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Antioxidant Activity of Isolated Phytoconstituents from *Casuarina equisetifolia* Frost (Casuarinaceae)

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Abstract: The aim of the present study was to isolate the active constituents responsible for antioxidant activity. Radical scavenging activities of chromatographically isolated compounds from methanolic extracts of wood, bark, fruit and leaf were measured by the 1, 1-Diphenyl-2- Picrylhydrazyl (DPPH) method. The structures of isolated compounds were confirmed by spectroscopic techniques comprising of UV, IR, ¹³C NMR, P-NMR, Mass spectral and Co-TLC studies. The compound ANA 01, ANA 02 and ANA 04 were isolated from bark and confirmed as catechin, ellagic acid and gallic acid, respectively. The leaf extract resulted in separation of compounds ANA 03 (quercetin). The free radical scavenging activity of the different isolated compounds from methanolic extracts of *Casuarina equisetifolia* increased in a concentration dependent manner. ANA 04 (gallic acid) exhibited very strong antioxidant activity and when compared to ANA 01 (catechin), ANA02 (ellagic acid), ANA 03 (quercetin) and ANA05 (lupeol). This study suggests that the *Casuarina equisetifolia* could be pharmaceutically exploited for antioxidant properties.

Key words: *Casuarina equisetifolia*, antioxidant activity, free radical, DPPH

INTRODUCTION

Casuarina equisetifolia (Casuarinaceae) is handsome tree with drooping branches, 10-50 m high (Mhaskar *et al.*, 2000), it is found in dry hill sides and open forests of India, Sri Lanka and to Australia.

The following phytoconstituents have been isolated from the plant so far; kaempferol, quercetin (EL-Ansari *et al.*, 1977), alicyclic acids (shikimic and quinic acid), amino acids (Madhusudanamma *et al.*, 1978), taraxerol, lupenone, lupeol, gallic acid, sitosterol (Rastogi and Mehrotra, 1998), catechin and gallocatechin (Roux, 1957; Madhulata *et al.*, 1985).

The plant is used as astringent (Mhaskar *et al.*, 2000), diarrhea (Chopra *et al.*, 1956), dysentery, cough, ulcers, toothache, lotion for swelling (Wealth of India, 1992) and diabetes (Prajapati *et al.*, 2003). Pharmacological investigations of bark and wood showed significant anticancer and anthelmintic activities (Aher *et al.*, 2006, 2008).

The biological activities, *viz.*, anticancer, antibacterial (Wealth of India, 1992) and hypoglycemic, antifungal (Han, 1998) of the leaf has been reported.

The DPPH assay was used in the measurement of scavenging ability of isolated test compounds. The DPPH radical is reduced in the presence of an antioxidant molecule, the electron becomes paired off showing the color change from blue to uncolored methanol solutions stoichiometrical; depending on the number of electron taken up (Sundararajan *et al.*, 2006; Choudhary, 2006).

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The phenolic phytoconstituents isolated in earlier studies prompted to evaluate antioxidant potential of *Casuarina equisetifolia*.

MATERIALS AND METHODS

Authentication of Plant Material

The plant specimen was collected from Gangapur dam locality, Nashik (M.S.) identified as *Casuarina equisetifolia* Linn. Family Casuarinaceae, Voucher No. ANA1, Ref. No. BSI/WC/Tech./2005/867 dated 22.12.2005 by P.S.N. Rao, Joint Director, Botanical Survey of India, Pune (M.S.).

Preparation of Plant Extracts

Coarsely powdered materials of leaf and bark (100 g each) were subjected to reflux with 250 mL of methanol for 4 h coded as MEL and MEB followed by subsequent filtration and evaporation to yield extract.

Separation and Isolation of Phytoconstituents by Chromatographic Methods

Gradient fractionation of acetone soluble part from methanolic bark extract by column chromatography was performed by the using of toluene: ethyl acetate: methanol (4:3:3) followed by methanol resulting in the isolation of compound designated as ANA 01(catechin), ANA 04 (gallic acid) and ANA 02(ellagic acid).

Methanolic extract of leaf was chromatographed with toluene: ethyl acetate: formic acid (5:4:1), followed by spraying with ferric chloride. The presence of quercetin was evidenced in UV light. The methanolic extract of leaf was processed by PTLC to isolate compound coded as ANA 03 (quercetin).

Characterization of Isolated Compounds

The isolated compounds were characterized with help of physical properties and the instrumental techniques.

Characterization of Compound ANA01

UV (MeOH) max: 219, 279 nm.

FT-IR: (KBr): 3820.10, 3391.85 (O-H), 2933.20, 2892.27, 1627.56 (C=O), 1522.37, 1471.52, 1289.96 (C-O-C), 1245.99, 1198.02 (C-O) and 868.70 cm^{-1} .

LC-ESI-MS: m/e: 290 (5%, M^+ , $\text{C}_{15}\text{H}_{16}\text{O}_6^+$), 289 (12%, M^+-1), 245 (32%, $\text{M}^+-\text{CH}_3\text{O}_2$), 205 (100%, $\text{M}^+-\text{C}_2\text{H}_6\text{O}_4$), 203 (40%) and 179 (5%).

^{13}C -NMR (DMSO, 300 MHZ): δ 156.59(C-7), 156.33(C-5), 155.51(C-3'), 144.99(C-4'), 130.75(C-8), 118.64(C-6), 115.26(C-6'), 114.64(C-9), 99.23(C-10), 95.27(C-5'), 94.02(C-2'), 81.12(C-3), 66.45(C-2) and 27.98(C-4).

^1H NMR (DMSO, 300 MHZ): δ 9.01(s), 6.72(1H,H-6,8,s), 6.68(1H,H-2',5',s), 6.61(1H,H-6',s), 5.88(s), 5.70(1H,H-5,7,s), 4.92(1H,H-3',4',s), 4.48(1H,H-3,s), 3.56(1H,H-3,s), 2.39(s),2.37(s) and 2.64(2H,H-4,d).

Characterization of Compound ANA02

UV (MeOH) max: 365.0, 254.0 nm.

FT-IR: (KBr): 3072.72, 3596.20(O-H), 1699.60(C=O), 1622.21(C=C), 1581.82, 1509.10, 1447.52, 1398.43(C-O), 1195.11 and 882.06 cm^{-1} .

LC-ESI-MS: m/e: 301.8(10%, M⁺-C₁₄H₆O⁺), 300.7(10%), 283.8(40%), 271.9(4%), 256.8 (50%, M⁺-H₂O+O₂), 228.9(100%, M⁺-CH₃O₄), 212.9(8%), 200.9(30%) and 184.9(60%).

¹³C-NMR (C₆D₂N, 300MHZ): δ 164.23(C-7,7'), 142.09(C-2,3,2',3'), 124.48(s), 123.11 (C-4,5,6,4',5',6') and 111.92(C-1,1').

¹H-NMR (C₆D₂N, 300 MHZ): δ 8.14(1H,H-1',s) and 5.28(1H,H-2,3,2',3',s).

Characterization of Compound ANA03

UV (MeOH) max: 255, 372 nm.

FT-IR: (KBr): 3405.97(O-H), 3100.53, 1667.12(C=O), 1627.56(C=C), 1522.37, 1471.52, 1319.97(C-O), 1262.97(C-O-C), 1198.94 and 868.70 cm⁻¹.

LC-ESI-MS: m/e: 301.7 (0.5%, M⁺,C₁₅H₁₀O⁺), 272.9 (6%),256.7 (15%), 229.2(8%), 192.8 (10%), 178.8(100%, M⁺-C₆H₆O₂) and 150.9(65%, M⁺-C₇H₆O₃).

¹³C-NMR (d₆-acetone, 300 MHZ): δ 177.70 (C-4), 165.95 (C-7), 162.87 (C-5), 158.58 (C-3), 149.13 (C-3'), 148.35 (C-4'), 146.59 (C-2), 137.61 (C-1'), 124.51 (C-9), 122.04 (C-10), 116.98 (C-8), 116.36 (C-6), 104.88 (C-6'), 99.60 (C-2') and 94.78 (C-5').

¹H-NMR (d₆-acetone, 300 MHZ): δ 7.74(1H,H-6,s), 7.73(1H,H-8,s), 7.65(1H,H-2',s), 7.62 (1H,H-5',s), 6.89(1H,H-6',s), 6.86(1H,H-3), 6.38(1H,H-7,s), 6.18(H-5,s) and 4.88(1H,H-3',4',s).

Characterization of Compound ANA04

UV (MeOH) λmax: 272.8 nm.

FT-IR: (KBr): 3285.28(O-H), 3072.72, 1703.34(C=O), 1615.63(C=C), 1501.90, 1446.96, 1339.41, 1245.99 and 867.96 cm⁻¹.

LC-ESI-MS: m/e: 169.9 (M⁺, C₆H₆O⁺), 168.9 (100%, M⁺-1) and 125 (40%, M⁺-COOH).

¹³C-NMR (d₆-acetone, 300 MHZ): δ 170.936 (C-7), 146.755 (C-3,C-5), 140.023 (C-4), 122.792 (C-1) and 110.79 (C-2,C-6).

¹H-NMR (d₆-acetone, 300 MHZ): δ 9.136 (1H,H-7,s), 7.08 (1H,H-2,H-6,s) and 5.011 (1H,H-3, H-4,H-5,s).

Antioxidant Assay

DPPH Radical Scavenging Activity

Preparation of Test Solutions for Isolated Compounds

Quercetin from leaf, catechin, gallic acid and ellagic acid from bark were dissolved in methanol (500 µg mL⁻¹ each).

DPPH Radical Scavenging Activity

DPPH scavenging activity was measured by spectrophotometric method (Choudhary, 2006; Ravishankara *et al.*, 2002). Methanolic solution of DPPH 100 µL (1.3 mg mL⁻¹) was added in 4 mL of methanol to give initial absorbance of 0.9 at 516 nm. To methanolic solution of DPPH (100 µL), test extracts dissolved in methanol were added at different concentrations (25-100 µg mL⁻¹). The decrease in absorbance of test mixtures was read at 516 nm against test blank and the percentage inhibition (Table 1) was calculated by using the formula:

$$\text{Inhibition (\%)} = \frac{\text{OD blank} - \text{OD of test}}{\text{OD standard}} \times 100$$

Statistical Analysis

Results were expressed as Mean±SD of three replicates and average value was considered. The IC₅₀ value was calculated using linear regression analysis of the percent inhibition obtained using

different concentrations. The regression equation was obtained and the concentration required to produce 50% inhibition (IC_{50}) was calculated.

RESULTS AND DISCUSSION

IC_{50} values showed by isolated polyphenolic compounds i.e., gallic acid, ellagic acid and catechin from bark justifies anti oxidant potential (IC_{50} $\mu\text{g mL}^{-1}$ 2.23, 3.67 and 12.14 > 14.10 (ascorbic acid)) supports the anticancer activity of bark (Acharya *et al.*, 2004; Aher *et al.*, 2008; Velavan *et al.*, 2007; Wealth of India, 1992).

Isolation of Phytoconstituents

The isolated constituents were characterized with help of the instrumental techniques as UV spectroscopy, IR spectroscopy, Mass spectrometry and NMR spectroscopy. Co-TLC of the isolated constituents with authentic compounds was also used as criteria for their identification. Methanolic extract of bark offered three compounds. These were identified as ANA01 (catechin), ANA02 (ellagic acid) and ANA04 (gallic acid), respectively. Methanolic extract of leaf showed the presence of ANA03 (quercetin).

Radical Scavenging Activity

The DPPH radical scavenging activity of the isolates increases with increasing concentration. The isolated compounds show more scavenging activity in comparison to ascorbic acid (Table 1).

The gallic acid from bark was found to most effective (IC_{50} 2.23 $\mu\text{g mL}^{-1}$) than catechin, ellagic acid from bark and quercetin from leaf (IC_{50} 12.14, 3.67 and 3.67 $\mu\text{g mL}^{-1}$) when compared with standard ascorbic acid (IC_{50} 14.10 $\mu\text{g mL}^{-1}$).

Oxidation is involved in decomposition of pharmaceutical preparation containing steroids, vitamins and antibiotics. Reactive Oxygen Species (ROS) are produced continuously in the cells as accidental by-products of metabolism which are cytotoxic and are important factors for several pathological conditions such as cardiovascular diseases, diabetes, inflammation and cancer etc. (Acharya *et al.*, 2004).

Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic etc. (Velavan *et al.*, 2007).

DPPH is one of the free radicals, stable at room temperature generally used for testing preliminary radical scavenging activity of a compound or a plant extract. The decrease in the concentration of DPPH radical due to scavenging ability of compounds isolated from *Casuarina equisetifolia* showed better activity.

Therefore, in this study, the antioxidant properties of the isolated compounds from methanol extracts of bark and leaf of *Casuarina equisetifolia* were examined for DPPH radical scavenging activity according to the method described and the results of the screening are shown in Fig. 1 as comparable with known antioxidant ascorbic acid. In terms of antioxidant activity, all the compounds investigated

Table 1: Antiradical activity for isolated compounds from various plant parts observed with DPPH

Isolated compound	Scavenging (%)				IC_{50} ($\mu\text{g mL}^{-1}$)
	5	10	15	20	
Catechin	12.59±0.76	57.91±0.65	65.66±0.75	68.97±0.65	12.14
Quercetin	51.97±0.26	67.59±0.28	91.19±0.38	92.52±0.34	3.61
Gallic acid	58.17±0.38	82.13±0.19	86.48±0.12	88.78±0.25	2.23
Ellagic acid	50.84±0.21	63.44±0.26	72.66±0.31	78.80±0.28	3.67
Ascorbic acid	15.64±0.09	34.51±0.11	51.45±0.04	73.87±0.01	14.10

Values are Mean of three replicates and expressed as Mean±SEM

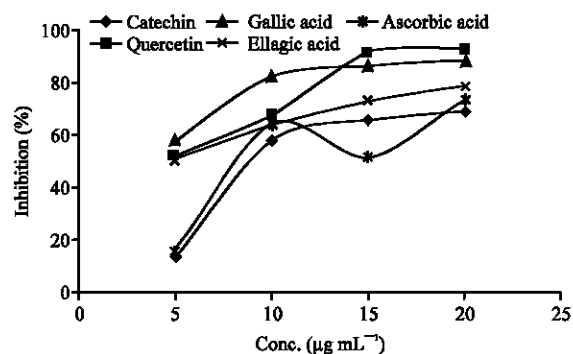


Fig. 1: DPPH scavenging activity of isolated compounds

exhibited a rather high degree of activity. In particular, compound gallic acid isolated from bark displayed the highest activities as antioxidant activity as removal of the stable radical DPPH (Fig. 1) and the lowest activity were found in catechin (from bark). As expected, the overall activity of the isolated compounds were higher than that of commercial antioxidant ascorbic acid, the reference antioxidant (Fig. 1).

In conclusion, the screening of antioxidant activity performed on *Casuarina equisetifolia*, which traditionally used as anti cancer and for swelling (Aher *et al.*, 2008; Wealth of India, 1992) shows that they are endowed with potentially exploitable antioxidant activities.

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