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A Comparative In Vitro Antioxidant Potential Of Various Extracts From Aerial And Underground Parts Of *Dalbergia Sissoo*.

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Abstract

Objective: To evaluate antioxidant and radical scavenging activities of aerial and underground parts of *Dalbergia sissoo* extracts.
Methods: Shed dried and powdered plant parts were initially extracted in methanol and subsequently partitioned in n-hexane, chloroform, ethyl acetate and 1-butanol successively. Antioxidant and radical scavenging potential of methanol extracts and fractions of each parts were evaluated using Total phenolic content (TPC) and Total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl and 2,2' –azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation radical scavenging, reducing power (potassium ferricyanide-trichloroacetic acid system), ferric ion reducing antioxidant potential, lipid peroxidation inhibition activity and total antioxidant activity assays.
Results: TPC and TFC values for methanol extracts and various fractions ranged from 0.23- 4mg/L gallic acid equivalents and from 30-545 mg/L rutin equivalent respectively, Methanol extracts and all fractions of roots and aerial parts showed higher TPC and TFC values. Methanol extracts and aqueous fractions of roots and aerial parts and n-butanol fractions of root showed lower EC_{50} values for 2, 2- diphenyl-1-picrylhydrazyl scavenging than the other plant extracts. The 2, 2' –azinobis , radical scavenging, total antioxidant potential and ferric ion reducing antioxidant potential values confirmed the presence of potent antioxidant potential values confirmed the presence of potent antioxidant principles in the methanol extract of roots. All the extracts of the roots showed high antioxidant and radical scavenging activities.
Conclusion: The crude methanol extract of root can be explored further for in vivo studies.

1. Introduction:

Oxidative stress, resulting from overproduction of reactive oxygen species, causes harmful effects on biomolecules like lipids, nucleic acids, proteins etc., leading to many pathological and neurological disorders. Evidence has accumulated that herbal extracts containing phyto antioxidants especially polyphenols, flavonoids, tannins and other related compounds have extensive biological properties including the positive health effects and reduction of the disease risk [1, 2]. Further, the phyto antioxidants are supposed to be safe since they occur in plants.

Dalbergia sissoo (family Fabacae) is a woody herb confined to warm areas of Africa and Asia. It is bitter and sour in taste, and is known to have immense therapeutic effects against various types of hematological, hepatic, neurological and inflammatory conditions [3]. The D. sissoo and other species of the same genus are considered to be anti-asthamatic, stimulant, antiseptic, antidote, antidysenterica, antihepatotoxic, antipyretic, diuretic and analgesic[4]. Phytochemical investigation on Dalbergia species has yielded a variety of chemical constituents including flavonoids, fatty acids, alkaloids, proteins and amino acids [5-8]. Dalbergia species have been reported to demonstrate antimicrobial, anticancer, antioxidant, and antihaemorrhagic activities [9-13].The objective of the present investigation is to analyse and compare the antioxidant properties and to quantify the phenolic contents of the fruit, root and aerial parts of Dalbergia sissoo. For this purpose, fruits, roots and aerial parts of the plant were extracted in methanol and subsequently fractionated in n-hexane, chloroform, ethyl acetate and1-butanol. Different assays were performed to investigate and compare the antioxidant and free radical scavenging potential of these extracts/fractions.

2. Materials and methods

2.1. Chemicals and equipment

All chemicals used were of analytical reagent grade. Rutin, ammonium molybdate, Folin-Ciocateu's reagent, Tween-20, ammonium thiocyanate, disodium phosphate, monosodium dihydrogen phosphate, trichloroacetic acid, linoleic acid, iron(III) chloride and iron(II) chloride were purchased from Merck (Germany); and 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, glacial acetic acid and tripyridyl-striazine (TPTZ) were purchased from MP Biomedicals (France). Absorbance readings were taken using UVD-3200 UV-vis spectrophotometer (Labomed, Inc., USA).

2.2. Collection of plant material

The plant material was collected from the desert area of Nasik District (India). Fruit, roots and aerial parts of the plant were manually separated, cut into small pieces, and left to dry at room temperature for one week. The dried parts of the plant were pulverized separately into fine powders which were used for extraction.

2.2. Preparation of methanol extracts

The dried powder of each part was soaked in methanol for 15 d with occasional shaking. The suspensions were filtered and the filtrate obtained was concentrated under reduced pressure using rotary evaporator. For fractionation, a part of the methanol extract of each part was suspended in water and extracted with solvents of increasing polarity to obtain hexane, chloroform, ethyl acetate, n-butanol and residual aqueous fractions. Each fraction was concentrated under reduced pressure and the residue obtained was used to make standard solution in respective solvent. All methanol extracts and the fractions were refrigerated at 4 °C until further analysis. Later, the methanol extracts and the fractions were subjected to different antioxidant and radical scavenging assays. All the measurements were taken in triplicate and the data are given as mean SEM for three determinations.

2.4. Total phenolic content (TPC) assay

The TPC of each part of the plant was estimated following an already reported procedure [14]. To 40 µL of the sample solution (3 mg methanol/fraction per 10 mL methanol), 3.16mL distilled water and 200 µL Folin-Ciocateu's reagent was added and mixed thoroughly. After incubation of 8 min, 600 µL of sodium carbonate solution was added and mixed. The mixture was then incubated at 40 °C for 30 min, and the absorbance readings were taken against a blank at 765 nm. The total phenols in these extracts were expressed as mg gallic acid equivalents (GAE) per gram of sample, using a calibration curve of a freshly prepared gallic acid solution.

2.5. Total flavonoid content (TFC) assay

The TFC in each part of the herb was determined following an already reported method [15]. To 0.3 mL of plant samples (3mg of each extract/fraction per 10 mL of methanol), 3.4 mL of 30% aqueous methanol was added followed by the addition of 150 µL NaNO2 solution (0.5 mol/L). After an interval of 5 min, 150 µL AlCl3 solution (0.3 mol/L) was added and mixed well. To this, 1 mL NaOH solution (1 mol/L) was added after an interval of 5 min. Finally, absorbance of the clear mixture was measured at 506 nm. TFC was estimated as µg of rutin equivalents (RE) per gram of sample using a calibration curve of rutin as a standard flavonoid

2.6. DPPH radical scavenging assay

The DPPH free radical scavenging activity of the fruit, root and aerial parts of D. Sissoo was determined following the method of Brand-Williams et al. with small modifications [16]. Ascorbic acid was used as a standard antioxidant. The working solution was prepared by diluting the DPPH stock solution (24 mg/100 mL methanol) with methanol to obtain an absorbance of (0.97依0.04) absorbance units at 516 nm. Briefly, in a test tube, 3 mL of the working solution was mixed with 100 µL of the plant extract/ fraction or the standard solution. The absorbance of the solution was then recorded at 516 nm for a period of 30 min. The percentage scavenging activity was calculated using the following formula:

Percent DPPH scavenging effect= {1-[DPPH] T=t/[DPPH]T=0}×100

Where $[DPPH]_{T=0}$ is the concentration of DPPH radical before reaction with antioxidant sample, $[DPPH]_{T=t}$ is the concentration of DPPH radical after reaction with antioxidant sample at time t. EC50 value for each part was also calculated using a concentration-response curve.

2.7. Phosphomolybdate assay

The phosphomolybdate antioxidant assay was carried out according to a reported procedure [17]. Briefly, 3 mL phosphomolybdate reagent was taken in a test tube, and to it, 300 µL of the plant extract/fraction or standard solution or methanol was added. The test tubes were capped with silver foil and incubated in water bath at 95 °C for 90 min. After the contents of the test tubes had cooled down, the absorbance of the test tube solution was measured at 765 nm against a blank. Ascorbic acid was used as a standard, and antioxidant activity was expressed as milligrams of ascorbic acid equivalents (mg AAE/g).

2.8. Ferric reducing antioxidant potential (FRAP)[16]

The so-called total antioxidant activity of each plant extract/fraction was measured by ferric reducing antioxidant power assay reported by Benzie and Strain [18].

The FRAP values of samples were expressed as µg/mL of AAE. Briefly, to 2.85 mL FRAP reagent, 150 µL plant extract/ fraction or standard antioxidant was mixed. The mixture was incubated for 30 min in dark after which its absorbance at 593 nm was noted.

2.9. Reducing power assay [17]

The reducing power assay was conducted according to the method of Oyaizu [19]. To 2.5 mL (1 mg/mL) of a plant extract/ fraction or standard solution, 2.5 mL sodium phosphate buffer (0.2 mol/L) and 2.5 mL potassium ferricyanide (1%) were mixed well. After incubating the mixture at 50 °C for 20 min, 2.5 mL trichloroacetic acid solution was added and the mixture was centrifuged at 650 r/min and 25 °C for 10min. The supernatant (5 mL) was mixed with 5 mL distilled water and 1 mL ferric chloride solution. The absorbance was measured at 700 nm. Gallic acid was used as standard antioxidant.

2.10. 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) decolorization assay [18]

The $ABTS^{\bullet+}$ decolorization assay was carried out according to the method developed by Re et al [20]. Stock solution of $ABTS^{\bullet+}$ was prepared by dissolving 0.038 g of $ABTS^{\bullet+}$ in 10 mL of distilled water, then 0.270 g of potassium persulfate was added in the solution. The stock solution of $ABTS^{\bullet+}$ was kept in dark for 18 h in order to generate deep colored radical containing solution. To 10 µL extract/fraction (1 mg/mL), 2.99 mL $ABTS^{\bullet+}$ working solution was mixed. The absorbance of the clear mixture was recorded at 734 nm after every 30 seconds for 8 min. Trolox was used as standard antioxidant. The percentage inhibition in the absorbance was measured using following formula:

Percent inhibition of ABTS at 734 nm= $(1-A_f/A_o) \times 100$

Where A_o and A_f are the absorbances at 734 nm before and after 8 min adding samples to 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) working solution, respectively.

2.11. Lipid peroxidation inhibitory assay

The lipid peroxidation value of various extracts was determined according to the method described by Mitsuda et al [21]. In a test tube, 100 µL of a plant extract was mixed with 2.4 mL of potassium phosphate buffer and 2.5 mL of linoleic acid emulsion. The mixture was incubated at 37 °C for 25 min. About 100 µL of this solution was regularly taken at 24 h intervals and dissolved in 3.7 mL of ethanol. Then it was reacted with 100 µL of ferrous chloride solution and then 100 µL of potassium thiocyanate solution was added and absorbance was measured at 500 nm. A 5 mL solution consisting of linoleic acid emulsion (2.5 mL) and potassium phosphate buffer (2.5 mL) was used as blank. Butylated hydroxyanisole was used as a standard antioxidant.[18]

3. Results

In case of aerial parts, the highest percent yield was observed for n-butanol 32.5% followed by aqueous 28.5%, ethyl acetate 22.5%, hexane 8.5% and chloroform fraction 7.8%. For roots, the highest percent yield 22.7% was calculated for hexane fraction which was followed by chloroform 22%, aqueous 21.9%, chloroform 17% and n-butanol 15.7%.

The TPC and TFC of the methanol extracts of roots and aerial parts of *Dalbergia sissoo* and their fractions in solvents of different polarity were determined and the results are shown in Figures 1 and 2. TPC and TFC values for methanol extracts and various fractions ranged from 0.23-4.30 mg/L GAE and from 30-545 mg/L RE, respectively. Overall, methanol extracts and all the fractions of root and aerial parts showed higher TPC and TFC values.[19]

Table No.1 Free radical scavenging activity as per DPPH assay of methanol extract of roots and aerial parts of D.sissoo and their fractions in different solvents and their EC_{50} Values

Sr.No.	Extract	Roots		Aerial Parts	
		% activity	EC_{50}	% activity	EC50
1	Methanol	43 ±0.02	11.00	34.00±0.01	09.0
2	Hexane	29±0.02	15.00	30.00±0.02	18.0
3	Chloroform	31±0.01	19.00	38.00±0.55	18.0
4	Ethyl acetate	37±0.03	17.00	35.00±0.02	16.0
5	n-butanol	26±0.04	19.00	30.00±0.12	11.0
6	Aqueous	31±0.02	12.00	33.00±0.07	09.0

The data are given as mean ±SEM for three determinations.

Methanol extracts and aqueous fractions of root and aerial parts and the n-butanol fraction of root showed lower EC50 values for DPPH scavenging than the other plant extracts.[20] A graphical comparison of DPPH scavenging ability of samples and their TPC and TFC are shown in Figure 3 to have a better view of any correlation between them.

Table 2 Lipid peroxidation inhibition activities (after 96 h of incubation) of various fractions of fruit, root and aerial parts of D. Sissoo

Sr.No.	Extract	Roots		Aerial Parts	
		% activity	EC_{50}	% activity	EC50
1	Methanol	43 ±0.02	11.00	34.00±0.01	09.0
2	Hexane	29±0.02	15.00	30.00±0.02	18.0
3	Chloroform	31±0.01	19.00	38.00±0.55	18.0
4	Ethyl acetate	37±0.03	17.00	35.00±0.02	16.0
5	n-butanol	26±0.04	19.00	30.00±0.12	11.0
6	Aqueous	31±0.02	12.00	33.00±0.07	09.0

Discussion

Dalbergia is a genus of 34 species which are confined to warm regions of almost all the continents. It has been reported to possess numerous phytochemical constituents, which have important role to prevent different types of diseases. In Pakistan, Dalbergia genus is present in D. Sissoo form and is known as Shisum It is a shrubby plant which is grown in the summer season.[21] Though Dalbergia genus is rich in phytochemicals, there are a very few reports on the antioxidant activities of this plant. The present study is the first ever study designed to explore a comprehensive antioxidant potential profile of fruit, root and aerial parts of D. Sissoo. The hydroxyl moieties present in the phenolic compounds are able to neutralize the unstable radicals by donating electron or hydrogen atom[22]. The TPC of the extracts were measured by Folin-Ciocalteu's reagent which is a mixture of phosphomolybdate and phosphotungstate. The reduction of tungsten and molybdenum oxides gives blue color to solution, which can be monitored spectrometrically at 765 nm. In the present investigation, the methanol extract of root showed the highest phenolic content (4.300$\pm$ 0.008) mg/ mL GAE. In case of fruit, n-butanol fraction exhibited the highest value of (3.20 $\pm$0.02) mg/mL GAE while in case of aerial parts, aqueous fraction superseded the other fractions with a value of (3.81$\pm$ 0.09) mg/mL GAE. The lowest TPC value (0.230$\pm$0.008) was found for n-butanol fraction of the aerial parts of the plant. The present study indicated an insignificant correlation between TFC and TPC of the extracts/fractions. This positive correlation between TPC and radical scavenging activity is also in line with the already reported facts that Folin-Ciocalteu's calorimetry method gives better correlation with the antioxidant assays [23]. TFC assay is also called aluminium chloride colorimetric method. TFC of methanol extracts and the fractions were calculated in accordance with an already reported procedure[24]. Hexane fraction of root showed the highest TFC value of 545.2 mg/mL RE. In fruit and aerial parts, the highest TFC values 200.1 mg/mL RE and 280.3 mg/mL RE were exhibited by aqueous fractions respectively. The lowest TFC value of 40.07 mg/mL RE was observed in case of n-butanol

In conclusion, the present study has indicated that in general, all the parts of D. Sissoo had high antioxidant and radical scavenging potential. In particular, the methanol extracts of root are more potent antioxidative and radical scavenging due to having high TPC and TFC as compared to other extracts/fractions of the fruit and aerial parts. Scavenging of DPPH and ABTS radicals, total antioxidant activity and FRAP assays also confirm greater potency of the methanol extract of roots. In general, extracts from all the parts showed considerable antioxidant and radical scavenging activity. However, the present study suggested that the crude methanol extract of root could be explored for further in vivo

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