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Activity Guided Isolation of Antioxidant Tannoid Principles from *Anogeissus latifolia*

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Abstract – Oxidative stress is an important causative factor in several human chronic diseases, such as atherosclerosis, cardiovascular disorders, mutagenesis, cancer, several neurodegenerative disorders, and the aging process. Phenolics and tannins are reported to be good antioxidants. *Anogeissus latifolia* (Combretaceae) bark has been used in the Indian traditional systems of medicine for curing a variety of ailments, but scientific validation is not available till date. Hence the present study was undertaken to isolate antioxidant compounds by activity-guided isolation. Inhibtion of diphenyl picryl hydrazyl (DPPH) and Xanthine oxidase along with photochemiluminescence assay were used as bioassay for antioxidant activity. Activity guided isolation was carried out using silica column and the compounds were quantified using HPLC. Ethyl acetate and butanol fraction exhibited potent antioxidant activity. Bioassay-guided isolation led to isolation of ellagic acid (**1**) and dimethyl ellagic acid (**2**) as the main active compounds, which along with gallic acid were quantified by HPLC. Thus we conclude that these three major tannoid principles present in *A. latifolia*, are responsible for the antioxidant potential and possibly their therapeutic potential.

Keywords – *Anogeissus latifolia*, Antioxidant, DPPH, Photochemiluminescence, Ellagic acid, Dimethyl ellagic acid, Gallic acid

Introduction

Anogeissus latifolia (Roxb.) Wall. Ex. Bedd. (Family Combretaceae) known as 'Dhava' in India, is a small or fairly large tree, commonly found in the forests of the sub-Himalayan region and Siwalik hills and throughout India upto 1200 m. It is an important timber and the leaves and bark are used for tanning. The bark has been used for curing various disorders in 'Ayurveda' the Indian codified system of medicine and also by the tribals. Ethnobotanically, the bark has been reported to be used in the treatment of various disorders like skin diseases (Roy and Chaturvedi, 1986), snake and scorpion bite (Mishra and Billore, 1983), stomach diseases (Jain and Tarafder, 1970), colic (Apparanantham and Chelladurai, 1986), cough (Balla *et al.*, 1982), and diarrhea (Ramachandran and Nair, 1981). We have earlier reported the antioxidant and wound healing activities of the crude extract of the plant (Govindarajan *et al.*, 2004a and 2004b). Little is known on the chemistry of *A. latifolia*. Leucocyanidin was isolated from bark of *A. latifolia* (Reddy *et al.*, 1965). Later two new glycosides of ellagic and flavellagic acid were reported (Deshpande *et al.*, 1976). Since the total extract of the plant has been shown to possess potent antioxidant activity, the aim of the present study was to isolate and characterize the main radical scavengers and antioxidant compounds through activity-guided fractionation from *A. latifolia* bark.

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Experimental

Plant material – Bark of *A. latifolia* was collected from Chitrakoot, Madhya Pradesh (India) during the month of October 2002. The plants were authenticated and the voucher specimen was deposited in the departmental herbarium of National Botanical Research Institute. Preliminary tests on the hydroalcoholic bark extract (Peach and Tracy, 1955) indicated the presence of tannins. Total phenolic content of the bark was determined spectrophotometrically by the Folin-Ciocalteau method using a calibration curve with tannic acid (Scalbert *et al.*, 1988).

DPPH scavenging activity – DPPH radical scavenging

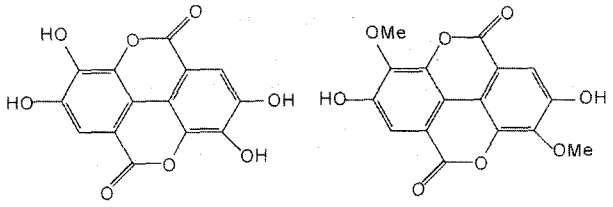
activity was investigated spectrophotometrically (Sreejayan and Rao, 1986). Briefly, to a methanolic solution of DPPH (100 μM, 2.95 ml), 0.05 ml of test compounds dissolved in methanol. Equal amount of methanol was added to the control. Absorbance was recorded at 517 nm after 20 minutes.

Photochemiluminescence assay – The quenching of free radicals by the extracts and compounds was evaluated by the Photochemiluminescence assay. The integral antioxidative capacity of the water soluble compounds (ACW) was measured by chemiluminescence assay using photochem (Analytic Jena AG, Germany). The extracts and the compounds (10 and 1 mg/ml in water respectively) were mixed with reagent containing luminol. The antioxidants were quantified due to their inhibitory effects on luminescence generation by comparison with ascorbic acid used as standard and the antioxidative capacity was calculated as equivalents units of ascorbic acid (from the calibration curve of ascorbic acid) and the results were expressed as nmoles ascorbic acid g^{-1} equivalents (Govindarajan *et al.*, 2004a).

Xanthine oxidase activity – The xanthine oxidase (XO) activity with xanthine was measured spectrophotometrically as previously reported using a spectrophotometer (Schmeda et al., 1996). Extracts and products were evaluated at 100 and 50 μg/ml respectively.

Activity guided fractionation – The air dried powdered material (2 kg) was macerated with petroleum ether to remove the fatty substances, the marc was further extracted with 50% aqueous alcohol for 3days and filtered. The extract was concentrated under reduced pressure and lyophilised (Labconco, USA) to get dry residue (188.8 g). The total extract was partitioned with chloroform, ethyl acetate, n-butanol and water yielding chloroform soluble (0.1% w/w), ethyl acetate soluble (2.19% w/w), butanol soluble (24.6% w/w) and an aqueous phase (73.11% w/w). Total extract and all the fractions were subjected to antioxidant activities and it was found that ethyl acetate soluble portion and butanol soluble portion showed potent antioxidant activity. The DPPH, Photochemiluminescence and xanthine oxidase bioassays showed highest activity in the butanol and ethyl acetate soluble fractions.

Isolation of compounds 1 and 2 – Five grams of the ethyl acetate soluble fraction was chromatographed over silica gel column with PE (Fr. 1); PE-EtOAc 9 : 1 (Fr. 2); PE-EtOAc 8 : 2 (Fr. 3); PE-EtOAc 7 : 3 (Fr. 4); PE-EtOAc 6:4 (Fr. 5); PE-EtOAc 1 : 1 (Fr. 6); PE-EtOAc 4 : 6 (Fr. 7); PE-EtOAc 1 : 9 (Fr. 8); EtOAc (Fr. 9); EtOAc-MeOH 9.5 : 0.5 (Fr. 10); EtOAc-MeOH 9 : 1 (Fr. 11); EtOAc-MeOH 8.5 : 1.5 (Fr. 12); EtOAc-MeOH 8 : 2 (Fr. 13); EtOAc-MeOH 7.5 : 2.5 (Fr. 14); EtOAc-MeOH 7 : 3 (Fr. 15-18); EtOAc-MeOH 6 : 4 (Fr. 19-21); EtOAc-MeOH 1 : 1 (Fr. 22-25); EtOAc-MeOH 4 : 6 (Fr. 26-27); EtOAc-MeOH 3 : 7 (Fr. 28-32); MeOH (Fr. 32-35). TLC analysis of the fractions was performed on precoated silica gel plate using ethyl acetate: formic acid: glacial acetic acid: water (10 : 1.1 : 1.1 : 2.6) as the mobile phase. After TLC comparison, fractions with similar patterns were combined. The free radical scavenging activity was located mainly in the fractions 9-13.



1 **2**

Fractions 10 and 11 on preparative TLC on silica gel using ethyl acetate-formic acid-glacial acetic acid-water (10 : 1.1 : 1.1 : 2.6) yielded two pure compounds **1** (0.54 g) and **2** (0.34 g), which were identified as ellagic acid and dimethyl ellagic acid respectively on the basis of spectroscopic data and co-chromatography with HPLC. Both these compounds were found to possess potent antioxidant activity. Both these compounds were confirmed by spectral data and co-chromatography.

High Performance Liquid Chromatography – HPLC analysis was performed using a Waters model (Water Corp, Milford, USA), equipped with a pump (Waters 515) with a Spherisor ODS2 column RP- 8 (250×4.6 mm, i.d., 5 μm pore size) and a Waters PCM Rheodyne injector with a 25 μl loop. Detection was done at 320 nm using 2996 PDA detector.

Butanol fraction and ethyl acetate soluble portion (1 mg/ml concentration) was eluted in HPLC using a binary gradient at a flow rate of 0.5 ml/min with water-methanol-formic acid (79.7 : 20 : 0.3 v/v) as solvent A and acetonitrile : formic acid (100 : 0.3 v/v) as solvent B using a gradient elution in 0-5 min. linearly at 100% of A, 5-6 min. with 100-80% A, 6-10 min. with 80-70% of A, 10-15 min. with 70-50% A, 15-20 min. with 50-20% A, 20-21 min. with 20-15% A, 21-21.5 min. with 15-10% A, 21.5-24 min. with 10-0% A, 24-34 min. with 0-100% A and isocratic till 40 min. with 100% of A. Gallic acid and **1** were quantified in butanol fraction while compound **2** as quantified in ethyl acetate fraction. The linearity of the HPLC method was investigated for tannins in the range

10-100 μg/ml at five concentration levels, using the successive dilutions of stock solutions (100 μg/ml). Calibration plots with correlation coefficient were obtained by reporting peak areas (relative units as given by the integrator) as a function of analyte concentrations.

Results and Discussion

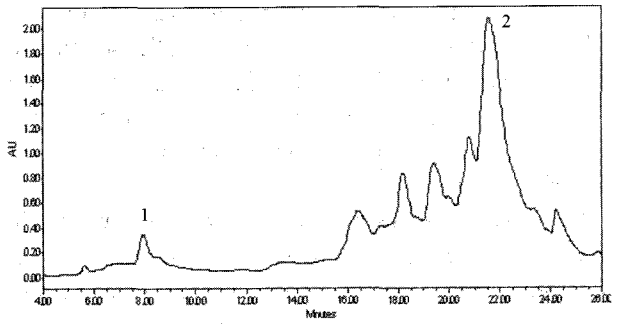
Preliminary analysis carried out on the crude *A. latifolia* extract indicates the occurrence of tannins, steroids and saponins. Total phenolics content of the bark determined spectrophotometrically by the Folin-Cicoalteau method was 12.7% w/w from dry starting material. Bioassay-guided isolation afforded compounds **1** and **2** as the major free radical scavengers from the ethyl acetate fraction. Dimethyl ellagic acid has been reported for the first time from the *A. latifolia* bark. The compounds were characterized by spectral data, micro-melting point and co-chromatography with a reference sample. The phenolic nature of ellagic acid makes a powerful antioxidant. Ellagic acid has been reported to inhibit CCl_4 induced toxicity and liver fibrosis in rats (Theismania and Kuttan, 1996). Barch *et al.*, (1996) studied the structure activity relationship of ellagic acid as a dietery anticarcinogen and found a variety of activities towards known cancer promoters. Ellagitannins have also been reported to possess potent antioxidant activity (Gyamfi and Aniya, 2002).

Identification of compounds

Ellagic acid (1) – UV λ_{max} MeOH (nm): 255, 362; IR ν_{max} cm^{-1}: 3380, 3270, 1720, 1690, 1610, 1575, 1485; ^1H NMR: δ 7.5 (s, H-4, H-9); ^{13}C NMR: δ 136.4 (s, C-1a, C-6a), 140.2 (m, C-2, C-7), 153.0 (s, C-3, C-8), 111.4 (d, J = 165 Hz, C-4, C-9), 107.7 (s, C-4a, C-9a), 112.3 (d, J = 10 Hz, C-4b, C-9b), 159.2 (d, J = 4 Hz, C-5, C-10); FAB-MS: pso. Ions 303 $[M + H]^+$. The data matched the reported spectral data of Nawwar and Soulman (1984) thus confirming ellagic acid.

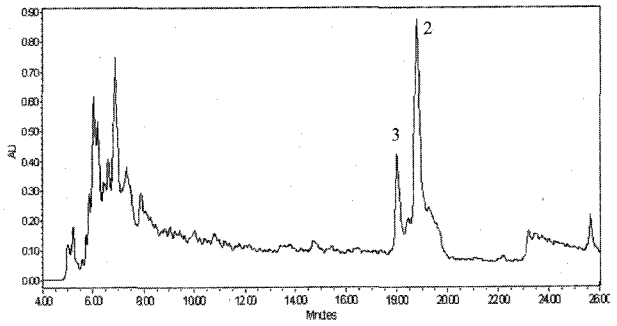
Dimethyl ellagic acid (2) – ^1H NMR spectrum showed the presence of one galloyl group by two proton singlets at δ 6.98, an ellagic acid moiety by the signals at δ 7.74 and 7.45 (s, 1H each), and six hydrogens of the xylose at 5.10 (d, J = 5, 5.8 Hz, 1H anomeric), 4.94 (t, J = 5, 7.3 Hz, 1H), 3.98 (dd, J = 5, 11 and 5 Hz, 1H) and 3.75-3.40 (m, 3H). The ^1H NMR also revealed the presence of two aromatic methoxy group at δ 4.04 and 3.92 (s, 3H each). Hence it was confirmed to be dimethyl ellagic acid (Taylor *et al.*, 1998).

Antioxidant activity – DPPH is a stable nitrogen centred free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents losing colour stoichometrically with the number of electrons consumed which is measured spectrophotometricallty at 517 nm. Photochem®, apparatus and phtochemiluminescence method allowed precise as well as time and cost effective determination of the integral antioxidative capacity of the *A. latiofila* extract and its fractions. Percentage inhibition of the xanthine oxidase was also observed while carrying out the activity guided fractionation.



(1 : gallic acid, 2 : ellagic acid)

Fig. 1. HPLC chromatogram of the butanol fraction.



(2: ellagic acid; 3: dimethyl ellagic acid)

Fig. 2. HPLC of the ethyl acetate fraction.

Quantification – HPLC chromatogram of the butanol fraction (Fig. 1) showed a number of peaks with the peaks at RT 7.946 of gallic acid and RT 21.405 of **1** being the major peaks. HPLC chromatogram (Fig. 2) of the ethyl acetate fraction showed a total of 10 peaks with the peak at RT 19.554 of ellagic acid being the major peak. Compounds **1**, **2** and gallic acid content in the bark drug were found to be 0.25, 0.16 and 0.98% w/w respectively from the dry starting material. The detection limits (LOD), calculated for a signal/noise ratio of 3, and was found to be 1.95, 1.22 and 0.76 μg/l for compounds **1**, **2** and gallic acid respectively. Free radical scavenging activity of the *A. latifolia* crude extracts, fractions and ellagic acid is presented in Table 1.

Table 1. Free radical scavenging activity of *Anogeissus latifolia* crude extract and fractions

Samples	% DPPH scavenging activity (50 mg/ml)	Photochemiluminescence assay nmoles ascorbic acid/equivalents	Xanthine oxidase (% inhibition)
Crude extract	85.26 ± 2.59	2.824	84.67 ± 2.97
Chloroform soluble fraction	45.36 ± 1.97	1.114	39.67 ± 2.57
Ethyl acetate soluble fraction	80.26 ± 2.25	2.221	75.28 ± 3.69
Butanol fraction	78.64 ± 1.67	2.172	71.48 ± 0.97
Aqueous fraction	64.23 ± 1.72	1.386	56.36 ± 1.28
Fraction 9	64.29 ± 1.79	1.487	64.22 ± 1.28
Fraction 10 (**1**)	95.23 ± 1.07	3.224	91.88 ± 1.02
Fraction 11 (**2**)	90.26 ± 1.47	2.778	86.17 ± 2.17
Fraction 12	92.14 ± 1.19	2.921	86.51 ± 2.17
Fraction13	82.37 ± 1.22	2.552	78.28 ± 2.62
Gallic acid	89.66 ± 0.86	2.812	86.99 ± 1.27

[Values are mean ± SE of 6 replicates]

In conclusion, tannins viz. ellagic acid and dimethyl ellagic acid were isolated and identified as main free radical scavengers of *A. latifolia* bark through activity guided fractionation. HPLC analysis showed that ellagic acid as the main component of the Ethyl acetate and butanolic fraction and gallic acid as the main component of the butanol fraction. The antioxidant activity of the plant extract may be in part one of the reasons for the potential medicinal use of the plant. Also, the compound(s) responsible for the activity may be tannins like ellagic acid, dimethyl ellagic acid and gallic acid.

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