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Phytochemical and antioxidant activity of *Diospyros kaki* Leaves

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Abstract
Medicinal plants have a significant contribution of ensuring health and wellbeing of the people throughout the world. From primitive periods, plant leaves, stems, flowers, seeds and roots were used for recovery and protection of various pathologic conditions as well as in beauty formulas, foods preparations and beverages. Therefore, the motive of this work was to determine the phenolic and flavonoid content, and antioxidant activity of methanol and ethyl acetate extracts of *Diospyros kaki* (Persimmon; Japan) leaves. The leaves were collected from the Modern Horticulture Centre, Zautola, Fulbagan, Natore, Bangladesh in October, 2021. The leaves were sun dried and crushed into coarse powder and extracted by methanol and ethyl acetate solvent. The phenolic content of methanol extract of *Diospyros kaki* leaves (DKLM) possessed 5.097 ± 0.245 mg of GAEs/gm of extract which was slightly greater than ethyl acetate extract of *Diospyros kaki* leaves (DKLEA) 2.226 ± 0.109 mg of GAEs/gm of extract and in case of flavonoid content both of the extracts possessed almost similar quantity. Again, the antioxidant capacity of DKLM was more than DKLEA and also DKLM displayed more reducing power capacity as compared to the DKLEA & it exhibited activity in a concentrated dependent manner. However, the effect of DKLM in DPPH radical scavenging capacity was significantly good as butylated hydroxytoluene (BHT). So further work is necessary to determine particular bioactive compound, evaluate their chemical characteristics, and pharmacological activities of this fruit. It also necessary to analyze other plant parts of persimmon.

Keywords: Medicinal plant, *Diospyros kaki* (Persimmon), Phytochemical, DPPH, Antioxidant

Introduction
Medicinal plants, which as a whole or any of its parts like root, stem bark, leaf, fruit, peel or seeds is used for treatment and therapeutic purposes because of the presence of bioactive substances [1-2]. This plant has been using in traditional medicines from prehistoric eras. This plant synthesizes these bioactive compounds such as phenolic compounds, alkaloids, terpenes, resins, tannins, volatile oils, for defense mechanism against different plant pathogens like bacteria, fungus, yeasts etc [3]. Medicinal plants are regarded as the backbone of traditional medicine because over 3.3 billion people are currently using it mainly in developing countries. Different tribes are solely depending on medicinal plants. A considerable number of active ingredients and excipients can be discovered from these types of plants. It also has a vital role of development of human cultures across the globe [4].

Persimmon belongs to the family of Ebenaceae and genus Diospyrous. Persimmon along with other species is edible fruit. The oriental persimmon (*Diospyros kaki*) is the most widely cultivated fruit among the persimmon species. It is cultivated in Asian countries including China, Japan and Korea. This tree grows up to 4.5-18 m tall and the leaves are 7–15 cm long. The leaf is deciduous and bluish-green color. The upper surface of leaf is leathery and glossy and the underneath is brown and silky. In the late fall the fruit matures and stay until winter in the tree. The color of ripe fruit is glossy light yellow-orange to dark red-orange. The different varieties have different intensity of color [5].

The global production of persimmon was 4.27 million tonnes in 2019 and China is the highest cultivar comprising of 75% of total persimmon. South Korea, Japan, Azerbizan and Brazil also are produces appreciable amount of persimmon [6].

In Bangladesh these fruits are grown newly in many areas. Therefore, the objective of this work was to evaluate the phytochemical and antioxidant activity of methanol and ethyl acetate extracts of *Diospyros kaki* (Persimmon; Japan) leaves.

Materials and Methods

Sample collection and extraction

About 2.5 kg of raw *Diospyros kaki* leaves were collected from the Modern Horticulture Centre, Zautola, Fulbagan, Natore in October, 2021. The collected leaves were botanically recognized by S.M. Quamruzzam (Director, Modern Horticulture & Germplasm Centre, Zautola, Fulbagan, Natore). After collecting the *Diospyros kaki* leaves were washed thoroughly in water and sun dried 4 days. To increase surface area and better preservation, it was crushed by grinding machine (Model: Miyako Electric Grinder MC-07, Origin: India) in the Department of Pharmacy, Pabna University of Science and Technology, Bangladesh and stored at room temperature (RT) in tightly-closed containers until solvent extraction.

Exactly 100 gm *Diospyros kaki* leaves powder was taken in a conical flask and mixed with 250 ml methanol and kept at room temperature for 4 hours with continuous stirring. Afterward, the liquid portion was decanted in a glass beaker and added another 250 ml methanol and the process was repeated. Again, 100 gm *Diospyros kaki* leaves powder was added in another conical flask and extracted with 250 ml ethyl acetate at room temperature for 4 hours with continuous stirring. Afterward, the liquid portion was decanted in a glass beaker and added another 250 ml ethyl acetate and the process was repeated. The sample (both) was extracted by three times to get the maximum extract. Then, the liquid portion of the both sample (sample + solvent) was filtered through Whatman No. 1 filter paper. The filtrate was then dried and obtained greenish color mass. The filtrate was collected in the glass beaker which was covered with aluminium foil for further analysis.

Chemicals

The used chemicals are: 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) (Tokyo Chemical Industry, Japan), Folin – ciocalteu reagent (Loba chemie Pvt. Ltd., Mumbai, India), Ethyl acetate (Daejung Chemicals & Metals Co. Ltd, Korea), Potassium ferricyanide (Smart Lab, Indonesia), Methanol (Pristine chem, Shyampur, Dhaka), Gallic acid (Wako pure chemicals Ltd., Japan), Potassium acetate (Merck, Germany), Sulphuric acid (Scharlab S. L., Spain), Aluminium chloride (Qualikems Fine Chem, India), Butylatedhydroxy toluene (BHT) (Merck, Germany). Besides, Sodium carbonate, Ammonium molybdate, Ferric chloride, Ascorbic acid were obtained from Sigma chemical company, USA and all the chemicals used in this study were of analytical grade.

Total Phenolic Content

The content of total phenolic of the extractives was determined according to Ainsworth EA, & Gillespie KM [7]. Here Folin - ciocalteu reagent (FCR) was used to oxidize the phenolic compounds of the plant and gallic acid (GA) was used to prepare the standard curve and the phenolic compound of the plants are expressed gallic acid equivalent. In short, 0.4 ml of the extract was added to 2.0 ml of FCR (diluted 10 times with water) reagent solution into each of the test tubes and the reaction was terminated using 2.0 ml of sodium carbonate (7.5%) solution. The tubes were vortexed for 20 seconds and after 20 minutes incubation at 25 ℃, the absorbance was measured at 760 nm using a spectrophotometer (Model: T 60, Origin: United Kingdom) against blank. The whole experiment was repeated three times.

Total Flavonoid Content

The content of total flavonoid of the extractives was estimated by aluminium chloride colorimetric method described by Chandra S, *et al*. [8]. According to this method, in 0.5 ml of the extract, 1.5 ml of methanol and 100 µl of 10% aluminium chloride solution were added. Then 100 µl of 1M potassium acetate solution & 2.8 ml of distilled water were added and left at room temperature for 30 minutes to complete the reaction. Absorbance of the mixtures was measured at 420 nm using a spectrophotometer (Model: T 60, Origin: United Kingdom) against blank. Flavonoid content of the sample was calculated on the basis of the standard curve for catechin acid and the results were presented as mg of catechin equivalents (CAEs)/gm of extract.

Total Antioxidant Capacity

The total antioxidant assay was evaluated according to the method explained by Prieto P *et al* [9] with some minor modifications reported by Mashwani Z, *et al*. [10]. The test is based on the changing of Mo (VI) to Mo (V) by samples and formation of green colored phosphate/Mo (V) complex at acidic pH. Briefly, aliquots 0.5 ml of sample was mixed with 3 ml of reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate into the test tubes. The test tubes were capped with cotton plug and incubated at 95 ℃ for 10 minutes to complete the reaction. After cooling at room temperature, absorbance of the resulting mixture was taken at 695 nm. Antioxidant capacity was determined from the standard curve of ascorbic acid. The result is expressed as mg of ascorbic acid equivalents (AAEs)/gm of extract. The average value was determined from three experiments.

Reducing Power Capacity

The reducing power capacity of the extractives was evaluated by the method described by Do QD, *et al* [11]. Concisely, 0.25 ml of sample solution at different concentrations (12.5 to 200 µg/ml) was mixed with 0.625 ml of 0.2 M phosphate buffer and 0.625 ml of 1% (w/v) solution of potassium ferricyanide. The ingredients were incubated after mixing. Incubation was done at 50 ℃ for 20 minutes. Then, 0.625 ml of 10% (w/v) trichloroacetic acid solution was added to terminate the reaction and the mixture was then centrifuged at 3000 rpm for 10 minutes. After which 1.8 ml supernatant was withdrawn from the test tubes and mixed with 1.8 ml of distilled water and 0.36 mL of 0.1% ferric chloride ($FeCl_3$) solution. Then absorbance was taken at 700nm (spectrophotometer Model: T 60, Origin: United Kingdom) against blank. Ascorbic acid was used as standard. Higher absorbance of the reaction mixture indicates increased reducing power.

DPPH Radical Scavenging Capacity

DPPH (2, 2- diphenyl- 1- picrylhydrazyl) was used to evaluate the free radical scavenging capacity of different extractives by Karadag A *et al*. [12]. In Brief, sample solution at different concentrations (12.5 to 200 µg/ml) was mixed with freshly prepared 0.004% of DPPH methanol solution. The reaction mixtures were shaken properly and allowed to stay for 30 minutes in the dark at room temperature. Then, the absorbance values were measured at 517 nm using a

spectrophotometer (Model: T 60, Origin: United Kingdom) against blank and converted into percentage of inhibition activity. Butylatedhydroxy toluene (BHT) was used as a standard compound. Lower value of absorbance means the maximal free radical scavenging activity of the reaction mixture.

Results

Total Phenolic Content

The phenolic content of the extracts was shown in table 1.

Table 1: Total phenolic content of methanol and ethyl acetate extract of *Diospyros kaki* leaves.

Sample Code	Conc. (µg/ml)	Absorbance	TPC (mg of GAEs /gm of extract)	APC (mg of GAEs/gm of extract) ± SD
DKLM	400	0.543	4.983	5.097±0.245
		0.537	4.928	
		0.586	5.378	
DKLEA	400	0.229	2.100	2.226±0.109
		0.250	2.293	
		0.249	2.284	

The results showed that total phenolic content of polar solvent methanol extract of *Diospyros kaki* leaves (DKLM) was 5.097±0.245 mg/gm and semipolar solvent ethyl acetate extract of *Diospyros kaki* leaves (DKLEA) was 2.226±0.109 mg/gm on 400 µg/ml sample concentration.

Total Flavonoid Content

Flavonoid content of the sample was calculated on the basis of the standard curve for catechin acid and the results are presented as mg of catechin equivalents (CAEs)/gm of extract (Table 2).

Table 2: Total flavonoid content of methanol and ethyl acetate extract of *Diospyros kaki* leaves.

Sample Code	Conc. (µg/ml)	Absorbance	TFC (mg of CAEs/ gm of extract)	AFC (mg of CAEs/ gm of extract) ± SD
DKLM	500	0.221	3.008	2.962±0.043
		0.215	2.924	
		0.217	2.952	
DKLEA	500	0.201	2.727	3.149±0.416
		0.232	3.163	
		0.260	3.558	

The table showed that total flavonoid content of DKLM and DKLEA was 2.962±0.043 mg/gm and 3.149±0.416 mg/gm on 500 µg/ml sample concentration. That means, DKLEA possessed higher flavonoid content than DKLM.

Total Antioxidant Capacity

From the standard curve of standard antioxidant ascorbic acid, antioxidant capacity of the extracts was calculated and expressed as mg of ascorbic acid equivalents (AAEs)/gm of extract.

Table 3: Total antioxidant capacity of methanol and ethyl acetate extract of *Diospyros kaki* leaves.

Sample Code	Conc. (µg/ml)	Absorbance	TAC (mg of AAEs/ gm of extract)	AAC (mg of AAEs/ gm of extract) ± SD
DKLM	500	0.145	17.533	17.222±1.688
		0.129	15.400	
		0.154	18.733	
DKLEA	500	0.093	10.600	13.222±2.272
		0.122	14.467	
		0.123	14.600	

The results demonstrated that total antioxidant capacity of DKLM was 17.222±1.688 mg/gm and DKLEA was 13.222±2.272 mg/gm on 500 µg/ml sample concentration. According to our results extracts on the leaves of *Diospyros kaki*, DKLEA had less antioxidant capacity than DKLM.

Reducing Power Capacity

There are different methods to assess antioxidant activity of plant products, among them reducing power capacity is widely used. But this method is mainly used for polyphenolic antioxidants. The reductants present in plant sample break free radical chains by giving hydrogen atom and exert antioxidant activity. In this assay, the sample (reductant) reduce Fe3+/ferricyanide complex to the Fe2+/ferrous form [13]. In this study, standard antioxidant ascorbic acid is used to compare the antioxidant activity of sample.

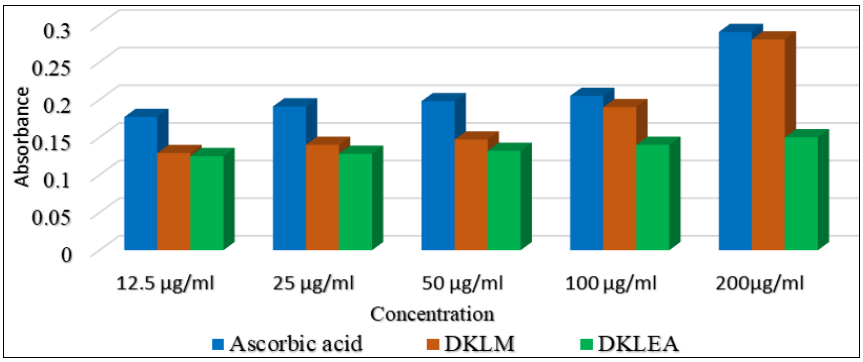

Fig 1: Reducing power capacity of *D. kaki* leaves

The above figure demonstrated that both sample extracts exert reducing power capacity which is directly related to its concentration. The more concentration, the more reducing capacity. Between the two extracts DKLM shows more reducing power capacity as compared to the DKLEA.

DPPH Radical Scavenging Capacity

The odd electron of DPPH is scavenges by the extracts and stabilizes DPPH radical. Here direct relationship between sample concentration and antioxidant activity was found. The results of the DPPH radical scavenging capacity of the extracts are presented in the figure 2.

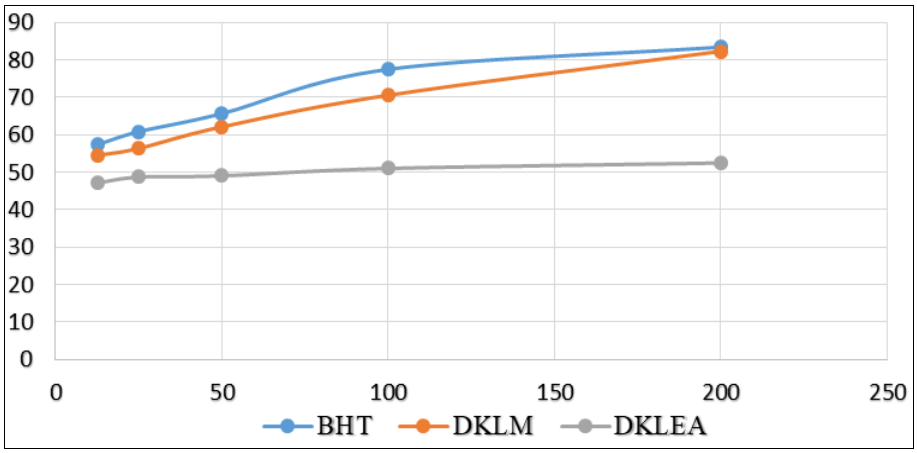

Fig 2: DPPH radical scavenging capacity of *D. kaki* leaves.

From the figure, we found that various concentrations (µg/ml) such as 12.5, 25, 50, 100 and 200 of methanol extract of *Diospyros kaki* leaves showed various percentage (%) of scavenging capacity 54.47, 56.36, 62.1, 70.56 and 82.31 respectively. Again, we found that various concentrations (µg/ml) such as 12.5, 25, 50, 100 and 200 of ethyl acetate extract of *Diospyros kaki* leaves showed various percentage (%) of scavenging capacity 47.13, 48.67, 49.02, 50.98 and 52.45 respectively. Both of the extracts showed DPPH radical scavenging capacity in a concentration dependent manner.

Discussion

It is beneficial for human being to eat plant derived foods particularly that contain antioxidant. So that the risk of getting chronic diseases will become decreased [14]. Phenolic compounds that are found in plant help to prevent against oxidative stress, inflammation, cancer, diabetes, heart disease, bacterial and fungal infection and so on [15-17]. In previous study, the entire phenolic content of *Diospyros kaki* leaves (aqueous extract) was found 75 mg GAE/gm. The study was conducted South korea and the leaves were collected during flowering time (May) [18]. In another study, phenolic content was found 58.06 mg GAE/gm [19]. Result obtained in our study showed that total phenolic content of methanol and ethyl acetate extract of *Diospyros kaki* leaves (after fruits collection) was 5.097±0.245 mg GAE/gm and 2.226±0.109 mg GAE/gm respectively.

Flavonoids are found in fruits and vegetables and has strong antioxidant potential [20]. Our findings reported that total flavonoid content of methanol extract of *Diospyros kaki* leaves was 2.962±0.043 mg CE/gm and ethyl acetate extract of *Diospyros kaki* leaves was 3.149±0.416 mg CE/gm. A study showed that flavonoid content (extracted by methanol) of *Diospyros kaki* leaves (obtained from Trabzon, Turkey) was 64.512±4.153 mg Quercetin/gm [21]. In another study conducted in Slovakia, Ceylan S, *et al*. reported that flavonoid content of 80% ethanol extract for twenty-four hours of *Diospyros kaki* leaves was 32 mg Quercetin/gm [22]. In comparison our results to those of older studies, it must be acknowledged that flavonoid content was very low from the previous study. The possible reasons may be due to difference on (1) solvent extraction process, (2) different solvent and (3) different standard compound. Because, in our study we used methanol extract & catechin as standard but they used 80% ethanol extract & quercetin as a standard and also extraction time was different.

In human body, antioxidant (*in vitro* and *in vivo*) act to prevent free radical damage. In the same time different free radicals are produced from metabolic process. The reduction of antioxidant initiates the generation of disease [23]. Our studied sample shows a strong antioxidant activity. During a study, extract on *Diospyros kaki* leaves grown in Slovakia, antioxidant capacity of 80% ethanol extract for 24 hours was 190 mg Trolox/gm of extract [22]. In another study, Martinez-Las Heras R, *et al*. showed that antioxidant capacity of *Diospyros kaki* leaves (extracted by boiling distilled water and conducted in Valencia, Spain) was 122±3 mg Trolox/gm [24]. On the opposite hand, this study has showed that total antioxidant capacity of DKLM was 17.222±1.688 mg AA/gm and DKLEA was 13.222±2.272 mg AA/gm.

Reducing is one of the mechanisms by which antioxidants exerts their effect [25]. Within the present study, AA showed a significantly higher reducing power capacity than DKLM and DKLEA. The results shows that DKLM gives slightly more reducing power capacity than DKLEA. But both of them possess significant reducing power capacity.

DPPH radical scavenging is an accepted mechanism for evaluation antioxidant potential of plant sample. In one study, the various concentrations of *Diospyros kaki* leaves (China) extracted by 70% ethanol (12.5, 25, 50, 100 and 200 µg/ml) showed antioxidant activities in a concentration dependent manner (14.48%, 21.15%, 32.06%, 51.28% and 68.73% respectively) in the DPPH radical scavenging assay [26]. In other study, Hossain A, *et al*. showed the antioxidant activities of the aqueous extract of different concentrations of *Diospyros kaki* leaves (South Korea) (12.5, 25, 50, 100 and 200 µg/ml) was 41%, 42%, 45%, 48% and 52% respectively [16]. During this study, DKLM had significant scavenging effects with increasing concentrations within the range of 12.5 – 200 µg/ml. However, the scavenging effects of DKLM was obtained similar that of BHT and slightly above DKLEA.

Conclusion

Because of low side effect and cost effectiveness, plant derived medicine and food supplements are gaining popularity [27]. The persimmon is rich of phytochemical and

antioxidants. It is established that fruits rich of antioxidant are good source of anticancer, antidiabetic, anti-arthritis and antimicrobial activities [28-29]. So further study is needed to identify individual compounds and evaluate their activity.

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