonoids, steroids and other natural compounds based on different fluorescence with different chemical reagents. These studies help in authentication of the plant since there is no work reported on their pharmacognostical investigation previously.

Table 1: Ash values and loss on drying (LOD) of the root and leaves of *F. strobilifera*

Ash values (% w/w)				Loss on drying (% w/w)
Plant Part	Total ash	Water-soluble ash	Acid-insoluble ash	LOD
Root	6.79,	1.77	2.06	6.68,
Leaves	5.34	2.47	0.86	8.76

Table 2: Extractive values of the root of *F. strobilifera*

Parameters	Values % (w/w)
Water soluble	14.6
Ethanol soluble	10.4
Butanol soluble	6.4
Methanol soluble	8.8
Dichloromethane soluble	5.0

Table 3: Results of fluorescence analysis of powdered root of *F. strobilifera*.

Drug + Reagent	UV light		Visible light
	Short (UV 254 nm)	Long (UV 366nm)	
Powder as such	Brown	Dark brown	Light brown
Powder + 1N NaOH (Aq)	Dark brown	Dark brown	Light brown
Powder + 1N NaOH (Alc)	Brown	Light brown	-
Powder + 1N HCl	Dark brown	Light brown	Light brown
Powder + NH_3	Light brown	Light purple	Light brown
Powder + 5% Iodine	Dark green	Dark green	Light yellowish brown
Powder + 5% $FeCl_3$	Dark yellowish green	Dark brown	Dark greenish brown
Powder + acetic acid	Light blue	Light orange	Light brown
Powder + 1N H_2SO_4	Dull green	Orangish brown	Dark brown
Powder + 1N HNO_3	Light yellow	-	Light yellowish brown

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Table 4: Results of fluorescence analysis of powdered leaves of *F. strobilifera*.

Drug + Reagent	UV light		Visible light
	Short (UV 254 nm)	Long (UV 366nm)	
Powder as such	Dark green	Dark green	Light green
Powder + 1N NaOH (Aq)	Light yellow	Light fluorescent green	Light yellowish green
Powder + 1N NaOH (Alc)	Light yellowish green	Light pink	Very Light green
Powder + 1N HCl	Light yellowish green	Light yellow	Fluorescent green
Powder + NH_3	Light yellowish green	Light blue	Light yellowish green
Powder + 5% Iodine	Dark yellowish brown	Dark yellowish brown	Dark yellowish brown
Powder + 5% $FeCl_3$	Dark brown	Dark brown	Greenish brown
Powder + acetic acid	Dark green	Light orange	Light green
Powder + 1N H_2SO_4	Dark brown	Orangish brown	Dark green
Powder + 1N HNO_3	Dark green	Dark green	Light yellowish brown

Table 5.1: *In vitro* antioxidant activity of different extracts of *F. strobilifera* roots and leaves

IC_{50} values ± SEM (µg/ml)*				
Plant extract	Yield of extract (%)	DPPH	Nitric oxide	*p*-NDA
$MeOH^R$	8.8 ± 0.5	11.4 ± 1.0	> 700	378.33 ± 3.4
$MeOH^L$	10.0 ± 0.5	38.0 ± 1.2	> 700	>1000
DCM^R	5.0 ± 0.6	19.0 ± 1.3	310.0 ± 3.1	>1000
$BuOH^R$	6.4 ± 0.8	125.0 ± 1.0	150.0 ± 2.8	>1000
Standards				
Ascorbic acid	-	2.69 ± 0.02	-	>1000
Rutin	-	5.83 ± 1.2	68.44 ± 1.4	205.83 ± 0.4

*SEM: ± standard error mean, average of three determinations, R-Root, L-Leaves.

Table 5.2. Total phenolic and total flavonol content of different extracts of *F. strobilifera* root and leaves

Plant Extract	Total Phenol conte ± SEM (% w/w)	Total Flavonol cont ± SEM (% w/w)
$MeOH^R$	13.75 ± 1.5	2.14 ± 0.07
$MeOH^L$	8.84 ± 1.2	3.26 ± 0.3
DCM^R	1.57 ± 0.9	0.64 ± 0.13
$BuOH^R$	8.07 ± 1.1	0.74 ± 0.4

*SEM: ± standard error mean, average of three determinations, R-Root, L-Leaves.

Preliminary phytochemical screening of the root and leaf of *F. strobilifera* showed the presence of phytosterols, lipids, phenolic compounds, carbohydrates, flavonoids and tannins. Subsequent quantification showed the presence of 13.75 and 8.84 % w/w phenolics (calculated as gallic acid), 2.14 and 3.26 % w/w of flavonol in methanol extract of root and leaf of *F. strobilifera* respectively (Table 5).

Plant phenolics including flavonoids are known to possess strong antioxidant properties [19]. The results of antioxidant activity of standards ascorbic acid, rutin and different extracts are shown in Table 5. The methanolic extract of root and leaf of *F. strobilifera* showed a concentration-dependent DPPH radical scavenging activity by bleaching it with IC_{50} values of 11.4 and 38µg/ml. DCM and BuOH extract of *F. strobilifera* root showed the IC_{50} values of 19 and 125 µg/ml by DPPH method. The Butanolic and DCM extract of *F. strobilifera* root showed good nitric oxide scavenging activity with an IC_{50} value of 150 and 310 µg/ml. Scavenging of hydroxyl radical by *p*-NDA method of methanolic extract of root showed IC_{50} value of 378.33 µg/ml. This free- radical scavenging activity can be attributed to the high amounts of flavonoids and phenolics present in different extracts of the plant. It also resulted in showing the lower IC_{50} values in *in-vitro* antioxidant studies. The lower IC_{50} values indicated the high antioxidant potency of the extracts. On the basis of the deep phytochemical findings it was found that the different extracts of *F. strobilifera* root and leaf were rich in polyphenolic compounds like phenolic compounds, flavonoids, steroids, flavonoids glycosides, tannins which may act as free radical scavengers. In view of this the present author has performed the free-radical scavenging activity of different extracts of *F. strobilifera* root and leaf. There is no such previous report of antioxidant activity of root and leaf in the literature. This work may serve as a model for detailed free radical scavenging property of this plant extract.

CONCLUSION

In conclusion, the extracts from the root and leaf of *F. strobilifera* possessed significant antioxidant activity. As there is no pharmacognostic work on record of this traditionally much valued drug, the present work was taken up with a view to lay down standards, which could be useful to detect the authenticity of this medicinally useful plant

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