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Investigation of the Phytoconstituents and Antioxidant Activity of *Diospyros malabarica* Fruit Extracts

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Abstract

Diospyros malabarica (Deshi Gab) belonging to the family "Ebenaceae" grows well in the humid tropical climate of Bangladesh. In order to investigate the phytoconstituents both qualitatively and quantitatively, the seed and flesh extracts of *D. malabarica* were prepared using two polar solvents (*i.e.*, water and 70% ethanol) and a nonpolar solvent (*i.e.*, hexane). The maximum yield was obtained for aqueous and ethanolic seed extracts indicating that most of the phytoconstituents present in *D. malabarica* fruit are polar. The qualitative phytochemical analysis of the extracts revealed the presence of diverse amount of phytoconstituents in the extracts. On the other hand, the quantitative phytochemical analysis for phenols, tannins, flavonoids, alkaloids, saponin, proteins, reducing sugar and vitamin C revealed that the maximum amount of phenols, tannins, flavonoids and reducing sugar were present in aqueous seed extract. However, the maximum amount of total protein and vitamin C was found in ethanolic seed extract. *D. malabarica* seed powder contained more amount of alkaloids (*i.e.*, 13.6%) than that of flesh (*i.e.*, 3.4%). However, the content of saponin was more in flesh extract (0.74%) when compared to that of seed extract (0.42%). *In vitro* antioxidant properties of the extracts were also investigated by DPPH free radical scavenging assay, FRAP assay and reducing power assay. The highest antioxidant activity was observed for aqueous seed extract followed by the ethanolic seed, ethanolic flesh, and aqueous flesh extracts. The lowest antioxidant activity was obtained for hexane flesh extract. Furthermore, the lowest (*i.e.*, 44.70 μg/ml) as well as the highest (2359.66 μg/ml) IC_{50} values were obtained for aqueous seed and hexane flesh extracts, respectively.

Keywords

Diospyros malabarica, Phytochemical Assay, Total Phenol Content, Total Tannin Content, Total Flavonoid Content, Antioxidant Activity

1. Introduction

Medicinal plants have excellent therapeutic effects because of the presence of various bioactive molecules including terpenoids, flavonoids, saponins, phenols, steroids, resins and so on [1] [2] [3]. It has been reported that these phytoconstituents provide defense mechanisms against bacteria, fungi, amoeba, and insects. They also act as effector molecules for many physiological functions and provide therapeutic benefits against many chronic diseases [4] [5] [6]. During metabolic processes, free radicals (*i.e.*, atoms or molecules possessing unpaired electrons), reactive oxygen species (ROS) (*i.e.*, oxygen-derived free radicals especially the superoxide anion (O_2^-), hydroxyl, peroxyl and alkoxyl) and non-free radicals (*i.e.*, hydrogen peroxide, hypochlorous acid, ozone and singlet oxygen) are generated through various endogenous processes (*i.e.*, respiration, stimulation of polymorphonuclear leucocytes, and macrophages) and exogenous factors (*i.e.*, ionizing radiation, smoking, pollutants, pesticides and organic solvents) [7] [8] [9]. Free radicals are highly reactive to proteins, lipids, and DNA [10] and bring about various chronic diseases and physiological abnormalities including inflammation and autoimmune diseases [11], aging, mutagenicity, carcinogenicity, altering cell signaling pathways and modulating gene expression [12] [13].

To tackle the menace of various free radicals and reactive oxygen species, potential antioxidants having ability to scavenge free radicals by donating their redox hydrogen are necessary [9]. Although the free radicals can be scavenged by both the natural (plant derived) and synthetic (chemically synthesized) antioxidants, the natural antioxidants are getting preference because they do scavenge free radicals without any side effects [14]. Several studies reported that the synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) show toxicity to human through DNA damaging and other toxic effects [15] [16]. The toxicity of these chemically synthesized antioxidants has necessitated the search of new natural antioxidants. Previously published reports showed that the plant-derived antioxidants have been known to protect human from several chronic diseases such as inflammation, autoimmune diseases, cancer, and tumor formation by inhibiting the propagation of free radical reactions [17]. Hence the present study was conducted to investigate the presence of natural antioxidants in different parts of *Diospyros malabarica* fruit.

D. malabarica belonging to the family "Ebenaceae" grows well in the humid tropical climate of Bangladesh. It is considered to be medicinally important because different parts of it, for instance—bark, showed anti-diabetic [18], anti-diarrheal [19], and anti-inflammatory activity [2]. Previous studies also re-

ported that *D. malabarica* is traditionally used for the treatment of ulcer, dysentery, intermittent fever and irregularities in the menstrual cycle [18] [20]. Furthermore, the unripe fruits are usually used as traditional medicine for the treatment of diarrhea and dysentery as well as to paint the undersides of boats to provide protection in water and thus act as a preservative. The infusion of the unripe fruit is also used to harden ropes and render them more durability in water [2]. Since extensive studies on different parts of *D. malabarica* fruits have not been carried out yet, different parts of *D. malabarica* fruits may contain pharmacologically active phytoconstituents with excellent antioxidant propensity. Herein, we prepared *D. malabarica* seed and flesh extracts using water, 70% ethanol (*i.e.*, polar solvent), and hexane (*i.e.*, non-polar solvent) to extract both polar and nonpolar phytochemicals. The phytoconstituents of the extracts were determined both qualitatively and quantitatively. The antioxidant activity of the extracts was investigated through DPPH free radical scavenging assay, FRAP assay, and reducing power assay.

2. Materials and Methods

2.1. Chemicals and Reagents

Absolute methanol, ethanol, hexane, bismath nitrate and HNO_3, H_2SO_4, HCl, NH_3, glacial acetic acid, Folin-Ciocalteu reagent, Na_2SO_4, KOH, bovine serum albumin, and Na-K tartarate were purchased from Merck, Germany. Chloroform, gallic acid, catechin, $NaNO_2$, and NaOH were bought from Ashland Inc., USA. Thiourea and 2,4-Dinitro phenylhydrazine were collected from Titan Biotech Ltd., India. NaCl, Na_2CO_3, iodine, and $Cu_2SO_4 \cdot 5H_2O$ were purchased from UNI-CHEM, China. Tannic acid, trichloroacetic acid (TCA), $AlCl_3$, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), KI, potassium ferricyanide, and arsenomolybdate color reagent were bought from SIGMA-ALDRICH, Germany. Glucose (Dextrose), $FeCl_3 \cdot 6H_2O$, sodium acetate, ascorbic acid, and $NaHCO_3$ were procured from VEGA, China. KCl, Na_2HPO_4, and KH_2PO_4 were obtained from Scharlab, Spain.

2.2. Sample Collection and Preparation

The unripe fruits of *D. malabarica* were collected from the local market of Mymensingh district, Bangladesh (24.7471˚N, 90.4203˚E) and were brought to the laboratory for analysis. After washing properly with water, the flesh and seed parts of the collected *D. malabarica* fruits were separated carefully and cut into small pieces. Both the flesh and seeds were then dried in a hot air oven at 60˚C to remove solvents (*i.e.*, ethanol, hexane, and water) and condense the phytoconstituents. Thereafter, the dried chips were ground into coarse powder using a blender and the powders were stored in hermetically sealed containers to store the extracts in aseptic conditions as well as to protect them from air, moisture and contaminants, with necessary markings in a cool, dark and dry place.

2.3. Preparation of Crude Extract and Fractionation

Crude extracts and their stock solutions preparation were prepared according to our previously established protocol [21] [22] [23]. About 5 g of powdered materials of both seed and flesh were taken in clean conical flasks and mixed with 100 ml of the respective solvents. Three solvents were used *viz.* distilled water, 70% ethanol (as polar solvents) and hexane (as nonpolar solvent). The flasks were kept in a water bath for 72 hours upon gentle shaking at 120 rpm. The crude mixtures were then filtered by Whatman No.1 filter paper and the solvents were evaporated at 60˚C. When the evaporation was completed, the extracts were stored in a dry and cool place for further study. Finally, 1% (10,000 μg/ml) of the extract stock solution was prepared using 0.9% NaCl solution.

2.4. Qualitative Screening of Phytoconstituents of the Extracts

2.4.1. Test for Saponins (Foam Test)

2 ml of distilled water was mixed with 1 ml of the extracts and then vortexed for few minutes. The formation of 1 cm thick layer of foam for 10 minutes indicated the presence of saponins [21].

2.4.2. Test for Tannins

1 ml of distilled water was mixed with 0.5 ml of the extracts and then 1 - 2 drops of $FeCl_3$ solution was added. Gallic tannin showed blue color whereas catecholic tannin showed greenish black color [21].

2.4.3. Test for Reducing Sugar or Carbohydrates (Fehling's Test)

Equal volume of Fehling's A (35 g $CuSO_4{\cdot}5H_2O$ in 500 ml distilled water) and Fehling's B (173 g Na-K tartarate and 50 g NaOH in 500 ml distilled water) were mixed thoroughly and incubated in water bath (65˚C) for a minute. Then equal volume of the various solvent extracts were added to the reagent mixture and boiled at 65˚C for 10 - 15 min. Initially yellow color was appeared and finally brick red precipitate of the cuprous oxide was obtained indicating the presence of reducing sugars [21].

2.4.4. Test for Glycosides

1 ml of glacial acetic acid was added to 1 ml of the sample extracts and then few drops of $FeCl_3$ solution was mixed with it. The appearance of brown colored ring at the top indicated the presence of glycosides [21].

2.4.5. Test for Phenols (Ferric Chloride Test)

1 ml of the extracts, 1 ml ethanol and few drops of $FeCl_3$ were mixed properly. The plant extracts containing phenolic compounds showed red, blue, green and purple color [21].

2.4.6. Test for Alkaloid (Dragendorff's Test)

Equal volume of the extracts and Dragendorff's reagent (bismuth nitrate + concentrated HCl + KI in distilled water) were mixed. The formation of orange-red

precipitate indicated the presence of alkaloids [13].

2.4.7. Test for Alkaloid (Wagner's Test)

Few drops of Wagner's reagent (1.27 g I_2 + 2 g KI in 100 ml distilled water) were added to 500 ml of various extracts. The mixtures were heated at 60˚C for 30 minutes. The appearance of reddish brown precipitate with Wagner's reagent indicated the presence of alkaloids [21].

2.4.8. Test for Flavonoids (Alkaline Reagent Test)

Few drops of 10% NaOH were added to 1 ml of the extracts. The addition of NaOH to the solution initiated the formation of intense yellow color and then the solution became colorless upon addition of few drops of diluted HCl which indicated the presence of flavonoids [21].

2.4.9. Test for Amino Acids and Proteins (Xanthoproteic Test)

2 - 6 drops of concentrated HNO_3 were added to 1 ml of the extracts. Then concentrated NaOH solution was added to neutralize the solution. The appearance of yellow or orange color indicated the presence of protein and amino acids in the sample extracts [21].

2.4.10. Test for Cardiac-Active Glycosides (Keller-Killani Test)

2 ml of glacial acetic acid containing one drop of ferric chloride ($FeCl_3$) solution was added to 5 ml of the extracts. Then 1 ml concentrated sulfuric acid was added to the mixture. Brown ring was formed at the interface which indicated the presence of deoxy sugar of cardenolides. A violet ring may appear beneath the brown ring and a greenish ring may also form gradually throughout the acetic acid layer [24].

2.4.11. Test for Terpenoids (Salkowski Test)

5 ml of the extracts were mixed properly with 2 ml of chloroform. Then 3 ml concentrated sulfuric acid was also added to the solution. The appearance of the reddish brown colour at the interface indicated the presence of terpenoids [13].

2.4.12. Test for Xanthoproteins

Few drops of concentrated HNO_3 and NH_3 solution were mixed separately with 1 ml of the extracts. Formation of reddish orange precipitate indicated the presence of xanthoproteins [25].

2.4.13. Test for Quinones

1 ml of alcoholic potassium hydroxide solution was mixed separately with 1 ml of each of the extracts. The formation of color ranging from red to blue gave the proof of the presence of quinones [26].

2.5. Quantitative Estimation of the Phytoconstituents

2.5.1. Determination of Total Phenolic Content (TPC)

The content of total phenolic compounds in *D. malabarica* seed and flesh extracts was determined by Folin Ciocalteu Reagent (FCR) method as described by

Polash, *et al.* [22] with little modification. Here, gallic acid was used as a standard. The stock solution of aqueous, ethanolic and hexane extracts of *D. malabarica* seed and flesh were diluted to different concentrations with saline solution (0.9% NaCl). 0.1 ml of the different concentrations of standards and sample extracts were taken into different falcon tubes. 500 µl FCR (10% v/v) and then 400 µl of 7.5% sodium carbonate were added to each tube. The mixtures were incubated for 1.5 hour at room temperature. The absorbance of the mixtures was measured at 765 nm by using UV-vis spectrophotometer (Optizen POP, Korea) against blank containing distilled water. Different concentrations of gallic acid were used to prepare a standard calibration curve that was used to determine the TPC value of the extracts. The TPC of the sample extracts were expressed as microgram of gallic acid equivalent per milliliter (µg GAE/ml).

2.5.2. Estimation of Total Tannin Content (TTC)

Total tannin content (TTC) of *D. malabarica* seed and flesh extracts was determined according to the protocol described by Tambe and Bhambar [27] with little modification and tannic acid was used as standard. Different concentrations of *D. malabarica* extracts were prepared with saline solution (0.9% NaCl). In this experiment, 0.1 ml of the different concentrations of standards and sample extracts were taken into different falcon tubes. Then 7.5 ml of distilled water was added to each tubes. 0.5 ml of 100% Folin-Ciocalteu's reagent was added to the above mixtures followed by and the addition of 1 ml 35% Na_2CO_3 and 0.9 ml of distilled water. The mixtures were incubated for 30 min at room temperature. Finally, the absorbance of the mixtures was measured at 725 nm by UV-vis spectrophotometer (Optizen POP, Korea) against a blank containing distilled water. The TTC of the extracts was expressed as microgram of tannic acid equivalent per milliliter (µg TAE/ml).

2.5.3. Estimation of Total Flavonoid Content (TFC)

Total flavonoid content (TFC) in *D. malabarica* seed and flesh extracts was determined by aluminium chloride ($AlCl_3$) colorimetric assay [12]. Here, the flavonoid content was expressed as catechin equivalent. 1 ml of the different concentrations of standard and sample extracts were taken into different tubes. Then 4 ml of distilled water was added to each tube followed by the addition of 0.3 ml of 5% sodium nitrite ($NaNO_2$) and incubated for 5 minutes. Then 0.3 ml of 10 % aluminium chloride ($AlCl_3$) was added to the above mixtures and incubated for 6 minutes at room temperature followed by the addition of 2 ml of 1M sodium hydroxide (NaOH). Immediately 2.4 ml of distilled water was added to each tube to make the total volume up to 10 ml. The solutions were mixed well and the absorbance of the mixtures was measured at 510 nm by UV-vis spectrophotometer (Optizen POP, Korea) against a blank containing distilled water. The TFC of the sample extracts was expressed as microgram of catechin equivalent per milliliter (µg CE/ml).

2.5.4. Determination of Total Alkaloids Content

Total alkaloids content in seed and flesh parts of *D. malabarica* was quantita-

tively determined via gravimetric analysis according to the method described in the previously published reports with little modification [14] [28]. Briefly, 5 g of each of the seed and flesh powder were taken into different conical flasks. In each flask, 200 ml of 20% acetic acid was added. These flasks were then shaken properly for mixing, covered with aluminium foil and then incubated for 4 hours at room temperature. After incubation, the mixtures were filtered and the volume of the filtrate was reduced to one-quarter of its original volume through evaporation. To this concentrated sample, 32% ammonium hydroxide (aqueous NH_3) was added drop-wise in order to precipitate the alkaloids. When the precipitation was completed, the whole solution was allowed to settle and the precipitate was collected by filtration using a pre-weighed filter paper. The percentage of total alkaloid content was calculated as:

$$\text{Percentage of total alkaloids } (\%) = \frac{\text{Weight of the residue}}{\text{Weight of sample taken}} \times 100$$

The alkaloid content of seed and flesh powder of *D. malabarica* was expressed as mg of alkaloid present per 100 g of sample powder.

2.5.5. Determination of Total Saponin Content

Total saponin content of *D. malabarica* seed and flesh extracts was estimated by double extraction gravimetric method described by Ezeabara, *et al.* [29] with few modifications. Briefly, 5 g of the powdered fruit parts were mixed with 50 ml of 20% aqueous ethanol solution and the mixtures were incubated at 55˚C for 90 minutes in a water bath with periodic agitation. After incubation, the mixtures were filtered. The residue was also extracted and filtered in the same manner. Both the extracts were then mixed together and the combined extract was reduced to 40 ml through evaporation at 90˚C. The concentrated extract was transferred to a separating funnel containing 40 ml dichloromethane and shaken vigorously. When the aqueous and organic layer were partitioned, the aqueous layer was separated. If the aqueous layer is not clear, re-extraction and partitioning should be done. The extracted aqueous portion was transferred to separating funnel where it was washed with 60 ml of 100% ethanol. After that, 50 ml of 5% NaCl solution was added to the mixture. After partitioning, the upper aqueous layer was separated from the funnel and evaporated at 60˚C in a pre-weighed glass dish. After complete evaporation, the dish was dried and reweighed. Saponin content was determined from the following formula:

$$\text{Percentage of total Saponin } (\%) = \frac{\text{weight of dish containing sample} - \text{weight of dish}}{\text{Weight of crude sample taken}} \times 100$$

Total saponin content of *D. malabarica* fruit extracts were expressed in percentage.

2.5.6. Estimation of Total Protein Content

Total Protein content of *D. malabarica* seed and flesh extracts were determined

by Lowry's method [30] [31]. Briefly, bovine serum albumin was used as a standard. 2 ml of different dilutions of standard and sample solution was taken into different test tubes or falcon tubes. 2 ml of alkaline copper sulphate reagent (analytical reagent) was added to the tubes and were incubated at room temperature for 10 min. Finally, 0.2 ml of Folin Ciocalteau reagent was added to each tube and incubated for 30 min at room temperature and the absorbance was measured at 660 nm with UV-vis spectrophotometer (Optizen POP, Korea) against a blank containing reagent and distilled water. The protein content of the extracts of various dilutions was expressed as microgram of BSA equivalent per ml of the sample extracts (μg BSAE/ml).

2.5.7. Estimation of Total Reducing Sugar Content

Total reducing sugar (carbohydrates) content of *D. malabarica* seed and flesh extracts was determined by Nelson-Somogyi method with little modification [32]. In this method, glucose was used as a standard. 1 ml of different dilutions of standards and sample solution were mixed properly with 1 ml of copper reagent. These solutions were boiled at 90˚C for 15 minutes and then cooled. After cooling down, 1 ml of arsenomolybdate color reagent was added to the mixture and the solutions were mixed well. The optical density was measured at 520 nm with UV-vis spectrophotometer (Optizen POP, Korea) against a blank containing reagent and distilled water. The glucose content of sample extracts of various dilutions were expressed as microgram of glucose equivalent per ml of sample extracts (μg Glucose/ml).

2.5.8. Estimation of Vitamin C

Vitamin C content of *D. malabarica* seed and flesh extracts was determined by 2,4-dinitrophenyl hydrazine method according to the protocol of Kapur, *et al.* [33] with little modification where 800 μg/ml ascorbic acid prepared with 5% trichloroacetic acid (TCA) was used as standard. 500 μl of different dilutions of standards and sample extract solutions were mixed properly with 100 μl of 2, 4-Dinitrophenyl hydrazine-thiourea-copper (DTC) solution and incubated at 37˚C for 3 hours in water bath. After incubation, 750 μl of ice-cold 65% sulfuric acid was added and the solutions were mixed thoroughly. The solutions were then kept at room temperature for 30 minutes. Finally, the optical density of the mixture was measured at 530 nm with UV-vis spectrophotometer (Optizen POP, Korea) against a blank containing reagent and 5% TCA. The ascorbic acid content of sample extracts of various dilutions was expressed as microgram of ascorbic acid equivalent (AAE) per ml of sample extracts (μg AAE/ml).

2.6. Antioxidant Activity

2.6.1. DPPH Free Radical Scavenging Activity Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of the aqueous, ethanolic, and hexane extracts of *D. malabarica* were determined according to the method described by Manzocco, *et al.* [34] with few modifica-

tions. Briefly, ascorbic acid prepared with methanol was used as a standard. 200 µl of the different concentrations of standards and sample extracts were mixed with 800 µl of DPPH solution (0.025% in methanol). The solutions were mixed well and the tubes were incubated for 30 minutes in the dark environment to complete the reaction. After incubation, the absorbance of the mixtures was measured at 517 nm by UV-vis spectrophotometer against a blank containing distilled water. The percentage of DPPH free radical scavenging activity was calculated from the following equation:

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test samples}}{\text{Absorbance of the control}} \times 100$$

Percentage of the free radical scavenging activity was plotted against the concentration of plant extracts. The concentration of the extract at which it inhibits 50% of the free radical formations (*i.e.*, IC_{50} value) were determined according to our previously published papers [21] [23]. IC_{50} was calculated from the following equation:

$$Y = a * X + b$$

This equation is called the linear regression equation. Here, Y = 50%, X = the concentration of the extracts at which 50% inhibition of free radical formation occurs, a = coefficient and b = constant. The IC_{50} value was plotted against the concentrations of extracts and a bar graph was generated. The extracts showing lowest IC_{50} value possessed the highest DPPH free radical scavenging activity.

2.6.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay of *D. malabarica* seed and fruit extracts was performed according to a method established by Benzie and Strain [35] [36] with little modification. Here the antioxidant activity was measured as the equivalent of the known antioxidant agent (*i.e.*, ascorbic acid). For the preparation of FRAP reagent, the three solutions (*i.e.*, 300 mM acetate buffer prepared with sodium acetate and glacial acetic acid, 10mM 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in distilled water solutions) were mixed freshly at a ratio of 10:1:1 and pre-warmed at 37˚C before using performing the reducing activity assay. Then the different concentrations of standards and sample extracts (0.2 ml) were taken into different tubes. FRAP reagent (1.5 ml, pre-warmed at 37˚C) was added to each tube and incubated for 4 min at 37˚C. Finally, the absorbance of the mixtures was measured at 593 nm using a UV-vis spectrophotometer (Optizen POP, Korea) against a blank containing FRAP reagent and distilled water. The antioxidant activity of the sample extracts was expressed as microgram of ascorbic acid (AA) equivalent per milliliter (µg AAE/ml).

2.6.3. Reducing Power Assay

The antioxidant potential of *D. malabarica* seed and fruit extracts was also performed by ferric reducing power assay according to Afsar, *et al.* [37] and Bag, *et al.* [15] with little modification. For this experiment, gallic acid was used as standard. 0.5 ml of the different concentrations of standards and sample extracts

were taken into different tubes. Then 0.5 ml phosphate buffer (0.2 M) and 0.5 ml potassium ferricyanide (1%) were added to each tube and the solution was mixed properly. The mixtures were incubated at 50˚C for 20 min. After incubation, 0.5 ml trichloroacetic acid (10%) was added to the reaction mixture in order to stop the reaction and was centrifuged at 3000 rpm for 10 minutes. After centrifugation, 0.5 ml of supernatant was pipetted out from the upper layer and taken into separate tubes 0.1 ml ferric chloride (0.1%) and 0.5 ml distilled water were added to the tubes. The absorbance of the mixtures was measured at 700 nm using UV-vis spectrophotometer (Optizen POP, Korea) against a blank containing distilled water. The absorbance of the standard and sample extracts were plotted against concentration to prepare a line graph from which the reducing power of the *D. malabarica* seed and fruit extracts could be determined. Increased absorbance of the reaction mixture indicated increased reducing power.

2.7. Statistical Analysis

Statistical analysis was carried out by GraphPad Prism 5.0 using one-way analysis of variance (ANOVA) test and the significance of the difference between means was determined by Duncan's multiple range test at ($P < 0.05$) significant level. All the analysis was carried out multiple times and the result was represented in mean ± SEM.

3. Results

3.1. Yield Coefficient

The percentage of yield indicates the amount of extracts obtained through the extraction procedure expressed in gram (g) of extracts obtained from per 100 gram (g) of crude powder and presented in Table 1.

The highest yield was obtained in aqueous seed extracts (40.6 gm per 100 gm of crude powder) and the lowest yield was obtained in hexane flesh extracts (1.8 gm per 100 gm of crude powder). The higher yield in aqueous as well as in ethanolic extracts indicate that most of the phytoconstituents present in *D. malabarica* fruit are polar. As a result, the phytoconstituents were easily extracted by polar solvent (*i.e.*, water and ethanol). The low yield in hexane extracts revealed that very low amount of nonpolar phytoconstituents are present in both the seed and flesh extracts of *D. malabarica* (Table 1). On the other hand, greater yield in seed extracts was obtained when compared to that of flesh extract which indicates that *D. malabarica* seed extracts are excellent source of phytoconstituents.

3.2. Qualitative Phytochemical Screening

The results of qualitative phytochemical screening of *D. malabarica* seed and flesh extracts are presented in Table 2.

The seed and flesh extracts of *D. malabarica* prepared using distilled water, and 70% ethanol showed the presence of high amount of tannin, reducing sugar, protein, cardiac-active glycosides, and quinones. In addition, these extracts also

Table 1. The percentage yield of *D. malabarica* seed and flesh extracts prepared using distilled water, 70% ethanol, and hexane.

SL No.	Extracts with solvents (5 gram in 100 ml)	Yield of the extract (in gram)	Percentage yield (%w/w)
1.	Aqueous seed extracts	2.03	40.6
2.	Aqueous flesh extracts	0.68	13.6
3.	Ethanolic seed extracts	1.94	38.8
4.	Ethanolic flesh extracts	0.73	14.6
5.	Hexane seed extracts	0.12	2.4
6.	Hexane flesh extracts	0.09	1.8

Table 2. Phytochemical screening of *D. malabarica* seed and flesh extracts prepared using distilled water, 70% ethanol, and hexane.

Test	Aqueous Seed Extract	Aqueous Flesh Extract	Ethanolic Seed Extract	Ethanolic Flesh Extract	Hexane Seed Extract	Hexane Flesh Extract
1. Saponin	++	+	+	++	+	+
2. Tannin	+++	+++	+++	+++	−	−
3. Reducing sugar	+++	+++	+++	++	−	+
4. Glycoside	++	+	+++	−	−	−
5. Phenol	++	++	++	++	−	−
6. Alkaloid (Dragendorff's test)	+	++	++	++	−	−
7. Alkaloid (Wagner's test)	+	++	++	++	−	−
8. Flavonoid	++	+	++	++	−	−
9. Protein	+++	++	+++	+++	−	−
10. Cardiac-active glycosides	+++	+++	+++	+	−	−
11. Terpenoids	−	++	−	++	−	−
12. Xanthoproteins	+	+	+++	++	+	−
13. Quinones	+++	−	+++	−	−	−

*N.B: +++ = high; ++ = moderate; + = low; − = negative.

showed the appearance of moderate amounts of phenol, terpenoids, saponins, flavonoids, alkaloids, and glycosides (Table 2). On the other hand, hexane seed, and hexane flesh extracts showed negative results in almost all the qualitative tests which indicates the absence of nonpolar phytoconstituents in the *D. malabarica* seed and flesh extracts.

3.3. Quantitative Phytochemical Assay

3.3.1. Determination of Total Phenol, Tannin and Flavonoid Content

Total phenol content (TPC), total tannin content (TTC) and total flavonoid

content (TFC) of *D. malabarica* seed and flesh extracts have been expressed as µg GAE/ml, µg TAE/ml and µg CE/ml, respectively. The data revealed that the aqueous seed extract showed the maximum amount of TPC, TTC, and TFC in a dose dependent manner followed by ethanolic seed extract (Figures 1-3). For all extracts, the concentration of TPC, TTC and TFC increased as the amount of the plant extracts was increased. The amount of TPC, TTC, and TFC in all the extracts always follow the same order: Aqueous seed > ethanolic seed > ethanolic flesh > aqueous flesh >> hexane extracts. This indicates that most of the phenolics, tannins and flavonoids were extracted through polar solvents (*i.e.*, water and ethanol). The phenol, tannin, and flavonoid contents in aqueous seed extract were significantly higher than that of aqueous flesh extract. Similarly, the phenol, tannin, and flavonoid contents in ethanolic seed extract were significantly higher than that of ethanolic flesh extract. Although the differences between aqueous seed and ethanolic seed extracts were very minuscule in terms of their phenol, tannin, and flavonoid contents (Figures 1-3). The highest TPC was found in aqueous seed extract (23 µg GAE/ml) and the lowest TPC was found for hexane flesh extract (1.15 µg GAE/ml) at a concentration of 100 µg/ml (Figure 1). Again, the highest TTC was found in aqueous seed extract (369 µg TAE/ml) and the lowest TTC was found in hexane seed extract (10.72 µg TAE/ml) at a concentration of 500 µg/ml (Figure 2). Furthermore, the highest TFC was found in aqueous seed extract (110 µg CE/ml) and the lowest TFC was found in hexane seed extract (2.81 µg CE/ml) at a concentration of 1000 µg/ml (Figure 3).

3.3.2. Determination of Total Alkaloid and Total Saponin Content

Total alkaloid and total saponin content of *D. malabarica* seed and flesh extracts were estimated by gravimetric analysis method (Table 3). The total amount of

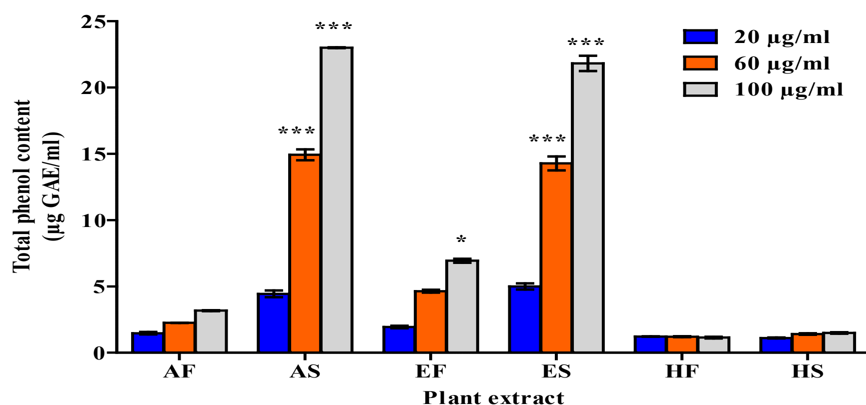

Figure 1. Estimation of total phenol content (TPC) of *D. malabarica* seed and flesh extracts. The graph shows TPC of three different concentrations (*i.e.*, 20 µg/ml, 60 µg/ml and 100 µg/ml) of all extracts expressed in µg GAE/ml. Here, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, ES = ethanolic seed extract, HF = hexane flesh extract and HS = hexane seed extract. The data have been presented as average value of different replications ± standard deviations (n = 3). Data were analyzed by GraphPad Prism 5.0 using ANOVA and significance level was determined (*$p < 0.05$, ***$p < 0.001$).

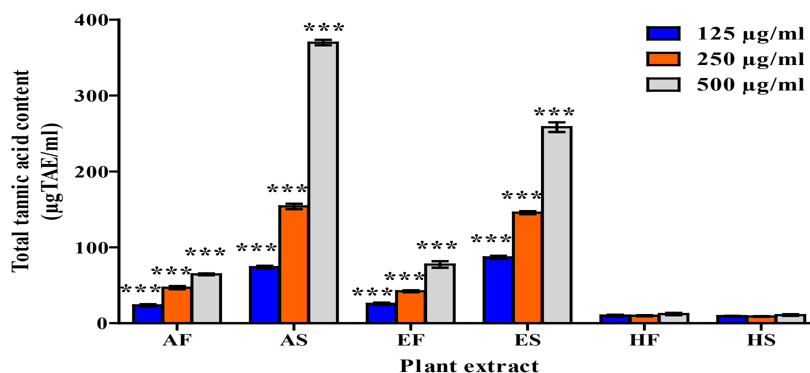

Figure 2. Estimation of total tannin content (TTC) of *D. malabarica* seed and flesh extracts. The graph shows total tannin content of three different concentrations (*i.e.*, 125 µg/ml, 250 µg/ml and 500 µg/ml) of both the seed and flesh extracts expressed in µg TAE/ml. Here, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, ES = ethanolic seed extract, HF = hexane flesh extract and HS = hexane seed extract. The data has been presented as average value of different replications ± standard deviations (n = 4). Data were analyzed by GraphPad Prism 5.0 using ANOVA and significance level was determined (***$p < 0.001$).

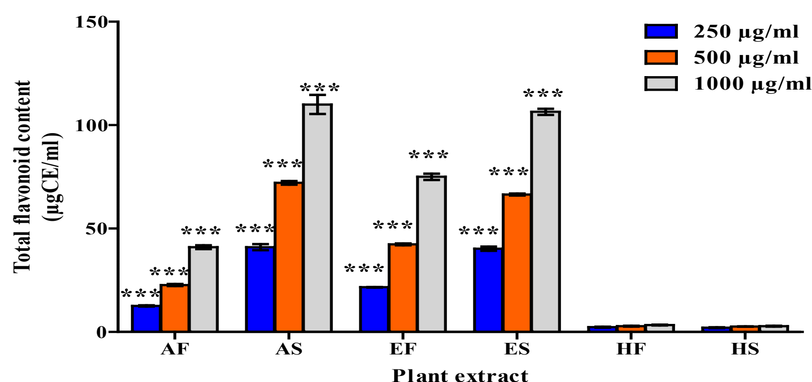

Figure 3. Estimation of total flavonoid content (TFC) of *D. malabarica* extracts. The graph shows total flavonoid content of three different concentrations (*i.e.*, 250 µg/ml, 500 µg/ml and 1000 µg/ml) of both the seed and flesh extracts expressed in µg CE/ml. Here, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, ES = ethanolic seed extract, HF = hexane flesh extract and HS = hexane seed extract. The data have been presented as average value of different replications ± standard deviations (n = 4). Data were analyzed by GraphPad Prism 5.0 using ANOVA and significance level was determined (***$p < 0.001$).

Table 3. Total Alkaloid and total saponin content of *D. malabarica* seed and flesh extracts.

D. malabarica Plant Parts	Alkaloid Content (gm)	Saponin Content (gm)
Seed	13.6 ± 0.92***	0.42 ± 0.05
Flesh	3.4 ± 0.40	0.74 ± 0.07

***Significance level ($p < 0.001$).

alkaloid was 13.6 gm in 100 gm of the seed powder of *D. malabarica*. On the other hand, the amount of alkaloid present in 100 of flesh powder was 3.4 gm,

that is, alkaloid content of seed was significantly higher than that of flesh. Therefore, *D. malabarica* seed is an excellent source of alkaloid. However, the amount of saponin was 0.42 gm and 0.74 gm in 100 gm of each of the seed and flesh powder of *D. malabarica*, respectively, that is, saponin content of flesh was not significantly higher than that of seed. Hence, *D. malabarica* fruit is a moderate source of saponin.

3.3.3. Estimation of Total Protein, Reducing Sugar and Vitamin C Content

Total protein, total reducing sugar, and total vitamin C content of *D. malabarica* seed and flesh extracts were determined in a dose dependent manner (**Figures 4-6**). Ethanolic seed extract showed the highest amount of total protein and total vitamin C content, whereas aqueous seed extracts showed the highest amount of total reducing sugar content. In all concentrations, the higher amount of proteins, reducing sugars, and vitamin C were extracted with polar (*i.e.*, water and ethanol) solvents which indicate that most of the proteins, reducing sugars and vitamin C present in *D. malabarica* seed and flesh were polar in nature. The protein, reducing sugar, and vitamin C content of the aqueous seed extract was significantly higher than that of the aqueous flesh extract. On the other hand, the protein, reducing sugar, and vitamin C content of ethanolic seed extract was significantly higher than that of the ethanolic flesh extract. The differences of protein, reducing sugar and vitamin C content between aqueous seed and ethanolic seed extracts were insignificant (**Figures 4-6**). The highest protein content was obtained in ethanol seed extract (920 μg BSAE/ml) and the lowest protein content was obtained in aqueous flesh extract (156 μg BSAE/ml) at a concentration of 600 μg/ml (**Figure 4**). The highest reducing sugar content was obtained in aqueous seed extract (694 μg GE/ml) and the lowest reducing sugar content was

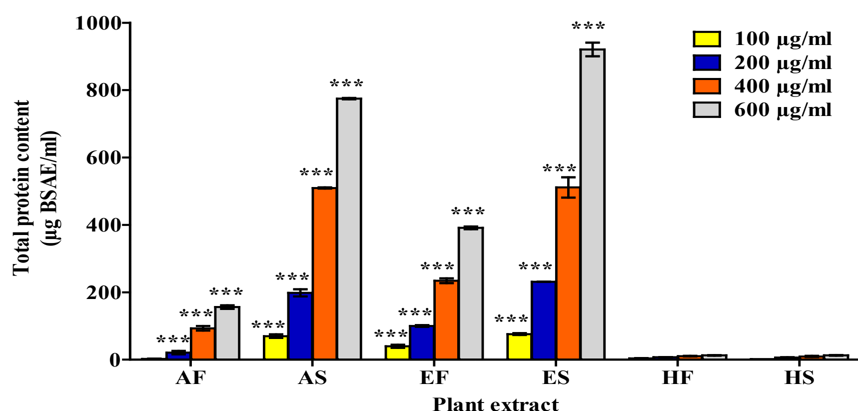

Figure 4. Estimation of total protein content of *D. malabarica* seed and flesh extracts. The graph shows total protein content of four different concentrations (*i.e.*, 100 μg/ml, 200 μg/ml, 400 μg/ml and 600 μg/ml) of both the seed and flesh extracts expressed in μg BSAE/ml. Here, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, ES = ethanolic seed extract, HF = hexane flesh extract and HS = hexane seed extract. The data have been presented as average value of different replications ± standard deviations (n = 3). Data were analyzed by GraphPad Prism 5.0 using ANOVA and significance level was determined (***p < 0.001).

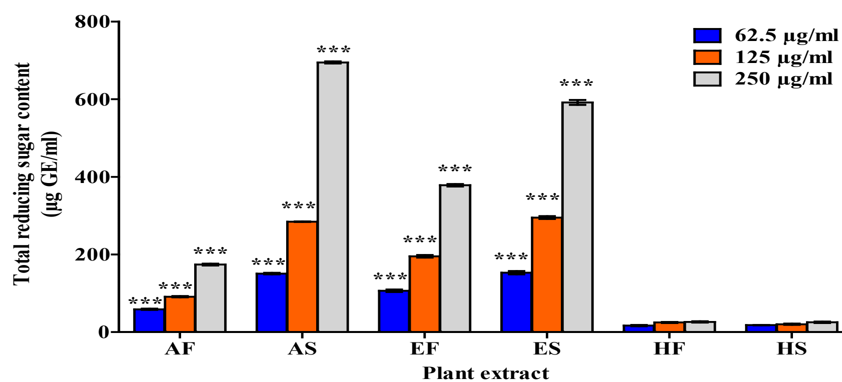

Figure 5. Estimation of total reducing sugar content of *D. malabarica* seed and flesh extracts. The graph shows total reducing sugar content of three different concentrations (*i.e.*, 62.5 µg/ml, 125 µg/ml and 250 µg/ml) of both the seed and flesh extracts expressed in µg GE/ml. Here, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, ES = ethanolic seed extract, HF = hexane flesh extract and HS = hexane seed extract. Data were analyzed by GraphPad Prism 5.0 using ANOVA and significance level was determined (***$p < 0.001$).

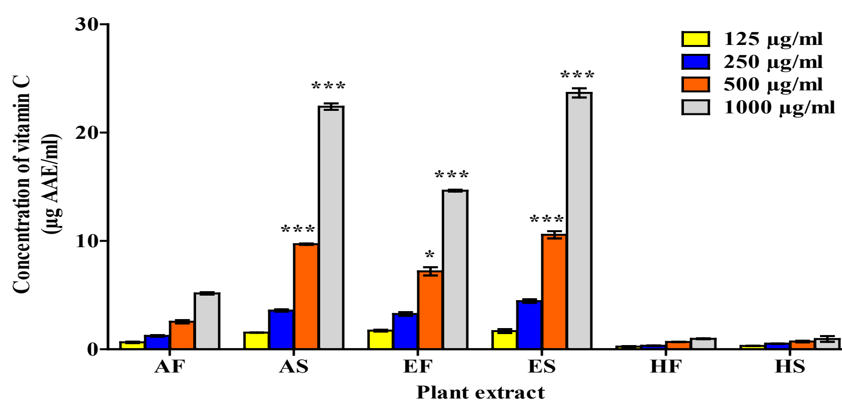

Figure 6. Estimation of ascorbic acid content of *D. malabarica* seed and flesh extracts. The graph shows vitamin C content of four different concentrations (*i.e.*, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml) of both the seed and flesh extracts expressed in µg AAE/ml. Here, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, ES = ethanolic seed extract, HF = hexane flesh extract and HS = hexane seed extract. Data were analyzed by GraphPad Prism 5.0 using ANOVA and significance level was determined (*$p < 0.05$, ***$p < 0.001$).

obtained in aqueous flesh extract (194 µg GE/ml) at a concentration of 250 µg/ml (Figure 5). The highest vitamin C content was obtained in ethanolic seed extract (23 µg AAE/ml) and the lowest vitamin C content was obtained in aqueous flesh extract (5 µg AAE/ml) at a concentration of 250 µg/ml (Figure 6). Hexane extracts showed very little or almost absence of protein, reducing sugar as well as ascorbic acid.

3.4. Antioxidant Activity Assay

3.4.1. DPPH Free Radical Scavenging Assay

DPPH is a stable free radical and that can accept an electron or hydrogen radical

to become a stable molecule. A freshly prepared DPPH solution is deep purple in colour with absorption maximum at 517 nm in the presence of an antioxidant that quenches DPPH free radicals and converts them into a colourless product [9]. Herein, all the *D. malabarica* fruit extracts were used for DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay and ascorbic acid was used as positive control. The highest scavenging activity was performed by aqueous seed extract, followed by ethanolic seed extract and showed significant free radical scavenging activity even at a very low concentration (5 µg/ml) (Figure 7(A)). On the other hand, both the aqueous and ethanolic flesh extracts showed significant free radical scavenging activity at 50 µg/ml (Figure 7(A)).

To determine the IC_{50} value (*i.e.*, the concentration of the plant extract required to inhibit the formation of free radicals by 50%) of *D. malabarica* seed and flesh extracts in terms of their DPPH free radical scavenging activity, the absorbance for various concentrations of different plant extracts was plotted against the concentration and found a line graph for each extract. The IC_{50} value was calculated from the line graph. The extracts having the lowest IC_{50} value had the highest DPPH free radical scavenging activity. The IC_{50} values of aqueous

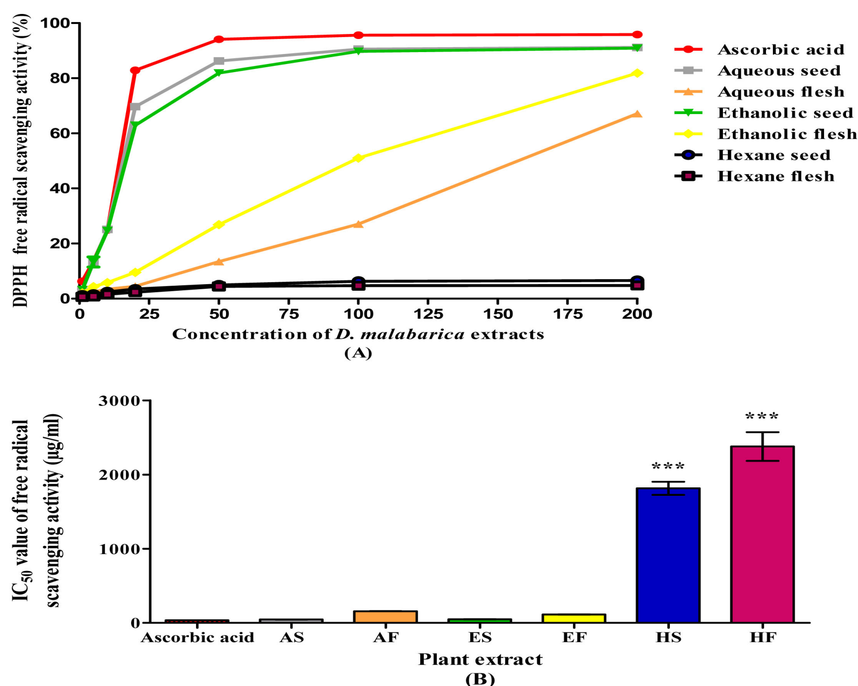

Figure 7. Antioxidant activity assay of *D. malabarica* extracts. (A) Dose response curve of percentage of scavenging of DPPH free radical by different seed and flesh extracts. Different concentrations of the extracts (1 - 200 µg/ml) are presented in the figure. The graph shows that the free radical scavenging activity is proportional to the concentration of extracts. (B) Comparison of IC_{50} value of different seed and flesh extracts of *D. malabarica.* The data were represented as an average value of different replications ± standard deviations (n = 3). Here, AS = aqueous seed extract, AF = aqueous flesh extract, ES = ethanolic seed extract, EF = ethanolic flesh extract, HS = hexane seed extract and HF = hexane flesh extract. Data were analyzed by GraphPad Prism 5.0 using ANOVA and significance level was determined (***p < 0.001).

seed, aqueous flesh, ethanolic seed, ethanolic flesh, hexane seed, and hexane flesh extracts were 44.50 μg/ml, 156.03 μg/ml, 49.12 μg/ml, 113.53 μg/ml, 1811.36 μg/ml, and 2359.66 μg/ml, respectively. However, the IC_{50} value of ascorbic acid was 32.42 μg/ml (**Figure 7(B)**). Therefore, it was shown that the IC_{50} values of aqueous seed, aqueous flesh, ethanolic seed and ethanolic flesh extracts were not significant with respect to ascorbic acid. However, the IC_{50} values of both hexane extracts were statistically significant when compared to ascorbic acid. The lowest IC_{50} value was obtained for aqueous seed extract (*i.e.*, 44.50 μg/ml) and is the most efficient free radical scavenger. On the other hand, the highest IC_{50} value was obtained for hexane flesh extract (*i.e.*, 2359.66 μg/ml) and is the least efficient free radical scavenger.

3.4.2. Ferric Reducing Antioxidant Power (FRAP) Assay

In FRAP assay, the antioxidant activity of *D. malabarica* fruit extracts was expressed in μg AAE/ml. Here, the reduction of a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex into its ferrous form (Fe^{2+}) in the presence of an antioxidant produces an intense blue color at low pH that can be monitored by measuring the absorbance at 593 nm [38]. Therefore, the change in absorbance is directly related to the reducing power of the electron donating antioxidants present in the reaction mixture [35]. The ferric reducing antioxidant potential (FRAP) for aqueous seed extract was significantly higher than that of aqueous flesh extract. Similarly, the FRAP for ethanolic seed extract was significantly higher than that of ethanolic flesh extract. However, the difference between aqueous seed and ethanolic seed extracts in terms of ferric reducing antioxidant potential was statistically insignificant (**Figure 8**). In the current study, the aqueous seed (7.31 μg AAE/ml) and ethanolic seed (5.18 μg AAE/ml) extracts showed significant antioxidant activity when compared to other extracts at a concentration of 25 μg/ml

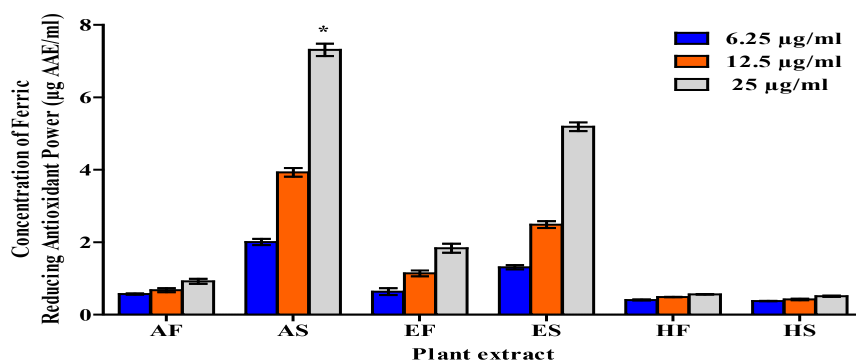

Figure 8. Antioxidant activity assay of *D. malabarica* extracts. The graph shows the antioxidant potential through FRAP assay of three different concentrations (*i.e.*, 6.25 μg/ml, 12.5 μg/ml and 25 μg/ml) of both the seed and flesh extracts expressed in μg AAE/ml. Here, AF = aqueous flesh extract, AS = aqueous seed extract, EF = ethanolic flesh extract, ES = ethanolic seed extract, HF = hexane flesh extract and HS = hexane seed extract. The data have been presented as average value of different replications ± standard deviations (n = 3). Data were analyzed by GraphPad Prism 5.0 using ANOVA and significance level was determined (*$p < 0.05$).

(Figure 8). In addition, the concentration of antioxidant activity increased as the amount of the plant extract (6.25 to 25 μg/ml) was increased.

3.4.3. Reducing Power Assay

The reducing power of the extracts may serve as an indicator of their potential antioxidant activity [9]. In this assay, the presence of antioxidant activity in the sample extracts may cause the reduction of Fe^{3+}/Ferric cyanide complex to ferrous form which can be monitored spectrophotometrically at 700 nm. The antioxidant potential of the different seed and flesh extracts were estimated from the differences of their absorption at various concentrations. Furthermore, the reducing power of the sample extracts increased with the increase of their concentrations (*i.e.*, 6.25 to 25 μg/ml) (Figure 9) and gallic acid was used as positive control. The maximum reducing power activity was obtained in the aqueous seed and ethanolic seed extracts followed by ethanolic flesh and aqueous flesh extracts.

4. Discussion

The present study was carried out to investigate the phytochemical constituents and antioxidant potential of *D. malabarica* seed and flesh extracts prepared using two polar (*i.e.*, distilled water, and 70% ethanol) solvents and a nonpolar (*i.e.*, hexane) solvent. The phytochemical analysis of the extracts was carried out qualitatively as well as quantitatively, that indicates the presence of different functional constituents in the extracts. For example, the qualitative phytochemical assay showed the presence of saponin, tannin, flavonoid, carbohydrate, glycoside, phenol and protein. On the other hand, the quantitative phytochemical analysis revealed that different extracts of *D. malabarica* seed and flesh contained different amount of phenolics, tannins, flavonoids, alkaloids, saponins, protein, reducing sugar and vitamin C. More specifically, the maximum amount of these phytoconstituents were found in the aqueous and ethanolic seed extracts. The phytochemical constituents detected in *D. malabarica* extracts are known for restorative benefits. For instance, plant polyphenols are found to

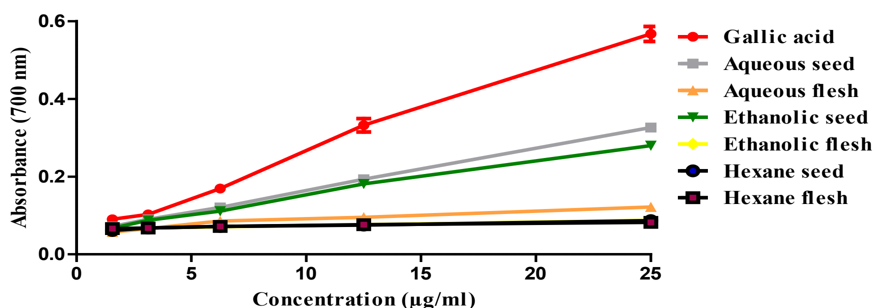

Figure 9. The dose-response curves for the reducing power of different concentrations of seed and flesh extracts of *D. malabarica*. The X-axis indicates different concentrations of gallic acid and *D. malabarica* seed and flesh extracts in μg/ml and the Y-axis indicates absorbance value at 700 nm.

be effective for the prevention of diverse oxidative stress associated diseases such as cancer, cardiovascular diseases, chronic inflammation and so on [13] [39] [40]. Tannins are also known to have antibacterial [41] [42], antiviral [43], and antitumor activities [43] [44]. Furthermore, plant derived alkaloids are known to show various biological activities including anti-inflammatory [45], antimalarial [46], and antibacterial [47] activity. Saponins are well known for their wide range of pharmacological and medicinal activities such as anti-ulcer, anticancer, antimicrobial and so on [29]. Vitamin C is known to be used as food additive, antioxidant, and reducing agent [33] [48]. Furthermore, previous research on *D. malabarica* bark reported the potential bioactivity (e.g., antioxidant activity) of the extract [18] [19].

Our data also support the antioxidant activity of *D. malabarica* fruit extracts. The aqueous and ethanolic extracts of *D. malabarica* seed and flesh showed free radical scavenging as well as reducing power activity, that is, antioxidant activity. The extracts showed antioxidant activity according to the following order: aqueous seed extract > ethanolic seed extract > ethanolic flesh extract > aqueous flesh extract >> hexane extracts. According to Aparadh *et al.* [49] and Hossain *et al.* [50], antioxidant activity of plant extracts depend on the presence of respective secondary metabolites, such as flavonoids, tannins and phenolics. Another study reported that vitamin C is the indicator of antioxidant activity [30]. The level of phenolics, tannins, flavonoids, and vitamin C in *D. malabarica* fruit extracts are in the order of aqueous seed > ethanolic seed > ethanolic flesh > aqueous flesh >> hexane extracts (**Figures 1-3**, and **Figure 6**). The antioxidant activity of the extracts follow the same trend as the secondary metabolites (**Figures 7-9**). Taken together, since these phytoconstituents are mainly responsible for the antioxidant activity, *D. malabarica* fruit extracts can be an important source of natural antioxidants.

5. Conclusion

Diospyros malabarica flesh and seed extracts have been prepared using two polar solvents (*i.e.*, water, and ethanol) and a nonpolar solvent (*i.e.*, hexane). The phytoconstituents including total phenol content, total tannin content, total flavonoid content, total protein content, total reducing sugar content, total ascorbic acid content, total saponin content, and total alkaloid content of all the extracts were determined through both qualitative and quantitative analysis. Some other phytoconstituents such as glycosides, terpenoids, resin and so on were determined only through qualitative analysis. *D. malabarica* flesh and seed extracts obtained from the polar solvents contained a greater amount of phytoconstituents when compared to their nonpolar solvent counterparts. More specifically, aqueous seed extract contained the highest amount of phytoconstituents except total content of protein and vitamin C. Furthermore, aqueous seed extract showed the highest DPPH free radical scavenging activity, ferric reducing antioxidant power (FRAP) activity and reducing power activity assay followed by ethanolic

seed extract. Therefore, *D. malabarica* seed is rich in different phytoconstituents with numerous therapeutic propensity and can be used as a potential source of natural antioxidants with other therapeutic applications including hepatoprotective efficacy. *D. malabarica* fruit can further be screened against various disease causing pathogens and can be a potential source of chemical and biologically important drug candidates.

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Conflicts of Interest

We declare no conflict of interest.

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