Studies on the Antioxidant Activities of *Desmodium gangeticum*

Raghavan GOVINDARAJAN,[a] Subha RASTOGI,[a] Madhavan VIJAYAKUMAR,[a] Annie SHIRWAIKAR,[b] Ajay Kumar Singh RAWAT, Shanta MEHROTRA,*,[a] and Palpu PUSHPANGADAN[a]

[a] *Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute; Lucknow-226 001, India: and* [b] *Pharmacognosy Department, College of Pharmaceutical Sciences; Manipal-576 119, Karnataka, India.*
Received March 31, 2003; accepted July 10, 2003

Desmodium gangeticum **is herbal species which is widely used in the indigenous system of medicine and is reported to contain flavone and isoflavanoid glycosides. In view of its wide use and it's chemical composition, this study was aimed at examining the antioxidant activity of the extract of *D. gangeticum*. The extract was studied for diphenyl picryl hydrazyl (DPPH), nitric oxide, ferryl-bipyridyl and hypochlorous acid scavenging activity along with lipid peroxidation. Nitric oxide was generated using sodium nitroprusside and was studied using Griess reagent. In order to study the iron chelating capacity of the extract, the percentage ferryl-bipyridyl inhibition was studied. Hypochlorous acid scavenging activity was tested by measuring the inhibition of 5-thio-2-nitrobenzoic acid oxidation. The extract was also studied for lipid peroxidation assay by thiobarbituric acid-reactive substances (TBARS) method using rat brain homogenate. The results indicate that *D. gangeticum* extract has potent antioxidant activity.**

Key words *Desmodium gangeticum*; diphenyl picryl hydrazyl (DPPH); nitric oxide; hypochlorous acid; ferryl-bipyridyl; lipid peroxidation

In the past few years natural antioxidants have generated considerable interest in preventive medicine. There is increasing evidence that oxidative stress, defined as an imbalance between oxidants and antioxidant in favour of the former, leads to many biochemical changes and is an important causative factor in several human chronic diseases, such as atherosclerosis and cardiovascular diseases, mutagenesis and cancer, several neurodegenerative disorders, and the aging process.[1)] The food industry also uses natural antioxidants as a replacement of conventional synthetic antioxidants in food by natural products that are considered to be promising and a safe source.[2)] As a result of which, much attention has been directed towards the characterisation of antioxidant properties of plant extracts/their fractions and identification of the constituents responsible for those activities.[3—5)]

Desmodium gangeticum (L.) DC. (Family Leguminaceae) is a small shrub of tropical region which has been used in Indian system of medicine as a bitter tonic, febrifuge, digestive, anticatarrhal, antiemetic, in inflammatory conditions of chest and various other inflammatory conditions due to 'vata' disorders.[6)] The aqueous extract of this species has been reported to show severe antiwrithing activity, moderate central nervous system (CNS) depressant activity and antileishmanial activity.[7,8)] Gangetin, a pterocarpnoid from *D. gangeticum* has been shown to possess anti-inflammatory and analgesic activities.[9)] Total alkaloids of this species showed anticholinesterase, smooth muscle stimulant, CNS stimulant and depressant responses.[10)] Earlier chemical studies on the *D. gangeticum* revealed the presence of alkaloids, pterocarpnoid, flavone and isoflavanoid glycosides.[9—11)] However there is no available information relating to the antioxidant properties of this species.

The present study aims to assess the antioxidant capacity of 50% aqueous alcoholic extract of *D. gangeticum*. Plant extracts were tested for different free radical scavenging activities including the 1,1-diphenyl picryl hydrazyl (DPPH), nitric oxide, hydrogen peroxide, hypochlorous acid, their capacity to reduce lipid peroxidation in rat brain homogenate, and their total antioxidant capacity.

MATERIALS AND METHODS

Chemicals 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, ethelene diamine tetracetic acid (EDTA), sodium nitroprusside, sulphanilamide, phosphoric acid, naphthyl ethelene diamine, ammonium molybdate, sodium phosphate, sodium hypochlorite, hydrogen peroxide and dimethyl sulphoxide (DMSO) were obtained from Sd fine chemicals (Mumbai, India). All other reagents used were of analytical grade.

Plant Material Aerial parts of *D. gangeticum* (L.) DC. were collected from Chitrakoot, Madhya Pradesh (India) during the month of October, 2001. The plants were authenticated and the voucher specimen was lodged in the herbarium of National Botanical Research Institute.

Preparation of Plant Extract Aerial parts of *D. gangeticum* (1 kg) were air dried at room temperature and powdered coarsely. The powder obtained (250 g) was macerated with 50% aqueous alcohol for a period of 24 h, filtered. The extract was concentrated under reduced pressure and lyophilised (Labconco, U.S.A.) to get 16.2 g (6.5%) of the extract.

DPPH Radical Scavenging Activity DPPH radical scavenging activity was investigated according to the method of Sreejayan and Rao,[12)] 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 concentration (2—10 mg/ml). Equal amount of methanol was added to the control. Absorbance was recorded at 517 nm at regular intervals of 30 s for 5 min. Ascorbic acid was used as a standard.

Total Antioxidant Capacity For total antioxidant capacity assay,[13)] 0.1 ml of the extract (10 mg/ml) dissolved in water was combined in a eppendorf tube with 1 ml of reagent

* To whom correspondence should be addressed. e-mail: pharmacognosy1@rediffmail.com

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.[14] Sodium nitroprusside (5 mM) in phosphate buffer saline was mixed with different concentrations of extract (2—10 mg/ml) dissolved in methanol and incubated at 25 °C for 30 min. After 30 min, 1.5 ml of the incubated solution were removed and diluted with 1.5 ml of Griess reagent (1% Sulphanilamide, 2% phosphoric acid, and 0.1% naphthyl ethelene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethelene diamine was measured at 546 nm along with a control. Ascorbic acid was used as a standard.

Hypochlorous Acid Scavenging Activity. Synthesis of Hypochlorous Acid For the assay, 75 μM HOCl was prepared immediately before use by adjusting a solution of NaOCl to pH 6.2 with dilute sulphuric acid. The concentration of HOCl was further determined spectrophotometrically at 235 nm using the molar extinction coefficient of 100 $M^{-1} cm^{-1}$.[15]

Synthesis of 5-Thio-2-nitrobenzoic Acid (TNB) TNB was prepared according to the described procedure.[16] Briefly to a 1 mM solution of DTNB in a 50 mM potassium phosphate buffer (pH 6.6) containing 5 mM EDTA, 20 mM sodium borohydride was added. The solution was incubated at 37 °C for 30 min. The concentration of TNB was determined by measuring the absorbance at 412 nm and using the molar absorbance coefficient of 13600 $M^{-1} cm^{-1}$.

Assay of Hypochlorous Acid Scavenging Activity The assay was performed at room temperature in a cuvette containing 40 μM TNB solution with or without extract (0.1, 0.5, 1, 2, 4 mg/ml). The absorbance at 412 nm was measured before and 5 min after the addition of hypochlorous acid (40 μM).[17] Lipoic acid was used as standard at 100 μM concentration.

Chelation of Fe^{2+} Ions Concentration of free iron ions (Fe^{2+}) was estimated using chelating agent 2, 2′-bipyridyl.[18] Briefly, the reaction mixture (1 ml) contained 50 μM $FeSO_4$, 50 μM NaCl (pH 7) and different concentrations of the extract and were incubated for 30 min, at the end of which 2 ml of 2,2′-bipyridyl (1 mM) was added. Absorbance of ferrous-bipyridyl complex was measured at 525 nm against the blank devoid of ferrous sulphate.

Hydrogen Peroxide Decomposition Hydrogen peroxide decomposition was determined according to the standard method.[19] The assay mixture contained 4 ml of H_2O_2 solution (80 mM) and 5 ml of phosphate buffer. 1 ml of the extract (10 mg/ml) was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. 1 ml portion of the reaction mixture was 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[20] 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.

Brain 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 1 week before the experiment. Animals were provided with rodent diet (Amruth, India) and water *ad libitum*. Male rat was fasted overnight and was sacrifised by cervical dislocation, and then whole brain was removed and weighed amount of brain was processed to get 10% homogenate in cold phosphate buffer saline (pH 7.4) using glass teflon homogeniser.

Assay of Lipid Peroxidation The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid-reactive substances (TBARS) by using the standard method[20] with minor modifications.[21] In brief, different concentration of extracts (200—1000 μg/ml) were added to the brain homogenate. Lipid peroxidation was initiated by adding 100 μl of 15 mM $FeSO_4$ solution to 3 ml of brain 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 TEP) and expressed as nmoles/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. Tocopherol at 100 μM concentration was used as standard.

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 defences. Potential antioxidant therapy therefore should include either natural free radical scavenging antioxidant enzymes or agents which are capable of augmenting the activity of these enzymes. Reactive oxygen species (ROS) has received considerable attention in the recent past because of its role in several pathological conditions including cancer, aging and atherosclerosis. ROS produced *in vivo* include superoxide radical $O_2^{\cdot -}$, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). $O_2^{\cdot -}$ and H_2O_2 can interact in presence of transition metal ions to yield a highly reactive oxidising species, the hydroxy radical.[22] 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.[23]

DPPH radicals react with suitable reducing agents losing colour stoichometrically with the number of electrons consumed which is measured spectrophotometricallty at 517 nm. As shown in Fig. 1, *D. gangeticum* extract strongly scavenged DPPH radical with the IC_{50} being 2.01 mg/ml. The scavenging was found to be dose dependent. Standard ascor-

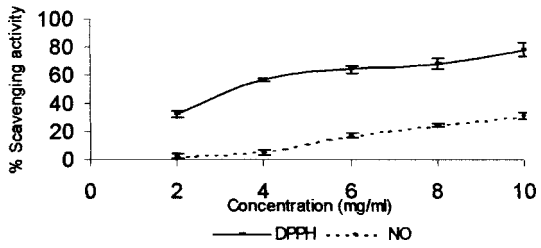

Fig. 1. Inhibitory Effect of *D. gangeticum* Extract on DPPH and Nitric Oxide Radical

Each value represents the mean±S.E. (n=6).

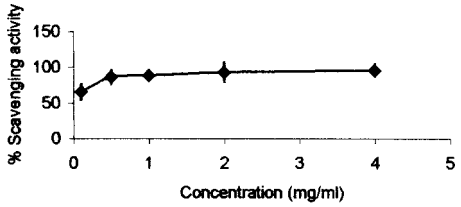

Fig. 2. Effect of *D. gangeticum* Extract on Oxidation of TNB by HOCl

Each value represents the mean±S.E. (n±6).

bic acid and was found to have 87.8% activity at 100 μM concentration.

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 ascorbic acid/g. Thus establishing the extract as an antioxidant.

D. gangeticum extract also moderately inhibited nitric oxide in dose dependent manner (Fig. 1) with the IC_{50} being 14.79 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. 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.[24] Thus the present study offers scientific evidence for the use of the plant in the indigenous system in inflammatory conditions. Standard ascorbic acid was found to have 79.8% activity at 100 μM concentration.

HOCl is produced *in vivo* by the oxidation of chloride ion catalyzed by neutrophil derived myeloperoxidase in the presence of H_2O_2 at the sites of inflammation.[25] *D. gangeticum* extract also exhibited HOCl scavenging activity as shown in Fig 2. Under the experiment conditions, the extract effectively scavenged HOCl in a concentration dependent manner with a protective effect of 96% at 4 mg/ml concentration. One of the major extracellular targets of HOCl is α_1-antiproteinase, the major circulating inhibitor of serine proteases such as elastase.[22] Thus the protective effects of the extracts *in vivo* during inflammation processess may be in part due to the HOCl scavenging ability. Standard lipoic acid was found to have 74.89% inhibition.

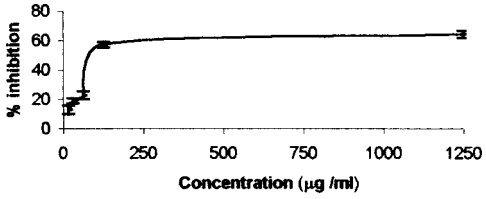

Fig. 3. Effect of Varied Concentration of *D. gangeticum* on Fe^{2+}-bipyridyl Complex Formation as Expressed by % Inhibition of Chromogen Formation

Each value represents the mean±S.E. (n=6).

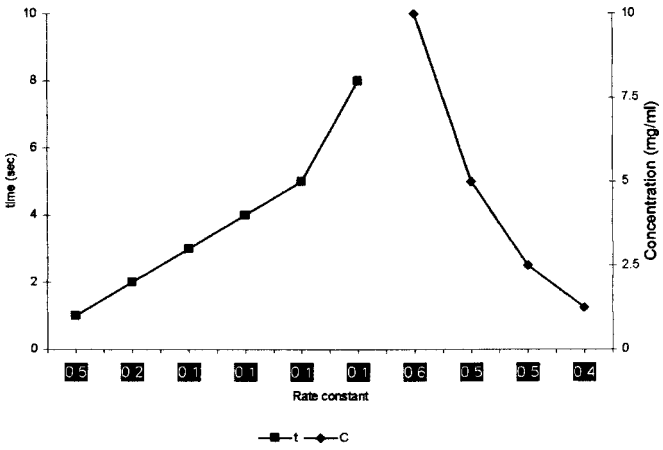

Fig. 4. Effect of *D. gangeticum* on Decomposition of H_2O_2

Each value represents the mean±S.E. (n=6), t=Time, c=Concentration.

Inorder to test the possibility of the change in Fe^{2+}/Fe^{3+} ratio, a separate experiment was performed. Effect of different concentrations of the extracts on inhibition of ferryl-bipyridyl (chromogen) formation has been depicted in Fig. 3. The extract inhibited the chromogen formation in a dose dependent fashion with the IC_{50} value of 115 μg/ml. Thus there is a possibility that the *D. gangeticum* extract chelating the ferrous form and thereby removing the free iron out of the reaction system. The other possibility could be change of ratio of Fe^{2+}/Fe^{3+}.

D. gangeticum extract also caused decomposition of the H_2O_2 in a dose dependent manner as shown in Fig. 4. Hydrogen peroxide is a weak oxidising 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 may of its toxic effects. 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.[26]

Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through OH Radical by Fenton's reaction. Figure 5 shows that the 50% aqueous alcoholic extract of *D. gangeticum* inhibited $FeSO_4$ induced lipid peroxidation in a dose dependent manner. IC_{50} values were found to be 0.64 mg/ml. The inhibition could be caused by absence of ferryl-perferryl complex

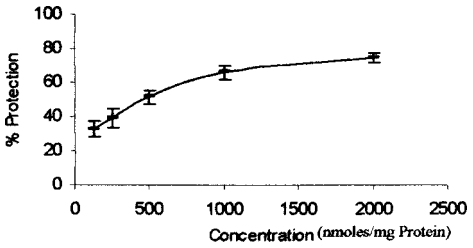

Fig. 5. Effect of *D. gangeticum* at Various Concentrations on Ferrous Sulphate Induced Lipid Peroxidation in Rate Brain Homogenate

Each value represents the mean±S.E. (n=6).

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 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.[18] 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.[22] 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. Standard tocopherol at 100 μM was found to have TBARS value of 0.06±0.001 μM/mg protein.

The results obtained thus indicate that *D. gangeticum* extract has potent antioxidant activity, achieved by scavenging abilities observed against DPPH, HOCl and lipid peroxidation. The plant has been reported to contain flavanoid and isoflavanoid glycosides[9—11] which are known antioxidants, hence the antioxidant activity of the hydroalcoholic extract of the plant also showed good antioxidant potential. Also the wide use of the plant in the Indian indigenous system of medicine as anti-inflammatory may be in part due to the antioxidant potential of the extract. Further, the isolation of the compounds responsible for the activity has to be taken up which may result in a modern drug from this plant.

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