Antioxidant Potential of *Anogeissus latifolia*

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***Anogeissus latifolia* is widely used in the Indian indigenous system of medicine and is reported to contain leucocyanidins and tannoid principles like ellagic acid and its derivatives. In view of its wide use and its chemical composition, this study was aimed at examining the antioxidant activity of the extract of *A. latifolia*. The extract was studied for total antioxidant capacity, hydrogen-donating ability, nitric oxide, superoxide scavenging activity, hydrogen peroxide decomposition activity along with lipid peroxidation. Integral antioxidative capacity was determined by chemiluminescence assay. The extract was also studied for lipid peroxidation assay by thiobarbituric acid-reactive substances (TBARS) method using rat liver homogenate. The results indicate that *A. latifolia* extract has potent antioxidant activity. Also to ascertain the possible reason for the potent activity, percentage of gallic acid was estimated and was found to be 0.95%, which could be one of the reasons for potent antioxidant activity exhibited by the plant.**

Key words *Anogeissus latifolia*; H-donating ability; chemilumiscence; high performance thin layer chromatography (HPTLC); lipid peroxidation; gallic acid

It is increasingly being realized that a majority of the disease of today are due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals or due to the excessive oxidative stress of the current life or due to the poor scavenging/quenching in the body due to the depletion of the dietary antioxidants.[1—3] Free radicals have been implicated in causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes *etc.* Reactive oxygen species (ROS) have been known to cause tissue injury through covalent binding and lipid peroxidation. Lipid peroxidative process has been shown to augment collagen synthesis and fibrosis.[4] Hence antioxidants have a role in inhibiting the fibrotic process induced during the cell damage of liver.[5] As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

Anogeissus latifolia (Roxb.) Wall. ex. Bedd. (Family Combretaceae) 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 was first examined by Reddy *et al.* who isolated (+) leucocyanidin.[6] Later ellagic acid and two new glycosides of ellagic and flavellagic acid were reported.[7] Ethnobotanically, the bark has been reported to be used in the treatment of various disorders like skin diseases,[8] snake and scorpion bite,[9] stomach diseases,[10] colic,[11] cough[12] and diarrhoea,[13] though till date no biological/pharmacological report on the plant or its extract has been published. Hence pharmacological validation of the ethnobotanical claims regarding the plant is essential to move towards the use of the plant as a drug. Since the two reports[6,7] suggest the presence of tannoid principles which are known antioxidants, we studied the antioxidant potential of the extract and also the percentage of gallic acid by high performance thin layer chromatography (HPTLC), which may be responsible for the antioxidant activity. HPLC and GC are efficient but time consuming methods; HPTLC on the other hand is relatively simple and a non expensive assay method, which does not require any experience, equipment or complex derivatization process.

Thus present study aims to assess the antioxidant potential of 50% aqueous alcoholic extract of *A. latifolia*. Plant extracts were tested for different free radical scavenging activities including the 1,1-diphenyl 2-picryl hydrazyl (DPPH), nitric oxide, hydrogen peroxide, their capacity to reduce lipid peroxidation in rat liver homogenate, radical scavenging potential using chemiluminescence and their total antioxidant capacity. Also the percentage of gallic acid present in the extract was evaluated using HPTLC.

MATERIALS AND METHODS

Chemicals Gallic acid, 1,1-diphenyl 2-picryl hydrazyl (DPPH), 1,1,3,3-tetraethoxypropane, 2-nitrobenzoic acid (DTNB) and pottasium superoxide were obtained from Sigma Chemical Co. (St. Louis, U.S.A.), ferrous sulphate ($FeSO_4$), trichloroacetic acid (TCA), thiobarbituric acid (TBA), acetic acid, ethylenediaminetetraacetic acid (EDTA), sodium nitroprusside, sulfanilamide, phosphoric acid, naphthyl ethylene diamine, ammonium molybdate, sodium phosphate, sodium hypochlorite, hydrogen peroxide and dimethyl sulfoxide (DMSO) were obtained from Sd. fine chemicals (Mumbai, India). All other reagents used were of analytical grade.

Plant Material and Extraction Bark of *Anogeissus 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. One kilogram of the plant material was air dried at room temperature and powdered coarsely. The powdered material (250 g) was macerated with petroleum ether to remove the fatty substances; the marc was further extracted with 50% aqueous alcohol for 3 d and filtered. The extract was concen-

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trated under reduced pressure and lyophilised (Labconco, U.S.A.) to get dry residue (23.6 g).

Hydrogen-Donating Activity Hydrogen donating activity was quantified in presence of stable DPPH radical on the basis of Blois method.[14] Briefly, to a methanolic solution of DPPH (100 μM, 2.95 ml), 0.05 ml of test compounds dissolved in methanol was added at different concentrations (2—10 mg/ml). Reaction mixture was shaken and absorbance was measured at 517 nm at regular intervals of 30 s for 5 min. Ascorbic acid was used as standard. The degree of discoloration indicates the scavenging efficacy of the extract.

Total Antioxidant Capacity Total antioxidant capacity was measured according to spectrophotometric method of Preito,[15] 0.1 ml of the extract (10 mg/ml) dissolved in water was combined in an eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

Nitric Oxide Scavenging Nitric oxide scavenging activity was measured spectrophotometrically.[16] Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of extract (2—10 mg/ml) dissolved in methanol and incubated at 25 °C for 30 min, then 1.5 ml of the incubation solution were removed and diluted with 1.5 ml of Griess reagent (1% Sulfanilamide, 2% phosphoric acid, and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylene diamine was measured at 546 nm along with a control.

Hydrogen Peroxide Decomposition Hydrogen peroxide decomposition was determined according to standard method.[17] The assay mixture contained 4 ml of H_2O_2 solution (80 mM) and 5 ml of phosphate buffer (pH 7.4). One milliliter of the extract (10 mg/ml) in water was rapidly mixed with the reaction mixture by a gentle swirling motion at room temperature. One milliliter portion of the reaction mixture was then blown into 2 ml of dichromate/acetic acid reagent at 60 s intervals. The decomposition of the hydrogen peroxide was determined based on the standard plot for H_2O_2 and the monomolecular velocity constant K for the decomposition of H_2O_2 was determined by the use of the following formula

$$K = 1/t \log_{10} S_0/S$$

Where, S_0 is the initial concentration and S is the final concentration of H_2O_2.

Superoxide Scavenging Activity Superoxide scavenging was carried out by using alkaline DMSO method.[18] Solid potassium superoxide was allowed to stand in contact with dry DMSO for at least 24 h and the solution was filtered immediately before use. Filtrate (200 μl) was added to 2.8 ml of an aqueous solution containing nitroblue tetrazolium (56 μM), EDTA (10 μM) and potassium phophate buffer (10 mM, pH 7.4). Sample extract (1 ml) at various concentrations (30—1500 μg/ml) in water was added and the absorbance was recorded at 560 nm against a control in which pure DMSO has been added instead of alkaline DMSO.

Lipid Peroxidation Inhibition. Liver Homogenate Male Sprague-Drawley rats (160—180 g) were purchased from the animal house of the Central Drug Research Institute, Lucknow, India. These were kept in the departmental animal house at 26±2 °C and relative humidity 44—55% light and dark cycles of 10 and 14 h respectively for one week before the experiment. Animals were provided with rodent diet (Amruth, India) and water *ad libitum*. Randomly selected male rats were fasted overnight and were sacrificed by cervical dislocation, dissected and abdominal cavity was perfused with 0.9% saline. Whole liver was taken out and visible clots were removed and weighed amount of liver was processed to get 10% homogenate in cold phosphate buffered saline, pH 7.4 using glass teflon homogeniser and filtered to get a clear homogenate.

Assay of Lipid Peroxidation The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid-reactive substances (TBARS) by using the standard method[19] with minor modifications.[20] In brief, different concentration of extracts (200—1000 μg/ml) in water was added to the liver homogenate. Lipid peroxidation was initiated by adding 100 μl of 15 mM $FeSO_4$ solution to 3 ml of liver homogenate (final concentration was 0.5 mM). After 30 min, 100 μl of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA. After 10 min tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated in a hot water bath at 85 °C for 30 min to complete the reaction. The intensity of pink coloured complex formed was measured at 535 nm. The values of TBARS were calculated from a standard curve (absorption against concentration of Tetraethoxy propane) and expressed as nmol/mg of protein. The percentage inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls not treated with the extracts.

Photochemiluminescence Assay For the determination of the integral antioxidative capacity (AC) of the water soluble substances in *A. latifolia* extract the method of photochemiluminescence (PCL) was used. Apparatus used was Photochem® with Standard kit ACW (Analitik jena AG), where the luminol plays a double role of photosensitizer as well as the radical detecting agent. Lyophilized extract was measured at 10 μg/ml concentration. A standard plot was plotted and the results were calculated in ascorbic acid equivalents (μmol/g).

Quantitative Densitometric Assay for Estimation of Gallic Acid One milligram of gallic acid standard was dissolved in 1 ml of methanol. Ten milligrams of the hydroalcoholic extract was dissolved in 1 ml of methanol. Five and 10 μl of the standard and the sample respectively were then applicated using the CAMAG Linomat IV applicator onto the precoated silica gel F254 plates (Merck) of 0.2 mm thickness plates. The plate was then eluted in solvent system toluene : ethyl acetate : formic acid (5 : 4 : 1). After elution, the plate was dried and scanned densitometrically using CAMAG TLC scanner 3 at 272 nm. The percentage of gallic acid in the extract was calculated by calibration using peak height ratio.

RESULTS AND DISCUSSION

Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defenses. In most diseases, increased oxidant formation is a consequence of the disease activity. Potential antioxidant therapy therefore should include either natural free radical scavenging antioxidant principles or agents, which are capable of augmenting the activity of the antioxidant enzymes. ROS are capable of damaging biological macromolecules such as DNA, carbohydrates or proteins. ROS is a collective term, which includes not only the oxygen radicals ($O_2^{\cdot -}$, and $OH^{\cdot}$) but also some non-radical derivatives of oxygen these include H_2O_2, HOCl and ozone (O_3). If human disease is believed to be due to the imbalance between oxidative stress and antioxidative defense, it is possible to limit oxidative tissue damage and hence prevent disease progression by antioxidant defense supplements.[21)]

DPPH is a stable nitrogen centered free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents, then losing colour stoichometrically with the number of electrons consumed, which is measured spectrophotometricallty at 517 nm.[17)] As shown in Table 2, *A. latifolia* extract strongly scavenged DPPH radical with the IC_{50} being 0.25 mg/ml. The scavenging was found to dose dependent.

The total antioxidant capacity of the extract was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at 695 nm. The total antioxidant capacity of the extract was found to be 149.91 nmol/g ascorbic acid. Thus establishing the extract as an antioxidant.

A. latifolia extract also moderately inhibited nitric oxide in dose dependent manner (Table 2) with the IC_{50} being 0.258 mg/ml. Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities.[22)] Studies in animal models have suggested a role for NO in the pathogenesis of inflammation and pain and NOS inhibitors have been shown to have beneficial effects on some aspects of the inflammation and tissue changes seen in models of inflammatory bowel disease.[22)] Thus establishing the usage of the plant in the Indian indigenous system as an anti-inflammatory agent.

A. latifolia extract also caused decomposition of the H_2O_2 in a dose dependent manner as shown in Fig. 1. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects.[23)] It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. Two types of enzymes exist to remove hydrogen peroxide within cells. They are the catalases and the peroxidases, which leads to ground state oxygen without any singlet oxygen. The extract may have decomposition of H_2O_2 activity due to any of these enzymes.[23)]

Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl–perferryl complex or through $OH^{\cdot}$ radical by Fenton's reaction. Table 1 show that the 50% aqueous alcoholic extract of *A. latifolia* inhibited $FeSO_4$ induced lipid peroxidation in a dose dependent manner. IC_{50} values were found to be 0.124 mg/ml. The inhibition could be caused by absence of ferryl–perferryl complex or by scavenging the OH radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. Iron catalyses the generation of hydroxyl radicals from hydrogen

Table 1. Effect of Hydroalcoholic Extract of *A. latifolia* at Various Concentrations on Ferrous Sulphate Induced Lipid Peroxidation in Rate Liver Homogenate

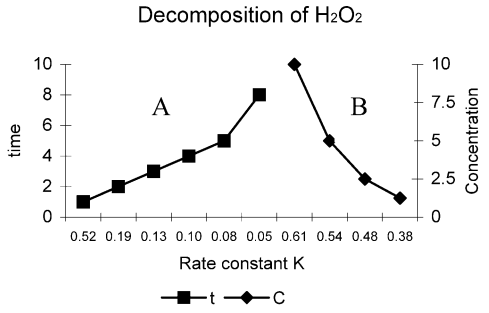
Concentration (μg/ml)	TBARS (nmol/mg protein)	% inhibition
Control	3.690±0.78	—
200	1.747±0.67	52.65±1.86
400	1.676±0.91	54.57±1.92
600	1.568±0.38	57.50±2.77
800	1.389±0.41	62.35±3.12
1000	1.199±0.47	67.51±3.09
Tocopherol	0.06±0.001	99.82±2.17

Values are mean±S.E. of 6 replicates.

Table 2. Radical Scavenging Capacity of Hydroalcoholic Extract of *A. latifolia*

Concentration (μg/ml)	DPPH scavenging %	Nitric oxide scavenging %	Superoxide scavenging %
Ascorbic acid (100 μM)	97.68±1.08	89.77±1.28	90.26±2.80
200	69.76±2.37	44.54±1.87	18.44±1.22
400	78.39±1.88	63.88±3.03	23.66±2.56
600	84.42±1.79	91.87±3.17	29.82±1.27
800	90.89±2.07	93.53±2.41	37.73±1.97
1000	93.23±2.78	—	52.69±1.72
IC_{50} (mg/ml)	0.25	0.258	1.024

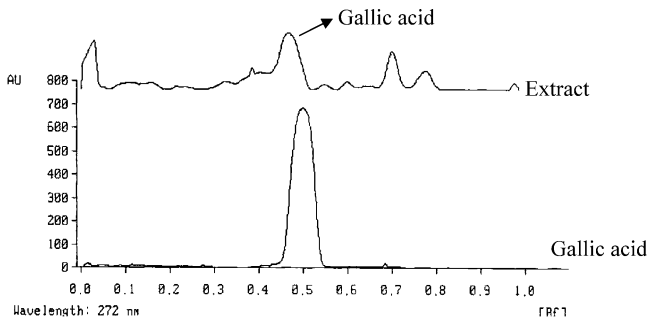
Values are mean±S.E. of 6 replicates.



t-time in seconds, c-concentration in mg/ml

Decomposition of hydrogen peroxide by *A. latifolia* extract at different concentrations with respect to the rate constant *K* (Graph B) and at different time intervals with respect to the rate constant *K* at 10 mg/ml concentration (Graph A). Rate constant was calculated as mentioned in material and methods.

Fig. 1. Decomposition of Hydrogen Peroxide by *A. latifolia*



Track 1 – standard gallic acid, track 2 – extract.

Mobile phase – Toluene: Ethyl acetate: Formic acid (5:4:1). Densitometrically scanned at 272nm.

Fig. 2. HPTLC Fingerprint Analysis of *A. latifolia* with Gallic Acid as Marker

peroxide and superoxide radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxides is produced.[21] Lipid hydroperoxide can be decomposed to produce alkoxy and peroxy radical they eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases.[19] Thus the decrease in the MDA level with the increase in the concentration of the extracts indicates the role of the extract as an antioxidant.

The extract also moderately scavenged superoxide radical with the IC_{50} values of 1.024 mg/ml.

Photochem® apparatus and method allowed precise as well as time and cost effective determination of the integral antioxidative capacity of the *A. latiofila* extract. Free radicals are generated in the instrument by means of photosensitizer. The free radicals thus generated were detected by their reaction with a chemiluminogenic substance. Luminol acts both as photosensitizer as well as the detecting reagent. In the presence of radical scavengers in the extract the intensity of the PCL was attenuated as a function of concentration. In this way the antioxidative capacity of the extract could be quantified. The antioxidative capacity was found to be 1.014 nmol ascorbic acid/g equivalents.

Since the plant has been reported to contain tannoid principles, we tried to investigate the presence the gallic acid in the extract and the gallic acid was found to be 0.95% w/w by HPTLC (Fig. 2) in the hydroalcoholic extract. The high percentage of the gallic acid in the extract justifies the potent antioxidant activity exhibited.

CONCLUSION

The present study aimed to evaluate the possible antioxidant activity of the *A. latifolia* extract used widely in Indian Indigenous systems of medicine but with no reported pharmacological data. The results obtained indicate that *A. latifolia* extract has potent antioxidant activity, achieved by scavenging abilities observed against DPPH, and lipid peroxidation. It showed high-H donating ability shown by the scavenging of DPPH radical. Thus the ethnobotanical claims of the plant being used in the stomach disorders and as a tonic may be in part due to the antioxidant activity. The percentage of gallic acid in the bark ascertains that the antioxidant activity may be due to the same. Further the pharmacological activity of the extract is to be carried out to validate the ethnobotanical claims.

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