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RESEARCH ARTICLE

EVALUATION OF ANTIOXIDANT ACTIVITY OF DIOSPYROS MELANOXYLON STEM EXTRACTS

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ABSTRACT

The present study was designed to identify antioxidant potential and phenolic content of *Diospyros melanoxylon* stem extracts. The antioxidant activity of the extracts was assessed by using various *in-vitro* tests such as DPPH, ABTS, and phosphomolybdenum assay. Among the all extracts in present study, the methanolic extract of *Diospyros melanoxylon* stem produced potent antioxidant activity with significant polyphenolics content. Thus, it is clearly indicated that the *Diospyros melanoxylon* stem can be used as potential source of natural antioxidant.

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INTRODUCTION

Free radicals have one or more unpaired electrons react easily with reactive oxygen species (ROS) to become radicals themselves. The excessive generation of ROS, induced by various stimuli and when exceed the endogenous antioxidant capacity of the organism, leads to a variety of pathophysiological conditions such as inflammation, diabetes, genotoxicity, and cancer. [1] As a result of this, much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation and to protect from damage due to free radicals. Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. [2] Recently, there have been great efforts to find safe and potent natural antioxidants from various plant sources. Plants have been used for several years as a source of traditional medicine to treat various diseases and conditions. [3] *Diospyros melanoxylon* is a member of the Ebenaceae family. The *Diospyros* is a genus of about 450-500 species of deciduous and evergreen tree. The majority are native to the tropics, with only a few species extending into temperate regions. They are commonly known as ebony or persimmon tree. During the survey of medicinal plants of The Similipal Biosphere Reserve, Mayurbhanj district of northeast Orissa, India Thatoi et al.38 reported that leaves and barks of *D. melanoxylon* used as diuretic, laxative, carminative, urinary and skin trouble and styptics. The decoction of bark used in diarrhea and dyspepsia; while dried Flowers used for urinary problems and skin diseases.

Shina and Bansal reviewed the phytochemical and Biological Studies of *Diospyros* species used in folklore medicine of Jharkhand[4]. Recently the antimicrobial activity[5], antidipogenic, hypolipidemic and antidiabetic[6] potential of bark were investigated. Despite used as medicinal plant, the stem of D. *melanoxylon* was not explored on modern scientific line. Thus, the present study was aimed to investigate the *in vitro* antioxidant activity of successive extracts of *D. melanoxylon* stem.

EXPERIMENTAL

Collection and extraction: *Diospyros melanoxylon* (DM) stem were freshly collected from the Mahur-Kinwat region of Nanded district, Maharashtra. The plant was authenticated by Dr. Vishal R. Marathe, Science College, Nanded, where a herbarium voucher specimen was deposited. The plant material was air-dried on the laboratory bench for five days and then ground to coarse powder and extracted with increasing polarity solvents with the help of soxhlation. The petroleum ether (60-80º) (PE-DM), chloroform (CH-DM), ethyl acetate (EA- DM), and ethanol (EO- DM) extracts successively were collected. The extracts were qualitatively evaluated for presence of different secondary metabolites using standard qualitative chemical tests. All the extracts were filtered, concentrated in a rotary evaporator and stored in desiccators for further use. Estimation of total phenolic content. Total phenolic content was estimated using the Folin-Ciocalteu method [7]. Test extract (100 µl) were mixed

thoroughly with 2 ml of 15% Na_2CO_3. After 2 min. 200 µl of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 760 nm against a blank. The standard calibration curve was prepared using gallic acid. Total phenolic content was expressed milligram of gallic acid equivalents (GAE) per gram of dried extract. It was calculated by the following formula:

$$T = \frac{C \times V}{M}$$

Where, T = Total phenolic compounds (mg/g of plant extract) in GAE; C = Concentration of gallic acid established from the calibration curve (mg/ml); V = Volume of extract (ml); M = Weight of plant extract (g)

Estimation of total flavonoids content: The flavonoids content was determined according to Kalaskar and Surana [8]. The different concentrations (20-100 µg/ml) of standard quercetin solutions (0.5 ml) were separately mixed with 1.5 ml of ethanol, 0.1 ml of 10% aluminium nitrate, 0.1 ml of 1 M sodium acetate and 2.8 ml of water. The resultant mixture was kept at ambient temperature for 40 min. The absorbance of reaction mixture was measured at 415 nm; calibration curve was plotted for concentration against absorbance. Same procedure was followed for the extracts. In the blank solution, the volume of 10% aluminium nitrate was substituted with the same volume of distilled water. The total flavonoid content in the extract expressed as milligram per gram of quercetin equivalents (QE) with formulae as mentioned for total phenolic content.

Antioxidant activity

DPPH radical-scavenging activity: Radical scavenging activity of DM stem extracts against stable DPPH was done according to the method of Ebrahimzadeh with minor modifications [9]. Different concentrations of each DM stem extracts were added, to an equal volume, methanolic DPPH (100mM) solution. Each of the extract or the reference standard solution was added separately in wells of the micro-titre plate. After 20 min at room temperature, the absorbance was measured at 517 nm using microplate spectrophotometer (BIO-Tek, USA. Model-96 well micro plate). Same procedure was followed for control by using methanol in place of extract. The percentageinhibition was estimated based on the percentage of DPPH radical scavenged using the following formula:

% Inhibition = [(Control absorbance – Sample absorbance) / (Control absorbance)] × 100

ABTS radical cation scavenging activity: ABTS radical cation scavenging activity was performed using the method reported by Fellegrin with slight modifications [10]. In brief, ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and kept overnight in the dark to yield a dark colored solution containing $ABTS^{\cdot+}$ radical cation. Prior to use in the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.700 at 734 nm. After the addition of 1.0 ml of diluted $ABTS^{\cdot+}$ to 10 µl of DP stem extract and standard incubated for 5 min and absorbance was measured at 734 nm. The percentage inhibition was calculated according to the formula used for DPPH activity. The antioxidant potential of extracts was expressed as IC_{50}, the concentration necessary for a 50% reduction of DPPH and $ABTS^{\cdot+}$ radicals.

Total antioxidant capacity by phosphomolybdenum method: The total antioxidant capacity of DM stem extracts were evaluated as reported by Saleh and Hameed [11]. An aliquot of 100 µl of extractsolutions were combined with 1 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). All tubes were capped and incubated in a boiling water bath at 95°C for 90 min. Tubes were allowed to cool at room temperature. Absorbance of the test and standard solutions was measured at 695 nm against blank containing 0.1 ml of distilled water and 1 ml of reagent. The standard curve for total antioxidant capacity was plotted using ascorbic acid standard solution (20-100 µg/ml) following aftersaid procedure. An antioxidant capacity was expressed as milimolar equivalents of ascorbic acid.

RESULTS AND DISCUSSION

Total phenolic contents (TPC) for DM stem extracts, obtained sequentially in different solvents, were determined spectrophotometrically using gallic acid as calibration standard. While Total phenolic contents (TFC) for DM stem extracts were determined spectrophotometrically using quercetin as calibration standard.

The TPC values varied over a wide range, *i.e.*, 3.03 – 33.64 mg GAE/g, among the extracts prepared in different solvents (Table 1). Highest TPC (33.64 mg GAE/g extract) was observed for ethanolic extract when compared with other solvents. The findings reveal that most of the phenolic compounds in DM stem are polar in nature, and thus more efficiently extractable by polar solvents. The highest yield in ethanol may be attributed to the chemical structure of phenolic compounds, which contain one or more hydrophilic hydroxyl groups. Moreover, the findings are in agreement with the observations of Matthaus, [12] who reported the high efficiency of polar solvents, *i.e.*, water and methanol. Total flavonoid contents ranged over 3.94–14.55mg QE/g extract (Table 1). The TFC was observed asPE-DMS < CH-DMS < EO-DMS < EA-DMS (Table 1). Highest TFC was recorded for ethyl acetate extract, while the lowest was for petroleum ether extract. Kalaskar and Surana [8] have reported the similar finding.

Table 1. Total phenolic and flavonoid content of *D. melanoxylon* stem extracts

Extracts	Total phenolic(mg/g gallic acid)	Total flavonoid(mg/g quercetin)
PE-DMS	3.03 ± 1.39	3.94 ± 0.52
CH-DMS	14.24 ± 1.39	6.97± 0.52
EA-DMS	20.61 ± 1.89	14.55 ± 0.91
EO-DMS	33.64 ± 0.91	12.12 ± 1.39

(n=3)

The DPPH and ABTS radicals are soluble in water and organic solvent, thus enabling thedetermination of antioxidant capacity of both hydrophilic and lipophillic compounds [13]. In DPPH radical-scavenging assay, radical-scavenging activity of the extracts from DM stem was estimated by comparing IC_{50} of the extracts andthose of ascorbic acid (Table 2).

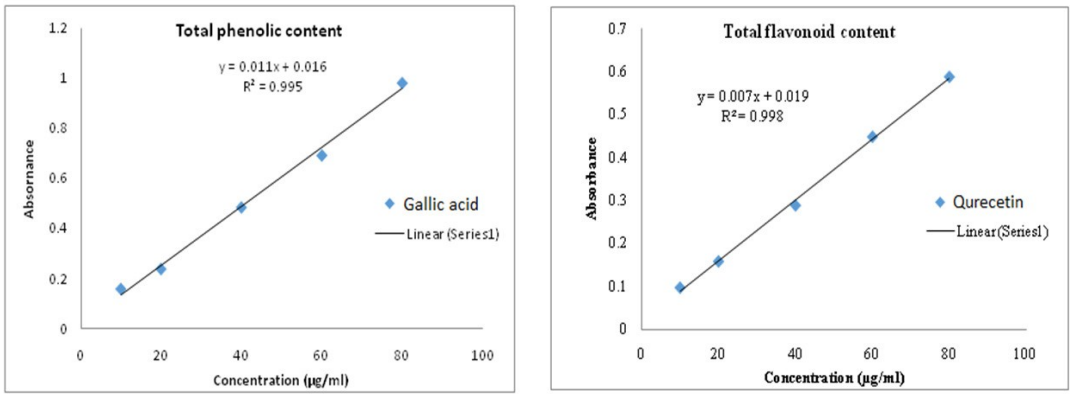

Figure 1. Calibration curve of standard gallic acid and quercetin

Table 2. Antioxidant effect (IC_{50}) on free DPPH radicals of *D. melanoxylon* stem extracts

Conc. of Sample (µg/ml)	PE-DMS	CH-DMS	EA-DMS	EO-DMS	As. Acid	Conc. of Std (µg/ml)
40	7.91	24.19	26.98	33.02	17.21	10
80	14.88	30.70	40.47	43.26	29.30	20
120	25.12	45.58	54.88	56.74	39.07	40
160	31.16	55.81	58.60	62.33	52.56	60
200	40.90	58.60	61.86	63.26	61.86	80
IC_{50} (µg/ml)	247.30	150.47	127.03	111.82	72.28	

Table 3. Antioxidant effect (IC_{50}) on ABTS radicals of *D. melanoxylon* stem extracts

Conc. of Sample (µg/ml)	PE-DMS	CH-DMS	EA-DMS	EO-DMS	As. Acid	Conc. of Std (µg/ml)
40	26.40	40.67	44.67	43.07	40.27	10
80	35.73	46.93	51.07	49.87	46.40	20
120	44.93	54.00	57.20	58.13	53.73	40
160	50.53	58.53	60.13	63.47	62.13	60
200	55.73	61.47	63.20	66.13	66.13	80
IC 50 (µg/ml)	247.30	150.47	74.70	79.13	31.94	

The observed differential scavenging activities of the extracts against the DPPH system could be due to the presence of different compounds in the extracts. The IC_{50} value for ethanol extract was 111.82, while petroleum ether extract had IC_{50} of 247.30. The IC_{50} of ascorbic acid was found as 72.28. A higher DPPH radical- scavenging activity is associated with a lower IC_{50} value. The scavenging ability of DPPH was in descending order from ethanol > ethyl acetate > chloroform > petroleum ether extract. All extracts from DM stem exhibited ABTS radical-scavenging activities to different extents in a concentration- dependent manner; although the activity levels of all of the tested samples were lower than that of ascorbic acid. Among all extracts, ethyl acetate and ethanol extract from DM stem exhibited the highest ABTS radical scavenging activity (IC_{50}) i.e. 74.70 and 79.13, respectively. In contrast, petroleum ether extract showed least ABTS radical scavenging capacity i.e. 247.30. The similar result was produced by ascorbic acid nearly at concentration 31.94 µg/ml (Table 3). The ABTS radical scavenging ability of samples can be ranked as ethyl acetate > ethanol > chloroform > petroleum ether extract. The DPPH and ABTS radical scavenging activity of the ethanol and ethyl acetate extract revealed high antioxidant activity, the possible reason might be the different contents and sorts of bioactive compounds including phenolics and other compounds responsible for antioxidant capacity. These results were consistent with the findings of many research groups, who reported such correlations between total phenolic content and free-radical scavenging activity. [14, 15]

The phosphomolybdenum assay is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity). The total antioxidant capacity of DM stem extracts was determined with reference to ascorbic acid (Figure 2). Total antioxidant capacity for ethanol and ethyl acetate extract were 11.01 and 6.87 mg of ascorbic acid equivalent/g of dry extract (AAE) respectively. While petroleum ether extract had less total antioxidant capacity i.e. 1.82 mg/g AAE (Table 4). The total antioxidant capacity of different extracts can be ranked in descending order as: ethanol > ethyl acetate > chloroform > petroleum ether extracts (Table 4). The ethanol extract demonstrated electron-donating capacity showing its ability to reduce Mo (VI) to Mo (V) and forms a green colour phosphomolybdenum V complex and act as chain terminators. Thus, transforming relative free radical species into more stable non-reactive products. The results obtained in this investigation reveal that the total antioxidant activity may be attributed to the presence of phenolics and flavonoids constituents in ethanol extracts. [16]

Table 4. Antioxidant effect (IC_{50}) on total antioxidant capacity of *D. melanoxylon* stem extracts

Extracts	Total antioxidant capacity
PE-DMS	1.82 ± 0.08
CH-DMS	2.51 ± 0.80
EA-DMS	6.87 ± 1.05
EO-DMS	11.01 ± 1.05

(n=3)

All *in vitro* antioxidant assay shown good correlation with total phenolic and flavono Iid content of extracts, which indicates the antioxidant activity of DM stem is due to polyphenolic compounds. The ethanol extract showed most potent antioxidant activity, because of presence of phenolic compounds, particularly flavonoids, hydrolysable and condensed tannins due to the presence of the hydroxyl groups.

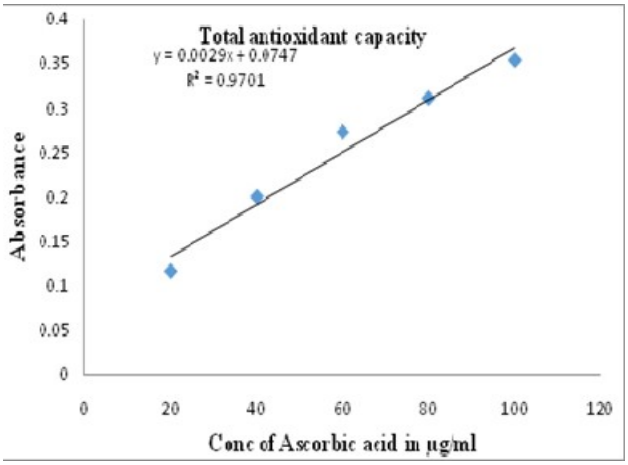

Figure 2. Standard calibration curve of ascorbic acid

CONCLUSION

The present investigation reveals that ethanol extracts of DM stem exhibit high antioxidant capability. This activity is attributed to high stem of total phenolic and flavonoid compounds. Consequently, our results suggest that the extract can be utilized as an effective and safe antioxidant source, although the antioxidant activities of ethanol extract was lower than that of ascorbic acid. It can be concluded that, stem of *D. melanoxylon* used in folklore practice for variety of diseases, can be used as an accessible source of natural antioxidants with consequent health benefits. Further scientific work is needed to ensure the other medicinal properties of the plant in correlation to antioxidant activity.

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