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IN VITRO EVALUATION OF PHYTOCHEMICAL PROFILE AND ANTIOXIDANT EFFECT OF CHLOROFORM AND PETROLEUM ETHER EXTRACTS OF *DENDROPHTHOE FALCATA* (LINN) LEAVES

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Abstract

Dendrophthoe falcata (Linn.) is a hemi parasitic plant and has been used as a traditional medicine against various diseases. The aim of our study was to evaluate phytochemical constituents and antioxidant potential of chloroform (CEDL) and petroleum ether (PEDL) extracts of the *D. falcata* leaves. Phytochemical screening was performed qualitatively by conventional methods. Total phenol (TPC) and total flavonoid content (TFC) were measured by folin-ciacaltue as well as aluminium trichloride methods, respectively. Antioxidant potential was evaluated by DPPH radical scavenging assay, ferric reducing power (FRP) assay and phosphomolibodenum methods (total antioxidant content). Both the extracts showed presence of major phytoconstituents. Also, phenolics and flavonoids were in remarkable level in both the extracts but in different extents. In DPPH radical scavenging assay, reference compound ascorbic acid (AA), and the extracts CEDL as well as PEDL have shown potent activity where 50% inhibition concentration (IC_{50}) value were 12.58±2.34, 36.38±5.33 and 33.42±3.15 µg/ml ; and in FRP assay, 50% effective concentration (EC_{50}) value were 25.11±2.10, 39.81±3.07and 28.18±2.11 µg/ml, respectively. Between the two extracts, PEDFL showed more total antioxidant content. Overall, the extracts are rich in bioactive phytochemicals, and have potent antioxidant effects.

Keywords: Dendrophthoe falcate; Antioxidant; Phytochemical Screening; Phenolics; Flavonoids

Introduction

Oxidative stress is the key factor for genesis and exacerbation of several disease such as asthma, diabetes, alzheimer, parkinson, cancer, atherosclerosis etc. which are major concern to the modern researchers [1,2]. It (Oxidative stress) is generated by reactive oxygen species (ROS) or reactive nitrogen species (RNS): superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$). The ROS are continuously produced inside the body as a metabolic byproduct mainly by mitochondria [3, 4]. Normal healthy people have sufficient endogenous antioxidant system to scavenge or to neutralize these free radicals [5, 6]. However, in case of disease condition an imbalance of normal antioxidant level has been discovered [6].

During the imbalance, level of free radicals become higher than its usual level that causes tissue injury by ROS and damages DNA, protein as well as important enzyme [5]. These events could consequently lead to generate several chronic diseases [6]. So, in that case it is necessary to eat antioxidant rich foods, or to supply synthetic antioxidant for ailment from the diseases.

But, the synthetic antioxidants such as ascorbic acid, butylated hydroxy-tolune (BHT) have not been succeed to use properly for toxicity or prooxidant activity [3]. For this reason, natural antioxidants are the main compound that may alleviate or inhibit disease exhibiting least toxicity [7]. Studies on herbal plants, vegetables, and fruits have indicated presence of antioxidants such as phenolics, flavonoids, tannins, and proanthocyanidins [8]. So, ingestion of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders [9]. *Dendrophthoe falcata* (Linn.) belongs to the family of Loranthaceace.

It is a perennial climbing woody hemi parasitic [10] and evergreen shrub that is frequently observed on different hosts plants. It comprises of 20 species where about 7 species are found in Indian subcontinent such as India, Sirilanka, China, Australia, Bangladesh, Malayasia, Myanmar, Thailand and Indo-china [11, 10]. The entire plant is used extensively in traditional system of medicine as an aphrodisiac, astringent, narcotic and diuretic. It is applied for the treatment of pulmonary tuberculosis, asthma, menstrual disorders, swellings, wounds, ulcers, strangury and psychic disorders [12]. It contains biologically active substances such as quercetin, tannins, *β*-sitosterol, *β*-amyrin, oleanolic acid [13]. Several active chemical constituents such as stigmasterol, kaempferol, quercetin–3-O-rhamnoside, rutin, and myricetin and their glycosides, (+)-catechin, leucocyanidin, gallic acid, chebulinic acid and some pentacyclic triterpenes: kaempferol-3-O-α-L-rhamnopyranoside and quercetin-3-O-α-L-rhamnopyranoside are isolated and identified from the plant [14]. The study was undertaken to evaluate phytochemical profile and antioxidant activity of chloroform and petroleum ether extract of *D. falcata* leaves which will unveil the rationality of use of the plant as traditional medicines. Therefore, the great efforts have been invested to exploit the perfect antioxidant drug protecting from different diseases.

Methods

Plant materials

As it is a parasitic plant, it was collected from *Swietenia fabrilis* tree in September, 2012 and was identified by an expert of the Bangladesh National Herbarium, Dhaka. A voucher specimen of the plant had also been retained with the accession number 39432. Leaves of the plant were separated from branchs and then cleaned, dried for one week and pulverized into a coarse powder using a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark, and dry place until further analysis.

Extract preparation

Approximately 400 g of powdered material was placed in a clean, flat-bottomed glass container and soaked in chloroform and similarly 400g of the powder was soaked in petroleum ether. Both the containers with its contents was sealed and kept for 7 days. Then extraction was carried out using ultrasonic sound bath accompanied by sonication (40 minutes). The entire mixture then underwent a coarse filtration by a piece of clean, white cotton material. The extract was then filtered through Whatman filter paper (Bibby RE200, Sterilin Ltd., UK). The filtrate was then concentrated by using a rotary evaporator and finally dried by electric oven at 45^0C temperature. The gummy extracts (chloroform 8.5g and petroleum ether 12.65g) were stored in an air tight container.

Drugs and chemicals

DPPH, quercetin, gallic acid, ascorbic acid were purchased from Sigma Aldrich, USA. Other chemicals and solvents were of highest analytical grade commercially available.

Phytochemical screening

Phytochemical screening was done qualitatively on

the extracts (CEDS and PEDS) by using previously described methods.

Test for alkaloids

0.4 g of extracts was stirred with 8 ml of 1% HCl in two separate test tubes. The mixtures were warmed and filtered. 2 ml of filtrate were treated separately with (a) few drops of potassium mercuriciodide (Mayer's reagent) and (b) potassium bismuth (Dragendroff's reagent). Turbidity or precipitation with either of these reagents was taken as evidence for existence of alkaloids [15].

Test for tannins

50 mg of the extracts was boiled in 20 ml of distilled water then filtered. A few drops of 0.1% $FeCl_3$ was added in filtrate and observed for color change. Appearance of brownish green or a blue-black coloration indicated the presence of tannins [16].

Test for saponins

The ability of saponins to produce emulsion with oil was used for the screening test. 20 mg of the extracts was boiled in 20 ml of distilled water in a water bath for five min and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for froth formation. 3 drops of olive oil were mixed with froth, shaken vigorously and observed for emulsion development [15].

Test for flavonoids

To perform the test 50mg of the extracts was suspended in 100 ml of distilled water to get the filtrate. 5 ml of dilute ammonia solution was added to 10 ml of filtrate followed by few drops of concentrated H_2SO_4. Presence of flavonoids was confirmed by yellow coloration [16].

Test for steroids

One ml of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids [15].

Test for reducing sugars

Both the extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates. Filtrates were treated with Benedict's reagent and heated on a water bath. Formation of an orange red precipitate indicates the presence of reducing sugars [17].

Test for terpenoids

Presence of terpenoids in the extracts was carried out by taking 5 ml (1 mg/ml) of CEDS and PEDS in test tubes. Then 2 ml of chloroform, followed by 3 ml of concentrated H_2SO_4 were added in the test tubes. A reddish brown coloration of the interface confirmed the presence of terpenoids [15].

Test for anthraquinones

200 mg of extract was boiled with 6 ml of 1% HCl and filtered. The filtrate was shaken with 5 ml of benzene, filtered and 2 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of a pink, violet or red colour in the ammoniacal phase indicated the presence of free hydroxyl anthraquinones [18].

Determination of phytoconstituents

Determination of total phenol content (TPC)

TPC of the extracts was measured according to the Folin-Ciocalteu method [19]. Briefly, the extract solution or standard gallic acid (0.5 ml) at different concentrations (ranging from 12.5 to 200µg/ml) was mixed with 2.58 ml of Folin-Ciocalteu reagent. After 30 min, 0.3 ml of saturated sodium carbonate solution was added to the mixture. The reaction mixtures were incubated at room temperature (25°C) for 20 min. The absorbance was measured at 760 nm with a spectrophotometer. TPC of the extracts was calculated from the regression equation of the standard gallic acid and expressed as gallic acid equivalents (mg of GAE/g of extract).

Determination of total flavonoid content (TFC)

1 ml of plant extract in methanol (200 µg/ml) was mixed with 1 ml aluminium trichloride in ethanol (20 mg/ ml and a drop of acetic acid), and then diluted with ethanol to 25 ml. The absorption at 415nm was read after 40 min. Quercetin (12.5 to 200 µg/ml), standard compound, was experimented with the same procedure. A blank samples were prepared using all the reagents except extract solution. TFC was determined from the standard calibration curve (r^2= 0.932) of quercetin and expressed as mg/g of quercetin equivalent [20].

Antioxidant ability assays

DPPH• radical scavenging activity

The DPPH free radical scavenging activity was measured by a established method described by Braca et [22]. Briefly, 0.004% w/v of DPPH radical solution was prepared in methanol and then 900 µl of this solution was mixed with 100 µl of extract or standard ascorbic acid solution (12.5 to 200 µg/ml)

and kept in a dark place for thirty minutes. Then absorbance was measured at 517 nm. Scavenging capacity of DPPH radicals (% Inhibition) was measured by the following formula and finally calculated 50% inhibition concentration (IC_{50}) using Ms Excell software.

$$\text{Inhibition (\%)} = (A_0\text{-}A_s)/A_0 \times 100$$

Where A_0=Absorbance of control group,
A_s= Absorbance of sample

Ferric-reducing power assay

The Fe^{3+}reducing power was determined by the method of Oyaizu [23] with slight modifications. Different concentrations of the extracts and standard ascorbic acid (12.5, 25, 50, 100, 200 µg/ml) were prepared. 1ml of both the extracts and standard ascorbic acid of all concentrations were taken in separate test tubes and were mixed with 2.5 ml of phosphate buffer solution (0.2 M, pH 6.6). Then 2.5 ml of potassium ferricyanide (1%) was added in each test tube, and incubated at 50°C for 30 min. After that, 2.5 ml of trichloroacetic acid (10%) was added to the mixture which was then centrifuged at 4000 rpm for 10 min. Finally, 2.5 mL of supernatant was mixed with 2.5 ml of distilled water and 0.1 ml of FeCl3 (0.1%) solution followed by incubation at 35^{0}C for 10 minutes. The absorbance was measured at 700 nm and the reducing powers of the extracts were compared with the standared ascorbic acid. From the calibration curve, median effective concentration (EC_{50}) value was calculated.The EC_{50} value (µg/ml) is the effective concentration giving an absorbance of 0.5 for reducing power and was obtained from the calibration curve.

Determination of total antioxidan content (TAC)

TAC of the extracts (CEDL and PEDL) was evaluated by phosphomolybdenum method described by Prieto et al. [21]. Briefly, 0.3 ml extracts (200µg/ml) was combined with 3ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min in water bath. Then the absorbance of the solution was measured at 695nm using a spectrophotometer against blank (methanol) after cooling to room temperature. Ascorbic acid have been used as standard antioxidant (r^2= 0.923) and total antioxidant capacities of the extracts were expressed as mg/g equivalents of ascorbic acid.

Statistical analysis

All the data are presentaded as mean ± SEM (n = 3). Statistical significance (p) calculated by ANOVA done in SPSS, Version 15.0, Folled by Dunnett ´s Test. $P^*<0.01$ were considered to be statistically significant. IC_{50} and EC_{50} values were calculated by using MS-excell program.

Results

Phytochemical screening

Various qualitative phytochemical tests were performed on CEDL and PEDL. The Phytochemical screening demonstrated the presence of alkaloids, tannins, saponins, flavonoids, steroids, reducing sugars and terpenoids (Table 1).

Total phenol and total flavonoid contents

The total phenol and total flavonoid contents of CEDL and PEDL were expressed as gallic acid and quercetin equivalents (mg/g), respectively (Table 2). TPC of CEDL and PEDL was 155.20 ± 6.38 and 182.69 ±7.12 mg/g Eq of gallic acid. TFC of CEDL and PEDL was 109.96 ± 5.90 and 132.63 ± 8.20 mg/g Eq of quercetin, respectively. TPC and TFC of the extracts were varied significantly ($P^*<0.01$) with each other.

In vitro antioxidant activity

DPPH free radical scavenging activity

Both the extracts have shown DPPH radical scavenging activity at concentration dependant manner. IC_{50} (50% inhibition concentration) value of the standard ascorbic acid was 12.58±2.34µg/ml whereas CEDS and PEDS showed 36.38±5.33 and 33.42±3.15µg/ml, respectively (Table 3).

Reducing power assay

Both the extract showed potent reducing power. It was found that the reducing capacity of each sample increased with the increase of concentration. Between the two extracts, the PEDS showed more reducing activity. EC_{50} value of standard ascorbic acid, CEDS and PEDS were 25.11±2.10, 39.81±3.07 and 28.18±2.11 µg/ml, respectively (Table 3).

Total antioxidant content

Total antioxidant content of the extracts have varied significantly (P<0.01) with each other. Between the two extracts, PEDS have shown more TAC. At 200 µg/ml concentration PEDS had 313.6 mg/g equivalent of ascorbic acid whereas CEDS had 220.58 mg/g equivalent of ascorbic acid (Figure 1).

Discussion

In recent years, there has been an increasing

interest in finding natural antioxidants from medicinal plants. Study have shown that ingestion of natural antioxidants enhances the immune defense and reduces risks of cancer, cardiovascular disease, diabetes and other diseases associated with ageing [3].The coordinate action of antioxidant system is very critical for the detoxification of free radicals. SOD reduces the concentration of highly reactive superoxide radical by converting it to H_2O_2 whereas CAT and GSH-Px decomposes H_2O_2 and protect the tissues from highly reactive hydroxyl radicals [24]. Phytochemical screening of CEDL and PEDL revealed presence of alkaloids, tannins, resins, saponins, flavonoids, terpenoids and steroids. Many flavonoids and terpenoids are potent antioxidants, and have anti-inflammatory, anti-bacterial, anti-viral and anti-cancer effects. Flavonoids and flavonols have an essential role in drugs particularly those involved in the reduction of cholesterol and fat, and in the reduction of the risk of coronary heart disease [25]. Phenolics have anti-oxidative, anti-diabetic, anti-carcinogenic, anti-microbial, anti-allergic, anti-inflammtory, anti-mutagenic activities and have prominent effect on neurodegenarative disorders [26]. Steroids are known to be important for their cardiotonic activities and possess insecticidal and anti-microbial properties while tannins are known to possess general antimicrobial and antioxidant activities. Saponins are used in treatment of hypercholesterolemia, hyperglycemia, and are used as antioxidants, anticancers, anti-fungal, anti-bacterial, anti-inflammatory and in weight loss [25].

Most abadon sources of polyphenols are food and food supplements of plant origin. The action of some flavonoids are based on their ability to chelate transition metal ions, thereby prevent the formation of radicals and scavenge lipid-alkoxyl and lipid-peroxyl radicals and regenerate α-tocopherol by reduction of α-tocopheryl radicals [26]. Significant amount of total phenols (155.20 ± 6.38 and 182.69 ± 7.12 mg/g Eq of gallic acid) and total flavonoids (109.96 ± 5.90and 132.63 ± 8.20 mg/g Eq of quercetin) were also presents in the CEDL and PEDL respectively (Table 2). Antioxidant capacity of an agent is based on both the free radical scavenging and the oxidation–reduction mechanisms which may be determined by several methods whereas phosphomolybdenum, DPPH radical and Fe^{3+} reducing power methods are the most commonly used and convenient to work [7]. In phosphomolybdenum method, the reduction level of Mo (VI) to Mo (V) by tested sample is determined by the subsequent formation of green phosphate Mo (V) compounds with a maximum absorption at 765 nm. The present study demonstrated that PEDL exhibited the highest antioxidant capacity for phosphomolybdate reduction. Recent studies have shown that many flavonoid and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants [5]. Significant amount of total antioxidants (313.60 ± 11.23 and 220.58± 10.55 mg/g Eq of ascorbic acid, respectively) was observed in both the extracts. The electron donation ability of natural products can be measured by 2, 20-diphenyl-1- picrylhydrazyl radical (DPPH) purple-coloured solution bleaching. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test (5). Median inhibitory concentration (IC_{50}) of CEDL and PEDL were 36.38±5.33 and 33.42±3.15 µg/ml, respectively. In reducing power assay, the yellow colour of the test solution changes to green depending on the reducing power of the test specimen. The presence of the reductants in the solution causes the reduction of the Fe^{3+} ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by absorbance measurement at 700 nm. Previous reports suggested that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain. Increasing absorbance at 700 nm indicates an increase in reducing ability. The antioxidants present in the extracts of the *D. falcata* leaves caused their reduction of Fe^{3+}ferricyanide complex to the ferrous form, and thus proved their reducing power [5]. Reducing power of the extracts increased in a concentration-dependent manner. Therefore, reducing power evaluation might be taken as an important parameter for the assessment of antioxidant activity [26].

The observed antioxidant property of the extracts could be attributed to polyphenol compounds such as ellagic tannins, ellagic acid and gallic acid (7). Antioxidant activities of medicinal plants have been attributed to their phenol content. Previous study showed positive correlation between the phenolic content with FRP and DPPH radical scavenging activities [6]. β-Sitosterol is a potent antioxidant which have shown to protect endogenous antioxidants from depletion of these by toxic substances [27]. Besides, D. falacata leaves also contain β-sitostirol, stigmasterol, kaempferol,

quercetin, rutin, (+) – catechin which may act as antioxidant of the extracts.

This in vitro assay indicates that the plant extracts (CEDL and PEDL) are significant source of natural antioxidants which might prevent the progression of oxidative stress on various organs and subsequent damage by free radicals. However, the specific compounds of the extracts which are responsible for antioxidant activity are still unclear. Therefore, further studies are necessary to isolate and identify the antioxidant compounds that will clarify the actual mode of action as antioxidant.

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Table 1. Phytochemical profile of chloroform and petroleum ether extracts of *Dendrophthoe falcata* leaves (CEDL and PEDL).

Phytochemical constituents	Test	CEDL	PEDL
Alkaloids	Mayer's test,Dragendroff's test	++	++
Tannins	Braymer's test	++	++
Saponins	Froth test	+	++
Flavonoids	NaOH Tests	+	++
Steroids	Liebermann-Burchard's test	++	++
Reducing sugars	Benedict's test	+	++
Terpinoids	Salkowski tests	+	++
Anthroquinone	Borntrager's test	-	-

+ = moderately present, ++ = extensively present, - = absence

Table 2. Total phenol, flavonoid content and extraction yield of CEDS and PEDS.

Samples	Total Phenol (mg/g Eq of gallic acid)	Total flavanoid (mg/g Eq of quercitine)	Extraction yield (%)
CEDL	$155.20 \pm 6.38^{*}$	$109.96 \pm 5.90^{*}$	2.12
PEDL	$182.69 \pm 7.12^{*}$	$132.63 \pm 8.20^{*}$	3.16

Each value is presented as mean ± SEM (n = 3). $P^{*}<0.01$ compared between the two groups. ANOVA is done in SPSS version 15.0

Table3. DPPH radical scavenging and reducing power of CEDL and PEDL.

Samples	DDPH radical scavenging assay			Reducing power assay (RPA)		
	Concentration (µg/ml)	Percent inhibition (Mean ± SEM)	IC_{50} (µg/ml)	Concentration (µg/ml)	Absorbance	EC_{50}
AA	12.5	41.69±4.89	12.58±2.34	12.5	0.39±0.03	25.11±2.10
	25	70.56±6.56		25	0.55±0.06	
	50	82.45±8.93		50	0.90±0.07	
	100	91.56±10.02		100	1.59±0.12	
	200	93.78±7.15		200	2.28±0.19	
CEDS	12.5	23.12±2.25	36.38±5.33*	12.5	0.17±0.02	39.81±3.07*
	25	42.42±4.12		25	0.39±0.02	
	50	63.36±6.18		50	0.58±0.08	
	100	80.2±9.35		100	0.86±0.12	
	200	84.13±5.10		200	1.07±0.14	
PEDS	12.5	35.08±2.34	33.42±3.15*	12.5	0.26±0.02	28.18±2.11*
	25	58.82±4.30		25	0.48±0.05	
	50	74.31±3.42		50	0.69±0.08	
	100	86.45±8.63		100	0.98±0.10	
	200	89.23±5.25		200	1.25±0.16	

Each value is PRESENTED as a mean ± SEM (n = 3). P*<0.05 compared between the two groups. ANOVA is done in SPSS version 15.0

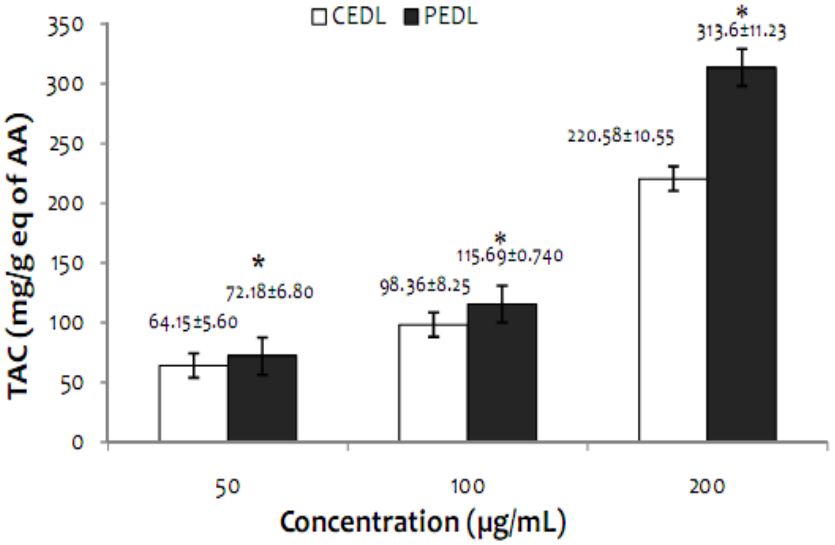

Figure 1. Total antioxidant content of CEDL and PEDL assessed by phosphomolybdenum method.