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Isolation and Characterization of Phytoconstituents from *Casuarina equisetifolia* (Casuarinaceae)

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The methanolic extracts of bark, wood, leaf and fruits of *Casuarina equisetifolia* were studied for isolation of tannins and flavonoids by thin layer chromatography (TLC), column chromatography and preparative-thin layer chromatography (P-TLC) and characterized by spectroscopic techniques (UV-visible spectroscopy, infrared spectroscopy, ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry). The tannins (catechin, ellagic acid and gallic acid) were isolated from bark and flavonoid (quercetin) and lupeol from leaf and fruit, respectively and identified with help of preliminary phytochemical methods, physical properties, spectroscopic data and Co-TLC with authentic standards.

Key Words: *Casuarina equisetifolia*, Gallic acid, Ellagic acid, Column chromatography.

INTRODUCTION

Casuarina equisetifolia (Casuarinaceae) is beautiful tree with droping branches, 10-50 m high[1]. It is found in dry hill sides of open forests in India, Sri Lanka and Australia[2]. The following phyto-constituents have been isolated from the plant so far; kaempferol, quercetin[3], alicyclic acids (shikimic acid and quinic acid), amino acids[4], taraxarol, lupenone, lupeol, gallic acid, β-sitosterol[5], catechin and gallocatechin[6,7]. The plant is used as astringent[1], in diarrhoea[8], cough, ulcers, toothache, lotion for swelling[9] and diabetes[10]. The biological activities, *viz.* anticancer, antibacterial[9], hypoglycemic, antifungal[2] of the leaf has been reported. Antibacterial activity (*S. aureus*) was showed by isolated compounds 24-ethyl cholest-5,22-dien-3β-ol, 24-ethyl cholest-5-en-3β-ol, 24-methyl cholest-5-en-3β-ol and cholesterol from leaves[11].

Literature reviewed suggested that the isolation and characterization of phytoconstituents from the plant has not been studied and henceforth in the present study the investigations were carried out.

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EXPERIMENTAL

Preparation of plant extracts: Coarsely powdered materials of leaf, bark, wood and fruit (100 g each) were subjected to reflux with 250 mL of methanol for 4 h coded as MEL, MEB, MEW and MEF followed by subsequent filtration and evaporation to yield extract.

Phytochemical screening: Preliminary phytochemical screening of wood, bark, fruits and leaves were tested for the presence of phytoconstituents[12-16].

Separation and isolation of phytoconstituents by chromatographic methods: Gradient fractionation of acetone soluble part (from the methanolic bark extract) was performed by eluting the column with toluene:ethyl acetate:methanol (4:3:3) followed by methanol over silica gel (60-80#), resulting in the isolation of compounds designated as **ANA 01** (catechin) and **ANA 02** (ellagic acid); **ANA 04** (gallic acid) from fractions no. 9-22 and 32-36, respectively. The separations of isolates were confirmed by spraying with ferric chloride (Table-1).

TABLE-1
TLC PATTERN OF COLUMN FRACTIONS FROM ACETONE SOLUBLE PART OF METHANOLIC EXTRACT OF BARK

S. No.	Test	Ferric chloride spray		
		Colour	Shape	R_f
1	Fr. No. 9-22	Green	Oval	0.74
		Black	Oval	0.64
2	Gallic acid	Black	Circular	0.64
3	Catechin	Green	Circular	0.74
4	Fr. No. 32-36	Blue	Oval	0.57
5	Ellagic acid	Blue	Oval	0.57

ANA 05 (lupeol) was identified from petroleum-ether extract of fruits by benzene: ethyl acetate (9:1) followed by spraying with vanillin-sulphuric acid reagent (Table-2). Methanolic extract of leaf was chromatographed by toluene:ethyl acetate:formic acid (5:4:1), followed by spraying with ferric chloride (Table-3). The presence of **ANA 03** (quercetin) was evidenced in UV light. The extracts were processed by preparative TLC (dimension: 20 cm × 20 cm, Merck, Germany) to isolate the compounds.

TABLE-2
TLC PATTERN OF PETROLEUM ETHER EXTRACT OF FRUIT

S. No.	Test	Vanillin sulphuric acid spray		
		Colour	Shape	R_f
1	Fruit	Pink	Kidney	0.29
			Elongated	0.47
			Moon	0.77
			Circular	0.64
			Circular	0.92
			Elongated	0.98
2	Lupeol	Pink	Circular	0.64

TABLE-3
TLC PATTERN OF METHANOLIC EXTRACT OF LEAF

S. No.	Test	Ferric chloride spray		
		Colour	Shape	R_f
1	MEL	Black	Elongated	0.97
		F. Black	Elongated	0.91
		F. Black	Elongated	0.81
		F. Black	Elongated	0.67
		F. Black	Elongated	0.24
		F. Black	Elongated	0.16
		F. Black	Elongated	0.08
2	Quercetin	Black	Elongated	0.67

Characterization of isolated compounds: The isolated compounds were characterized with help of physical characters and spectroscopic analysis (UV spectroscopy, IR spectroscopy, Mass spectrometry and NMR spectroscopy).

Characterization of compound ANA01: Crystalline pale brown powder, soluble in acetone and alcohol, m.p. 175-177 ºC, R_f value 0.74 [toluene (4):ethyl acetate (3):methanol (3)]. UV (MeOH) max: 219, 279 nm; FT-IR: (KBr, ν_{max}, cm^{-1}): 3820, 3391 (O-H), 2933, 2892, 1627 (C=O), 1522, 1471, 1289 (C-O-C), 1245, 1198 (C-O) and 868; LC-ESI-MS: m/e: 290 (5 %, M^+, $C_{15}H_{16}O^+_6$), 289 (12 %, M^+-1), 245 (32 %, M^+-CH_3O_2), 205 (100 %, M^+-$C_2H_6O_4$), 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: Crystalline pale yellow powder, slightly soluble in methanol, water and soluble in pyridine; m.p. 339-341 ºC, R_f value: 0.63 [ethyl formate (5):dichloromethane (5):formic acid (2):acetic acid (2)]. UV (MeOH) max: 365, 254 nm; FT-IR: (KBr, ν_{max}, cm^{-1}): 3072, 3596 (O-H), 1699 (C=O), 1622 (C=C), 1581, 1509, 1447, 1398 (C-O), 1195 and 882; LC-ESI-MS: m/e: 301.8 (10 %, M^+-$C_{14}H_6O^+_8$), 300.7 (10 %), 283.8 (40 %), 271.9 (4 %), 256.8 (50 %, M^+-$H_2O^+O_2$), 228.9 (100 %, M^+-CH_3O_4), 212.9 (8 %), 200.9 (30 %) and 184.9 (60 %); ^{13}C NMR (C_6D_5N, 300 MHz): 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 (C_6D_5N, 300 MHz): δ 8.14 (1H, H-1', s) and 5.28 (1H, H-2, 3, 2', 3', s).

Characterization of compound ANA03: Crystalline yellow powder, soluble in acetone and alcohol; m.p. 215-217 ºC, R_f value: 0.67, [toluene (5):ethyl acetate (4):formic acid (1)]. UV (MeOH) max: 255, 372 nm; FT-IR: (KBr, ν_{max}, cm^{-1}): 3405 (O-H), 3100, 1667 (C=O), 1627 (C=C), 1522, 1471, 1319 (C-O), 1262 (C-O-C), 1198 and 868; LC-ESI-MS: m/e: 301.7 (0.5 %, M^+, $C_{15}H_{10}O^+_6$), 272.9 (6 %), 256.7

(15 %), 229.2 (8 %), 192.8 (10 %), 178.8 (100 %, M^+-$C_6H_6O_2$) and 150.9 (65 %, M^+-$C_7H_6O_3$); ^{13}C NMR (d_6-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'); ^{1}H NMR (d_6-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: Crystalline colourless powder, soluble in methanol and distilled water; m.p. 235-237 ºC, R_f value: 0.64 [toluene (4):ethyl acetate (3):methanol (3)]. UV (MeOH) λ_{max}: 272.8 nm; FT-IR: (KBr, ν_{max}, cm^{-1}): 3285 (O-H), 3072, 1703 (C=O), 1615 (C=C), 1501, 1446, 1339, 1245 and 867; LC-ESI-MS: m/e: 169.9 (M^+, $C_6H_6O^+_5$), 168.9 (100 %, M^+-1) and 125 (40 %, M^+-COOH); ^{13}C NMR (d_6-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); ^{1}H NMR (d_6-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).

Characterization of compound ANA05: Crystalline white powder, soluble in petroleum ether, acetone and alcohol; m.p. 215-217 ºC, R_f value: 0.64 [benzene (9):ethyl acetate (1)]. UV (MeOH) max: 211.4 nm; FT-IR: (KBr, ν_{max}, cm^{-1}): 3325, 3083, 2937, 1642 (C=C), 1451, 1378 (O-H, bend) and 1106; LC-ESI-MS: m/e: 426.7 (0.5 %, M^+, $C_{30}H_{50}O^+$), 410.4 (12 %, M^+-H_2O), 366.0 (5 %), 353.2 (6 %), 339.4 (20 %), 325.5 (2 %), 311.4 (4 %), 299.4 (27 %), 285.3 (52 %), 271.3 (80 %, M^+-C_9H_{19}), 257.3 (100 %, M^+-$C_{10}H_{21}$), 243.2 (80 %, M^+-$C_{11}H_{23}$), 229.4 (75 %), 215.3 (60 %), 201.3 (40 %), 189.3 (25 %), 175.4 (52 %), 159.3 (25 %) and 137.2 (7 %); ^{13}C NMR (d_6-acetone, 300 MHz): δ 151.48 (C-20), 124.55, 109.97 (C-25), 78.49 (C-3), 56.233 (C-5), 51.271 (C-9), 48.989 (C-18), 48.778 (C-19), 43.68 (C-17), 43.518 (C-14), 41.592 (C-8), 40.572 (C-22), 39.504 (C-4), 38.938 (C-1), 37.886 (C-13), 36.219 (C-10), 35.069 (C-16), 28.531 (C-21), 28.272 (C-23), 28.175 (C-15), 25.957 (C-12), 21.604 (C-11), 19.451 (C-30), 19.046 (C-6), 18.270 (C-2), 16.586 (C-24), 16.392 (C-28), 16.068 (C-25), 14.903 (C-27); ^{1}H NMR (d_6-acetone, 300 MHz): δ 4.70 (1H, H-29, d), 4.56 (1H, H-27, dd), 3.32 (1H, H-3, s), 3.13 (1H, -3, s), 2.83 (2H, H-1, 2, 6, 7, 11, 12, 15, 16, m), 2.44 (H-20, H-21, sex), 1.69 (3H, H-22, 23, 24, 25, 26, 28), 1.07 (s), 0.99 (s), 0.86 (multiplet) and 0.76 (s).

RESULTS AND DISCUSSION

Phytochemical screening: A qualitative phytochemical analysis was performed for the presence of carbohydrate, protein, steroid and tannin in all plant parts. In addition, the phytoconstituents reported were alkaloid (bark), saponin (bark, fruit) and flavonoid (fruit, leaf).

Separation and isolation of phytoconstituents: Acetone soluble fraction (from methanolic extract) of bark resulted in column chromatographic separation of **ANA01** (catechin), **ANA02** (ellagic acid) and **ANA04** (gallic acid) (Table-1). Methanolic extract of leaf and petroleum extract of fruit were chromatographed

(Tables 2 and 3) for identification of **ANA03** (quercetin) and **ANA05** (lupeol) and isolated by preparative TLC.

Structural elucidation of isolated compounds

ANA01 (Catechin): λ_{max} from UV spectrum indicated the presence of conjugation and two chromophores which is a specific character of flavonoids. FT-IR spectra resulted in the presence of functional groups hydroxyl (-OH) stretch, C-H stretch of alkenes, C=O stretch for lactone and aromatic benzonoid ring. ^{1}H NMR and ^{13}C NMR showed the aromatic proton and hydroxyl proton and presence of 15 carbons in structure. The molecular weight of compound (m/e 289) was confirmed by mass spectrum. The data correlates the structure of the isolated compound to phenyl propanoid.

ANA02 (ellagic acid): λ_{max} from UV spectrum indicated the presence of conjugation and two chromophores which is a specific character of flavonoids. FT-IR spectra resulted in presence functional groups hydroxyl (-OH) stretch, C-H stretch of alkenes, C=O stretch for lactone and aromatic benzonoid ring. ^{1}H NMR and ^{13}C NMR showed the aromatic proton and hydroxyl proton and presence of 14 carbons in structure. The molecular weight of compound (m/e 302) was confirmed by mass spectrum. The data correlates the structure of the isolated compound to phenolic acid ester.

ANA03 (quercetin): λ_{max} from UV spectrum indicated the presence of conjugation and two chromophores which is a specific character of flavonoids. FT-IR spectra resulted in presence of functional groups hydroxyl (-OH) stretch, C-H stretch of alkenes, C=O stretch for lactone and aromatic benzonoid ring. ^{1}H NMR and ^{13}C NMR showed the aromatic proton and hydroxyl proton and presence of 15 carbons in structure. The molecular weight of compound (m/e 302) was confirmed by mass spectrum. The data correlates the structure of the isolated compound to phenyl propanoid flavanol.

ANA04 (gallic acid): λ_{max} from UV spectrum indicated the presence of conjugation and chromophore. FT-IR spectra resulted in presence of functional groups hydroxyl (-OH) stretch, C-H stretch of alkenes, C=O stretch for acid and aromatic benzonoid ring. ^{1}H NMR and ^{13}C NMR showed the aromatic proton, acidic proton and hydroxyl proton and presence of 7 carbons in structure. The molecular weight of compound (m/e 170) was confirmed by mass spectrum. The data correlates the structure of the isolated compound to phenolic acid.

ANA05 (lupeol): λ_{max} from UV spectrum indicated the absence of conjugation and chromophore. FT-IR spectra resulted in presence of functional groups hydroxyl (-OH) stretch, C-H stretch of alkenes and C=C stretch for cycloalkene. ^{1}H NMR and ^{13}C NMR showed the aliphatic protons and hydroxyl proton and presence of 30 carbons in structure. The molecular weight of compound (m/e 426) was confirmed by mass spectrum. The data correlates the structure of the isolated compound to cyclic terpenoid.

The work on screening of the potential of isolated compounds for assessment of antioxidant, antimicrobial and anticancer activities is in progress.

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