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Research Article

IN VITRO ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF LEAF EXTRACTS OF *DALBERGIA SISSOO* (ROXB.)

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ABSTRACT

Oxidative stress is the state of imbalance between the level of antioxidant defense system and production of reactive oxygen species. Antioxidants are an ancillary defense against oxidative stress. Natural antioxidants such as polyphenols and flavonoids present in the plants scavenge harmful free radicals from our body. The aim of the present study was to determine total phenolic content and in-vitro antioxidant activity in methanolic and ethanolic leaf extract of *Dalbergia sissoo* known for its medicinal properties in folk medicine. The antioxidant activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. The Antioxidant activity was compared with ascorbic acid as standard antioxidant. Quantitative analysis of antioxidative components like total phenolic content was determined by spectrophotometric method and expressed in gallic acid equivalent. The ethanol extract had the highest total phenolic content followed by methanol. The ethanolic crude extracts showed very promising antioxidant activity compared with methanolic extract. The EC_{50} value for ethanolic extract was found to be 106.3µg/ml. The total phenolic content was found out to be 0.185±1.87mg/gm GAE. The findings of the present study suggested that polyphenols present in *Dalbergia sissoo* extract may be responsible for the antioxidant activity. *Dalbergia sissoo* (Roxb.) could be a potential natural source of antioxidants and could have greater importance as therapeutic agent in preventing or slowing oxidative stress related degenerative diseases. Further investigations need to be carried out to isolate antioxidant compounds present in the plant extract and evaluate in vivo antioxidant activity.

Keywords: *Dalbergia sissoo*, Total Phenolic Content, Oxidative stress, Antioxidants, DPPH Method.

INTRODUCTION

Oxidative stress occurs when the generation of free radicals and active intermediates in a system exceeds the system's ability to neutralize and eliminate them. It is the state of imbalance in pro-oxidant/antioxidant homeostasis that leads to the generation of toxic reactive oxygen species (ROS). Oxidative metabolism is essential for the survival of cells. A side effect of this dependence is the production of free radicals and other reactive oxygen species that cause oxidative changes. [1, 2] Free radicals are chemical species, which contain one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. The family of free radicals generated from the oxygen is called reactive oxygen species (ROS). ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (O_2^-) and hydroxyl radicals ($OH\cdot$), as well as non free radicals (H_2O_2) and singlet oxygen. There is increasing evidence for the involvement of such species in a variety of normal in vivo regulatory systems. Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (ROS) now appears the fundamental mechanism underlying a number of human neurodegenerative disorders, diabetes, inflammation, viral infections, autoimmune pathologies, cardiovascular disorders and digestive system disorders .When an excess of free radicals is formed, they can overwhelm protective enzymes such as superoxide dismutase, catalase and peroxidase and cause destructive and lethal cellular effects (*e.g.*, apoptosis) by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration. [3-5]

The most effective way to eliminate free radicals that cause oxidative stress is the use of natural antioxidants present in the plants. that scavenge harmful free radicals from our body.[6] Although the mammalian body has certain defense mechanisms to combat and reduce oxidative damage, epidemiological evidence indicates that the consumption of foodstuffs containing antioxidant phytonutrients notably flavonoids and other polyphenolics is advantageous for our health.[7] Antioxidants are an ancillary defense against oxidative stress. Antioxidant means "Against oxidation". Antioxidants work to protect lipids from peroxidation by free radicals. Antioxidants delay or prevent the oxidation of a given substrate by free radicals.[8] Many plants with antioxidant potential possess flavonoids and Phenolic compounds.[9-12] There is currently immense interest in natural antioxidants and their role in human health and nutrition. Several medicinal plants (Rasayana) have also been extensively used in the Indian traditional (Ayurveda) system of medicine for the treatment of number of diseases.[13] However, majority of plants have not yet been screened for such activity.

The Indian medicinal plant *Dalbergia sissoo* belongs to family Fabaceae is popularly known as Indian rosewood tree. The genus consists of 300 species among which 25 species occur in India. *Dalbergia sissoo* is a medium to large tree of about 25 meters high with grey-yellow trunk, longitudinal crack, and downcast twig. Leaves are leathery, pinnately compound, with about five alternate leaflets. Leaf stalk (petiole) measures about 15 cm long, each leaflet widest at the base, to 6 cm long with a

fine pointed tip. Flowers are whitish to pink, fragrant, nearly sessile, up to 1.5 cm (0.59 in) long and in dense clusters 5–10 cm (2.0–3.9 in) in length. The plant has ability to fix nitrogen from the atmosphere through bacteria located in nodules present in the root system.

All parts of the *Dalbergia sissoo* are traditionally used in treating different diseases. Traditionally used for aphrodisiac, abortifacient, expectorant, anthelmintic and antipyretic, emesis, ulcers, leucoderma, dysentery, stomach troubles and skin disease.[14, 15] So, in order to contribute further to the knowledge of Indian traditional plants, our present study is focused on *Dalbergia sissoo* to determine their antioxidant and free radical scavenging properties. The literature survey showed scanty information available on these plants and thus prompted us to analyze these common Ayurvedic plants. The in-vitro antioxidant activity has been reported in bark of the plant.[16] Our interest was to find out natural antioxidant from the extract of leaves of *Dalbergia sissoo* using in vitro antioxidant assays.

MATERIALS AND METHODS

Collection of Plant Material

The plant material required for the experiment was collected from mandsaur district in the month of May in summer. The collected species was identified and authenticated by Dr. S.N. Mishra, Principal scientist (MAP) KNK College of Horticulture Mandsaur (M.P.)

Preparation of Powder

The disease free plant leaves were collected and dried under shade. These dried leaves were pulverised to get coarse powder, sieved using 40 mesh and stored in an airtight container. The powdered material was used for further investigation.

Chemical Reagents

All chemicals and reagents used were of analytical grade or purest quality. Methanol, gallic acid, folin coicalteu's reagent, sodium carbonate (Na_2CO_3), distilled water, ascorbic acid and DPPH were procured from store department of B.R. Nahata College of Pharmacy, Mandsaur (M.P.), India.

Extraction of Plant Material

200g of the coarse powder material was weighed and extracted with methanol and ethanol by cold maceration for 24 hours with occasional shaking. The extracts were then filtered, concentrated and dried.

Phytochemical Screening

Phytochemical examinations were carried out for all the extracts as per the standard methods. Phytoconstituents such as alkaloids, carbohydrates, glycosides, saponins, phenols, tannins, flavonoids, proteins and amino acids were determined.[17]

Quantitative Determination of Total Phenolic Content (Folin-Ciocalteu Assay)

Plant polyphenols, a diverse group of phenolic compounds (flavanols, flavonols, anthocyanins, phenolic acids, etc.) possess an ideal structural chemistry for free radical scavenging activity. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain breaking function) and from their potential to chelate metal ions.Total phenolic content of extract were determined using folin-ciocalteu assay. Gallic acid was used as a standard.[18-20]

Preparation of Standard Solution

Stock solution of gallic acid (1mg/ml) was prepared by dissolving 100mg of accurately weighed gallic acid in methanol and volume was made up 100ml with methanol in a volumetric flask. The aliquots (0.1ml, 0.2ml, 0.3ml, 0.4ml, 0.5ml and 0.6ml) of stock solution were transfer to 10ml volumetric flask and volume of each was adjusted to 10ml with methanol, to obtain standard solution containing 10µg/ml, 20 µg/ml, 30µg/ml, 40µg/ml, 50 µg/ml and 60µg/ml of gallic acid respectively. The reaction samples were prepared by mixing 0.5ml methonolic solution of each concentration, 2.5ml of 10% folin-ciocalteu reagent and 2.5ml of 20% sodium bicarbonate dissolved in water. Blank was prepared containing 0.5ml methanol, 2.5ml of 10% folin-ciocalteu reagent, 2.5ml of 20% Na_2CO_3 solution and 5ml of methanol. The standard solutions were then incubated in a thermostat at 40ºC for 30 minutes; the absorbance was determined using UV spectrophotometer at λ max 765nm. Calibration curve was prepared by plotting absorbance against concentration.

Preparation of Sample Solution

100 mg of the extract of the sample was weighed accurately and dissolved in 100 ml of methanol. The reaction samples were prepared by mixing 0.5ml methonolic solution of each extract, 2.5ml of 10% folin-ciocalteu reagent and 2.5ml of 20% sodium bicarbonate dissolve in water. Blank was prepared containing 0.5ml methanol, 2.5ml of 10% folin-ciocalteu reagent, 2.5ml of Na_2CO_3 and 5ml of methanol. The test solutions were then incubated in a thermostatic at 40ºC for 30 minutes, the absorbance was determined using UV spectrophotometer at λ max 765nm. These data were used to estimate the total phenolic content in different solvent leaf extracts using a standard calibration curve of gallic acid. The results were expressed as gallic acid equivalent (GAE).Three replicates were made for each sample.

The concentration of total phenolic compounds in the extract was determined by using the formula:

$$T=CV/M$$

Where, T= Total phenolic content mg/gm of plant extract in GAE,
C= Concentration of gallic acid from the calibration curve,
V= volume of the extract in ml,
M= weight of the pure plant methanol extract.

In-Vitro Antioxidant Activity-DPPH Free Radical Scavenging Assay

The antioxidant activity of methanolic and ethanolic leaf extract was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. This method is based on the reduction of DPPH in methanolic solution in the presence of a hydrogen–donating antioxidant. This results in the formation of the non radical form DPPH-H. The molecule 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize. The delocalization of electron also gives rise to the deep violet color, characterized by an absorption band in methanolic solution centered at about 517 nm. When a solution of DPPH is

mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color (Figure 1), which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517nm. The intensity of the yellow colour depends on the amount and nature of radical scavenger present.[21]

DPPH Method

DPPH solution (0.2mM) was prepared in methanol. From the stock solution of the extract (1mg /ml) different dilutions were prepared using methanol to get solutions of concentration 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml, 250 µg/ml and 300µg/ml. From each dilution 1ml of solution was mixed with 2ml of DPPH solution in test tubes. The absorbance of the solutions were determined using UV spectrophotometer after 10 minutes at 517 nm. Ascorbic acid was used as a standard. [22] The tests were carried out in triplicate. Percent inhibition of scavenging of the DPPH free radical was measured using the equation-

% DPPH radical scavenging activity = (Absorbance of Control – Absorbance of Sample) x 100 / (Absorbance of Control)

The extract concentration providing 50% inhibition (EC_{50}) was calculated from graph of % inhibition plotted against concentration.

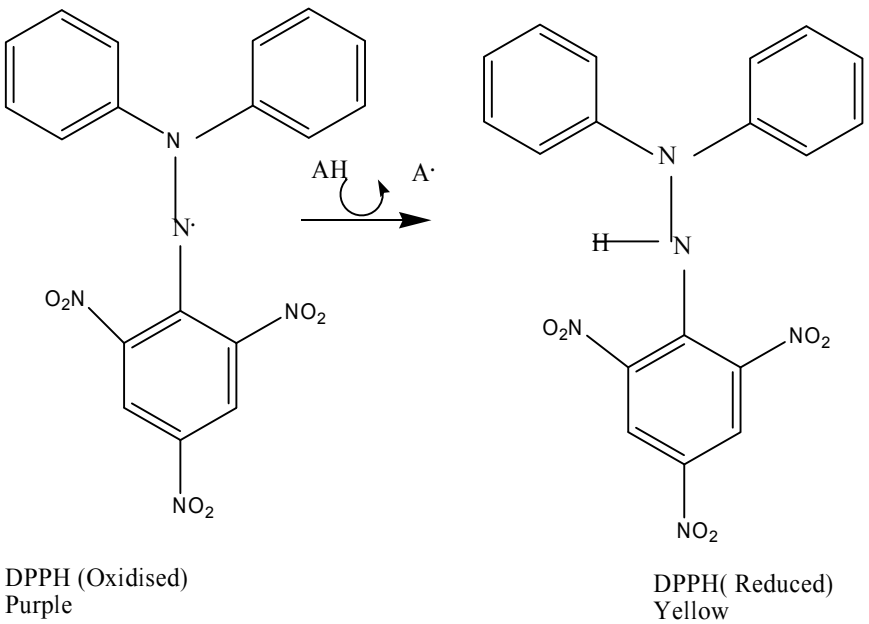

Figure 1 DPPH Radical

Table 1: Phytochemicals Detected in Leaf Extract of *Dalbergia sissoo*

Phytochemicals	Test name	Presence/Absence	
		Methanolic extract	Ethanolic extract
Alkaloids	Mayer's Test	-	-
	Mayer's Test	-	-
	Dragendorff's Test	-	-
	Hager's Test	-	-
Carbohydrates	Molisch's Test	+	+
	Benedict's Test	+	+
	Fehling's Test	+	+
Glycosides	Modified Borntrager's Test	+	+
Saponins	Froth Test	+	+
	Foam Test	+	+
Phenols	Ferric Chloride Test	+	+
Tannins	Gelatin Test	+	+
Flavonoids	Alkaline Reagent Test	+	+
	Lead acetate Test	+	+
Proteins and Amino acids	Xanthoproteic Test	+	-
	Ninhydrin Test	+	-

'+' Indicates present; '_' Indicates absent

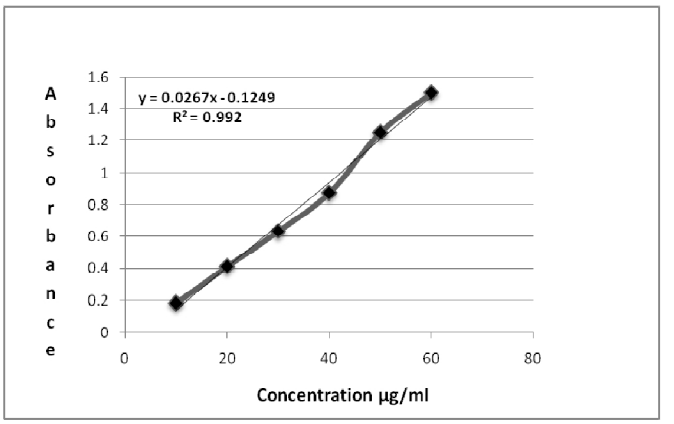

Figure 2: Standard Curve of Gallic Acid

Table 2: Total Phenolic Content in Extracts of *Dalbergia sissoo* Leaves

S.NO.	Extracts	Absorbance at 765 nm	Concentration GAE (mg/g)
1	Ethanol	0.851	0.185 ±0.035
2	Methanol	0.823	0.182 ±0.007

Table 3: EC_{50} Value

Extract	EC_{50} (µg/ml)
Ethanolic	106.3
Methanolic	815.53

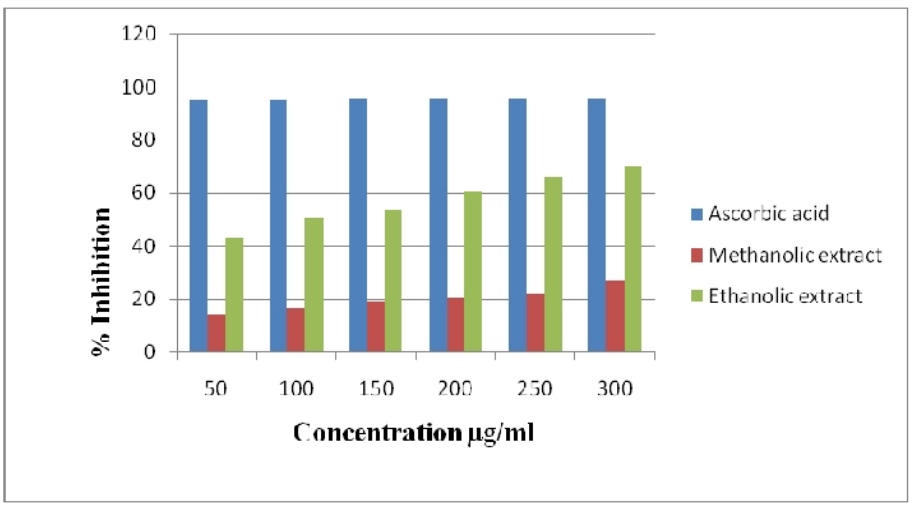

Figure 3: Comparison of In-Vitro Antioxidant Activity of *Dalbergia sissoo* Leaf Extracts with Standard Ascorbic acid

RESULTS AND DISCUSSION

Phytochemical Screening

The results of the preliminary phytochemical screening are presented in Table 1. The methanolic extract showed the presence of almost all major secondary metabolites such as carbohydrates, flavonoids, proteins, amino acids, glycosides, tannins, phenolics, saponins except alkaloids. Phenolics and flavonoids are the natural compounds which can influence the nutritive value of different foodstuffs used by humans. These phytoconstituents are known for their antioxidant properties.[23]

Total Phenolic Content

Many plant extracts have been reported to have multiple biological effects, including antioxidant properties due to their phytoconstituents including phenolics. The antioxidant activity of phenolics is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals.[24] Leaf extracts of *Dalbergia sissoo* are rich in phenolic content. Total polyphenol content was estimated with folin-ciocalteu colorimetric method. This reagent is a mixture of phosphotungstic and phoshomolybdic acids. It is reduced during the oxidation of phenols to a mixture of blue oxides of tungsten and molybdenum. The color produced, whose absorption is maximum between 700 and 750nm, is proportional to the amount of polyphenols present in plant extracts. The total phenolic contents were reported as mg of gallic acid equivalent per gram of dry extract. The total phenolic content was calculated using calibration curve of gallic acid shown in Figure 2. The ethanoilc crude extract of *Dalbergia sissoo* leaf shown a high phenolic content compared to methanolic extract, as presented in Table 2. On the basis of the fact that phenolics are

known for their ability to trap free radicals, it appeared important to us to evaluate antioxidant property of methanolic and ethanolic extract.

In-Vitro Antioxidant Activity

The in-vitro antioxidant activity of methanolic and ethanolic leaf extract of *Dalbergia sissoo* was evaluated by DPPH method using ascorbic acid as standard. The model of scavenging the stable DPPH radical is widely used method to evaluate free radical scavenging ability of various samples.[25] The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron or hydrogen donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The readings in the Figure 3 shows that ascorbic acid has potent antioxidant effect. These results are in agreement with the findings of Hasan *et al.* (2009) and Raghavendra *et al.* (2013) who reported that ascorbic acid has antioxidant activity evaluated by DPPH method.[26, 27] All the tested samples (ethanolic & methanolic extracts) revealed scavenging effects on DPPH presented in Figure 3. The EC_{50} values are shown in Table 3. All the leaf extracts of *D.sissoo* exhibited a significant dose-dependent inhibition of DPPH activity. However activity shown by ethanolic extracts was more than methanolic extract. The EC_{50} for Ethanolic extract was 106.32µg/ml and for Methanolic extract was 815.53 µg/ml. The antioxidant activity of the leaf extract were shown in the following order: ascorbic acid >ethanolic extract >methanolic extract.

CONCLUSION

The results of the present study revealed that an ethanolic extract of *D.sisoo* had the high phenolic content and exhibits high scavenging power activities compared to methanolic extracts. The in vitro assay indicates that *Dalbergia sissoo* (Roxb.) could be a potential natural source of antioxidants and could be used as a natural additives in food and pharmaceutical industries. Further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the in vivo antioxidant activity of this extract needs to be assessed prior to clinical use.

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