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PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY OF *DIOSPYROS EBENUM* J. KOENIG EX RETZ., LEAVES EXTRACT

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ABSTRACT: Phytochemical screening and antioxidant activities with different solvents extracts of *Diospyros ebenum* were investigated. The phytochemical screening of this study indicated the presence of alkaloids, carbohydrates, coumarins, flavonoids, glycosides, phenols, proteins, saponins, steroids, tannins, and terpenoids. The methanolic extract of *D. ebenum* was significantly higher phenolic content when compared to hexane and diethyl ether extracts. In order to effectiveness (IC_{50}) of the plant extracts the potent inhibitors of methanolic extract followed the methods (H_2O_2, RPA and TBA). This showed that *Diospyros ebenum* with different solvents extracts especially the methanolic extract may be a potent source of extraction of natural antioxidant and its use in the management of disease associated with oxidative stress was justified.

INTRODUCTION: Medicinal plants have been playing a vital role on the health and healing of man since down of human civilization. The damage caused by free radicals and their resulting oxidative stress on the living cells has been extensively studied in recent years. Free radicals have been demonstrated to be main initiator for many diseases such as cancer, Alzheimer's disease and rheumatoid arthritis [1]. For this reason, there has been intensive study of the antioxidant properties of plant extracts and isolated phytochemicals with a view to identifying potentially useful antioxidant treatments.

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The concept of disease-chemoprevention has been regarded as one of the most processing avenues for disease control. In recent years, considerable effort has been directed towards identifying naturally occurring substances that can protect against oxidative stress. There has been a worldwide trend towards the use of natural phytochemicals present in fruits, vegetables, oil seeds, teas, herbs, berry crops and beans. Natural antioxidants have a wide range of biochemical activities including inhibition of ROS generation, direct or indirect scavenging of free radicals and alteration of intracellular redox potential [2].

Diospyros is a large genus of trees or shrubs, belonging to family Ebenaceae which are widely distributed in both the hemispheres. It is a slow-growing medium sized tree up to 30 m tall and up to 90 cm in diameter. Bole straight, with buttresses up to 2 m high; crown dense. Bark surface scaly, fissured, black to grey-black.

In traditional medicine, *D. ebenum* fruits are used as a medicine for snake bite, diarrhea, biliousness, ulcer and eaten in times of famine. It has resistant to insect attack and fungi and very durable. It is mainly exported to China for furniture and to Europe as fancy wood. It finds use in sports goods, musical and mathematical instruments, ornamental carvings and turnery. The leaves of the plant proved antioxidant and antibacterial activity [3]. The objective of the present study was to evaluate phytochemical content and antioxidant activity from *Diospyros ebenum* leaf extracts.

MATERIALS AND METHODS:

Collection of Plant Leaf Material: The plant leaf materials of *Diospyros ebenum* were collected from Karaiyankadu, Thiruthuraipoondi Taluk, Thiruvarur District, Tamil Nadu.

Preparation of Plant Leaf Extract: The collected leaves were dried at room temperature and made as fine powder using grinding machine. 50 g of the powdered leaves were weighed and taken into 500 ml conical flask in which 200 ml of distilled water was added. The mixture was kept for 12 h with constant agitation at 30 minutes intervals. The extract was filtered using Whatman no. 1 filter paper [4].

Qualitative Phytochemical Analysis: The phytochemical analysis was carried out from *Diospyros ebenum* leaves with standard methods. The qualitative chemical composition of crude extracts using commonly employed, precipitation and coloration reaction to identify the major natural chemical groups such as alkaloids, carbohydrates, coumarins, flvanoids, glycosides, phenols, proteins, quinones, saponins, steroids, tannins and terpenoids were analysed. General reactions in these analyses revealed the presence or absence of these compounds in the test plant extracts [5].

Test for Alkaloids: About 2 ml of Mayer's reagent was taken and added to 1 ml of leaves extract of *Diospyros ebenum.* The formation of green precipitate indicated due to the presence of alkaloids.

Test for Carbohydrates: About 3 ml of the leaf extract was taken and added 2 ml of Molisch's reagent and the resulting mixture shaken. 2 ml of concentrated sulfuric acid was poured carefully down the side of the test tube. Formation of a red or dull violet color at the inter-phase of the two layers was indicating positive test.

Test for Coumarins: About 3 ml of 10 % NaOH was added to 2 ml of plant extracts and yellow colour was observed in positive results.

Test for Flavonoids: The plant extracts were treated with few drops of sodium hydroxide solution. The formation of intense yellow colour which becomes colourless on addition of dilute acid indicated in the presence of flavonoids.

Test for Glycosides: About four ml of plant leaf extract solution was dried till 2 ml and added 1-2 ml of Ammonium hydroxide and shaken well. The appearance of cherish red color indicated due to the presence of glycosides.

Test for Phenols: About 2 ml of plant extract, 2 ml of distilled water followed by 10 % $FeCl_3$ solution was added. Bluish black colour indicates the presence of phenol.

Test for Proteins: About 2 ml of plant leaf extract, 1 ml of 40% sodium hydroxide and few drops of 1% copper sulphate were added and formation of violet colour indicated the presence of peptide linkage molecules were performed and light blue color was represented.

Test for Quinones: The 2 ml of plant extract were added few drops of concentrated HCl, a yellow colour precipitate appeared for the presence of quinones.

Test for Saponins: Few drops of water and two drops of coconut oil were added to leaves extract of *Diospyros ebenum* and formation of layer or foam indicated the presence of saponins**.**

Test for Steroids: Acetic acid (2 ml) was added to 2 ml of leaves extract of *Diospyros ebenum* and boiled then allowed to cooled and add sulphuric acid for the formation of upper green colour layer is positive and presence of steroids were observed.

Test for Tannins: About five ml of the plant leaf extract was placed in a test tube and then 2 ml of 5 % of $FeCl_3$ solution was added. A greenish-black precipitate was indicated in the presence of tannins.

Test for Terpenoids: About two ml of chloroform was mixed with the plant extract and evaporated on the water path then boiled with 2 ml of H_2SO_4 concentrated. A grey color produced and indicated the terpenoids.

Quantitative Phytochemical Analysis:

Estimation of Alkaloids: The leaf extract (1g) with 20 ml of ethanol and 20% H_2SO_4 (1:1 v/v) was added. The filtrate (1 ml) was added to 5 ml of 60% H_2SO_4. After 5 min, 5 ml of 0.5% formaldehyde in 60% H_2SO_4 was mixed with the mixture and allowed to stand for 3 h. The absorbance was read at 565 nm [6].

Estimation of Carbohydrate: The estimation of polysaccharide content, 1ml of sample solution was taken and added 1 ml of 5% phenol and then added 5 ml of concentrated sulphuric acid mixed well and leave for 10 min. The absorbance measured at 488 nm against blank, compared it with standard glucose [7].

Estimation of Flavonoids: The plant extract (1 g) with 20 ml of ethylacetate for 5 min and filtered. To the filtrate added 5 ml of diluted ammonia was added and shaken for 5 min. The upper layer was collected and the absorbance read at 490 nm [8].

Estimation of Glycosides: One gram plant extract dissolved with 50 ml of distilled water and filtered. Taken one ml filtrate then added 4 ml of alkaline pirate solution. The mixture was boiled for 5 min and allowed to cool. The absorbance was read at 490 nm [9].

Estimation of Phenols: The phenols were determined by slightly modified Folin and Ciocalteu method. The 200µl of the plant extract with 800 µl of Folin Ciocalteu reagent was mixed and 2 ml of 7.5% sodium carbonate added. The total content was diluted to 7 volumes with distilled water and finally kept the tubes for 2 h of incubation in dark. The absorbance was measured at 765 nm. The results were calculated and tabulated [10].

Estimation of Proteins: The one ml of the plant extracts were added 3 ml of Bradford's reagent and incubated in dark for 5 min. The absorbance was measured at 595 nm. Bovine serum albumin was diluted (0.1 mg/ml to 0.5 mg/ml) are used as standard solutions [11].

Estimation of Saponins: The test leaf extracts (1 g) with 10 ml of petroleum ether and decanted into a beaker. Another 10 ml of the petroleum ether was added into the beaker and the filtrate evaporated into dryness. The residue was dissolved in 6 ml of ethanol. The solution (2 ml) was put in a test tube and 2 ml of chromagen solution added into it. It was left to stand for 30 min and the absorbance was read at 550 nm [12].

Estimation of Steroids: One ml leaf extracts were taken into conical flask and added Sulphuric acid (4N, 2 ml) and iron (III) chloride (0.5% w/v, 2 ml), followed by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water-bath maintained at 70 ± 20 ºC for 30 minutes with occasional shaking and diluted with distilled water. The absorbance was measured at 780 nm against the reagent blank [13].

Estimation of Tannins: The leaf extract (1g) with 50 ml of methanol and filtered. The filtrate (5 ml), 0.3 ml of 0.1N ferric chloride in 0.1N HCl and 0.3 ml of 0.0008 M of potassium ferricyanide were added and the absorbance read at 720 nm [14].

Estimation of Terpenoids: The leaf extract (1g) with 50 ml of ethanol and filtered. The filtrate of 2.5 ml of 5% aqueous phosphomolybdic acid solution was added and 2.5 ml of concentrated H_2SO_4 was gradually added and mixed. The mixture was left to stand for 30 min and then made up to 12.5 ml with ethanol. The absorbance was taken at 700 nm [15].

Antioxidant Activity of *D. ebenum*:

Hydrogen Peroxide Scavenging Capacity: The ability of the *Diospyros ebenum* leaf extracts by scavenge hydrogen peroxide was determined according to the standard method. The hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The plant extracts (100 µg/ml) in distilled water were added with hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined [16].

The percentage of hydrogen peroxide scavenging of *Diospyros ebenum* extracts and standard compounds were calculated:

$$\% \text{ Scavenged } H_2O_2 = (AC - AS)/AC \times 100$$

Reducing Power Assay: About one ml of the leaf extract containing (100, 200, 300, 400 and 500 µg/ml) in 1ml of the deionized water mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml potassium ferrocyanide (1%). The mixture was incubated at 50 ºC for 20 min. 2.5 ml of TCA (10%) and centrifuged at 3000 rpm. The upper layer of the solution was mixed with 2.5 ml distilled water and $FeCl_3$ (0.5 ml of 0.1%). The absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated the higher reducing power. The absorbance compared with the standard ascorbic acid (concentrations 100, 200, 300, 400 and 500 µg/ml) [17].

The percentage increased in reducing power was calculated using the following equation

Increase in reducing power (%) = Absorbance of (Control – Test) × 100 / Absorbance Control

Lipid Peroxidation Assay by TBA Method: The different concentration of leaf extracts (100, 200, 300, 400 and 500 µg/ml) were added to 1 ml of 20% aqueous trichloroacetic acid and 2 ml of 0.67% aqueous thiobarbituric acid. After boiling for 10 min, the samples were cooled. The tubes were centrifuged at 3,000 rpm for 30 min. Absorbance of the supernatant was evaluated at 532 nm in a spectrophotometer [18].

Percentage of lipid peroxidation activity = Absorbance of (Control – Test) × 100 / Absorbance Control

RESULTS AND DISCUSSION: In the present investigation suggested that the *Diospyros ebenum* medicinal plant was considered as the potential antioxidant activity in biological system.

The qualitative phytochemical compound like alkaloids, carbohydrates, coumarins, flavonoids, glycosides, phenols, proteins, quinones, saponins, steroids, tannins and terpenoids were analyzed with different solvents of aqueous, methanol, hexane and diethyl ether *Diospyros ebenum* leaf extracts respectively **Table 1**.

TABLE 1: QUALITATIVE PHYTOCHEMICAL COMPOUNDS OF *DIOSPYROS EBENUM*

Phytochemical compounds	Inference			
	Aqueous	Methanol	Hexane	Diethyl ether
Alkaloids	+	+	-	+
Carbohydrates	+	+	+	+
Coumarins	-	-	-	-
Flavonoids	+	+	+	-
Glycosides	-	-	-	+
Phenols	-	-	-	+
Proteins	+	+	+	+
Quinones	-	-	-	-
Saponins	+	+	+	+
Steroids	+	-	+	+
Tannins	-	+	-	+
Terpenoids	+	+	+	-

(+) Present, (-) absent

The methanolic fruit extract yield of 29 *Diospyros* species reported [19]. The maximum quantitative phytochemicals like alkaloids, carbohydrates, flavonoids, proteins, saponins, steroids, tannins and terpenoids were represented with 18.3 ± 0.14, 32.2 ± 0.12, 22.5 ± 0.23, 23.1 ± 0.15, 32.5 ± 0.18, 14.0 ± 0.16 and 19.4 ± 0.10 mg/g recorded with respective methanolic leaf extract of *Diospyros ebenum*.

Simultaneously, the minimum quantity phytochemical results were recorded in hexane leaf extract of *Diospyros ebenum* as 17.4 ± 0.15, 12.7 ± 0.22, 12.3 ± 0.20, 15.3 ± 0.18, 13.5 ± 0.14 and 10.2 ± 0.08 mg/g recorded with carbohydrates, flavonoids, proteins, saponin, steroids and terpenoids were represented **Table 2**. The phytochemical screening of the crude extracts revealed the presence of active entities that elicit a major pharmacological response [20].

Plant substances continue to serve as viable source of drugs for the world population and several plant-based drugs are in extensive clinical uses. The activities of medicinal plants were due to the safe, compared with costly synthetic drugs that have adverse effects. Many previous studies have reported that polyphenols of various plants and herbs are beneficial to animal and human health.

Antioxidant activity of *D. ebenum* extracts was determined by 2,2-diphenyl-1- picrylhydrazyl (DPPH). It was found that methanol 70% extract from *D. ebenum* stems was the most active as antioxidant, it causes scavenging effect by 89.6% with comparison with the standard Green tea extract (96.4%) and also the other extracts prove antioxidant effect, EtOAc extract has scavenging effect of free radical by 70.9% and for BuOH extract was 69.7% and for Aqueous extract was (47.8%) compared with the standard Green tea extract (96.4%). The significant antioxidant activity of methanol 70% extract of *D. ebenum* stems is may be due to the presence of bio-active phytochemicals as flavonoids, tannins, carbo-hydrates and triterpenes. These chemical compounds and especially phenolic compounds proved previously antioxidant effect in which flavonoids as one of the most diverse and widespread group of natural compounds. These compounds characterized with a broad spectrum of chemical and biological activities including radical scavenging properties [21]. The total phenolic content was highest in the methanolic extract and lowest in the petroleum ether extract whereas flavonoid content was highest in the petroleum ether extract and lowest in the aqueous extract. Various assays are used to test antioxidant activity but the most widely used methods are those that involve generation of free radical species that are then neutralized by antioxidant compounds [22].

TABLE 2: QUANTITATIVE PHYTOCHEMICAL COMPOUNDS OF *DIOSPYROS EBENUM*

Phytochemical compounds	Quantity (mg/g)			
	Aqueous	Methanol	Hexane	Diethyl ether
Alkaloids	15.2±0.15	18.3±0.14	-	22.1±0.15
Carbohydrate	14.0±0.24	32.2±0.12	17.4±0.15	20.0±0.18
Flavonoids	16.2±0.15	22.5±0.23	12.7±0.22	-
Glycosides	-	-	-	24.1±0.15
Phenol	-	-	-	25.2±0.16
Protein	19.4±0.11	23.1±0.15	12.3±0.20	23.4±0.18
Saponin	16.5±0.14	32.5±0.18	15.3±0.18	24.1±0.11
Steroids	17.3±0.11	-	13.5±0.14	30.3±0.24
Tannins	-	14.0±0.16	-	26.4±0.19
Terpenoids	13.0±0.15	19.4±0.10	10.2±0.08	-

Values are expressed in mean ± error

The DPPH radical is commonly used as substrates to evaluate antioxidant activity; it is a stable free radical that can accept an electron or hydrogen radical to become a stable molecule. Nandhakumar and Indumathi [23] also reported that organic extracts and components of *Teucrium orientale* L. var. showed significant antioxidant activities. In the present investigation, the maximum antioxidant activity were recordered in hydrogen peroxide scavenging assay was 0.28 ± 0.09, 0.33 ± 0.14, 0.39 ± 0.13, 0.43 ± 0.11 and 0.47 ± 0.15% of 100, 200, 300, 400 and 500 µg/ml methanolc leaf extract of *Diospyros ebenum* than compared to other antioxidant activity **Table 3**.

TABLE 3: ANALYSIS OF ANTIOXIDANT ACTIVITY OF *DIOSPYROS EBENUM* WITH METHANOL EXTRACT BY VARIOUS METHODS

Different concentration (µg/ml)	Percentage of activity (%)					
	Standard (ascorbic acid)	Hydrogen peroxide scavenging (H_2O_2) assay	Standard (ascorbic acid)	Reducing power assay	Standard (ferric thiocyanate)	Lipid peroxidation assay
100	0.18±0.13	0.28±0.09	0.17±0.10	0.23±0.07	0.21±0.14	0.24±0.08
200	0.27±0.12	0.33±0.14	0.23±0.14	0.34±0.11	0.23±0.10	0.30±0.10
300	0.28±0.11	0.39±0.13	0.19±0.13	0.37±0.12	0.25±0.12	0.33±0.11
400	0.32±0.10	0.43±0.11	0.13±0.11	0.37±0.12	0.27±0.10	0.43±0.14
500	0.36±0.16	0.47±0.15	0.24±0.15	0.42±0.13	0.29±0.16	0.45±0.12
IC_{50}	16.51	19.75	24.92	25.24	35.5	12.76

Standard mean ± error

A number of studies have focused on the biological activity of phenolic compounds which are potential antioxidants and free radical scavengers [24]. In the present investigation suggested that the

determination of antioxidant activity were carried out by three methods of hydrogen peroxide scavenging, reducing power assay and lipid peroxidation assay of methanol and diethyl ether leaf extract of *Diospyros ebenum*. Among the three methods, the reducing power assay showed excellent antioxidant activity was 0.24±0.08, 0.26±0.07, 0.28±0.09, 0.32±0.09 and 0.37±0.12% of diethyl ether leaf extract of *Diospyros ebenum* with different concentration of 100, 200, 300, 400 and 500µg/ml. The maximum percentage of IC_{50} value was observed in lipid peroxidase activity, here the values were decreased the IC_{50} values were increased **Table 4**.

TABLE 4: ANALYSIS OF ANTIOXIDANT ACTIVITY OF *DIOSPYROS EBENUM* WITH DIETHYL ETHER EXTRACT BY VARIOUS METHODS

Different concentration (µg/ml)	Percentage of activity (%)					
	Standard (ascorbic acid)	Hydrogen peroxide scavenging (H_2O_2) assay	Standard (ascorbic acid)	Reducing power assay	Standard (ferric thiocyanate)	Lipid peroxidation assay
100	0.16±0.10	0.20±0.06	0.14±0.14	0.24±0.08	0.25±0.14	0.20±0.07
200	0.19±0.12	0.21±0.07	0.19±0.13	0.26±0.07	0.20±0.16	0.23±0.08
300	0.20±0.16	0.22±0.07	0.24±0.15	0.28±0.09	0.19±0.17	0.24±0.08
400	0.25±0.14	0.26±0.10	0.32±0.12	0.32±0.09	0.22±0.11	0.26±0.14
500	0.27±0.13	0.30±0.09	0.29±0.13	0.37±0.12	0.25±0.15	0.37±0.14
IC_{50}	22.62	11.93	14.16	13.59	24.82	35.32

Standard mean ± error

The antioxidant properties of flavonoid depend on their structure, particularly hydroxyl position in the molecule and their ability as electron donor to a free radical [25]. Tannins are complex polyphenolic compounds widely found in many plants. Similarly, polyphenols, tannin have been shown to possess antioxidant [26]. Tannins act as scavengers of reactive oxygen species, peroxide decomposers and quenchers of singlet oxygen, electron donors, and inhibitors of lipoxygenase. Triterpenes proved antioxidant effect, it has been shown that ursolic acid, oleanoic acid and other triterpenoids were efficient protectors against lipid peroxidation also carbohydrates have shown a good antioxidant effect, sugars from Pomegranate fruit and pomegranate juices proved a significant antioxidant effect [27]. Pourmorad *et al*. [28] reported that flavonoids act as potential antioxidant agents through breaking the free radical chain reaction by donating groups of natural constituents found in the plants.

CONCLUSION: The qualitative and quantitative phytochemical like alkaloids, carbohydrates, flavonoids, glycosides, phenols, proteins, saponins, steroids, tannins and terpenoids were represented respectively. According to the antioxidant properties of *Diospyros ebenum* with high concentration of leaf extract has extraordinary performance of antioxidant properties by various methods. The free radical scavenging activity has been elevated and ascorbic acid as a standard for hydrogen peroxide and reducing power assay with aqueous and methanol extract has to be released.

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