

Antioxidant activity of *Desmodium gangeticum* and its phenolics in arthritic rats

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Accepted September 12, 2006

Total alcoholic extract of *Desmodium gangeticum*, which exhibited significant anti-inflammatory activity, was evaluated for the possible mode of action by studying its antioxidant potential in adjuvant-induced arthritic rats. Activity guided fractionation and isolation were carried out. The phenolics fraction showed maximum potency. Solid phase extraction followed by preparative HPLC of the active phenolic fraction yielded for the first time two potent antioxidant compounds, caffeic acid and chlorogenic acid, from this plant. The biological antioxidant defense system, involving superoxide dismutase, glutathione and catalase, showed a significant increase with their levels close to the normal control with a decrease in the lipid peroxide content upon administration of *D. gangeticum* extract (100 mg kg⁻¹) and its phenolics (50 mg kg⁻¹) in arthritic rats, thereby indicating the extracts antioxidant property under arthritic conditions.

Keywords: *Desmodium gangeticum* (Fabaceae), antioxidant potential, arthritis, phenolics, caffeic acid, chlorogenic acid

Reactive oxygen intermediates (ROI) produced by activated phagocytes in inflamed joints have been implicated, along with prostanoids, leukotrienes and proteases, as mediators of inflammation and pathogenesis of tissue destruction (1). At the onset of inflammation in rheumatoid arthritis (RA), there is a rapid drop in the serum iron level, followed by increased iron deposition in the synovial fluid. Such an increase in metal ion availability in the synovial fluid is thought to accelerate ROI mediated reactions and thus contribute to disease severity (2). Many drugs commonly used in the day to day treatment of RA are believed to mediate their therapeutic actions by multiple mechanisms, one being the reduction of oxidant damage at sites of inflammation by drugs acting either as ROI scavengers or inhibitors of ROI production by phagocytes (3). Under arthritic conditions, granulocytes and macrophages accumulate in the affected area and produce large amounts of superoxide and hydrogen peroxide radicals (4); estimation of these active species in disease-induced and drug-treated animals helps in assessing the free radical scavenging property and indirectly the anti-arthritic potential of the plant drug.

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Desmodium gangeticum (L.) DC. (*Fabaceae*) is a small shrub of tropical regions that has been used as a bitter tonic, febrifuge, digestive, anticatarrhal and antiemetic in inflammatory conditions of the chest and other organs (5). *D. gangeticum* has also been reported to contain alkaloids, flavone and isoflavanoid glycosides. Total alkaloids of this species showed anticholinesterase, smooth muscle stimulant, CNS stimulant and depressant responses (6, 7). The plant has been reported to contain gangetin, a pterocarpnoid shown to possess anti-inflammatory and analgesic activities (8). In our earlier paper, we reported the potent *in vitro* antioxidant activity of the 50% hydroalcoholic (total) extract (9). Phenolics are reported to be good antioxidant and anti-inflammatory agents. Since the plant under investigation is reported to contain both alkaloids and flavones along with isoflavanoid glycosides, we have undertaken the present study to evaluate its total extract and the phenolic fractions for *in vivo* antioxidant capacity in arthritic rats to ascertain certain therapeutic claims of the plant and identify the compounds responsible for the activity by measuring the ability to inhibit ferrous sulphate induced lipid peroxidation, glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in rat tissues and also to understand the mechanism behind the anti-inflammatory activity of the plant.

EXPERIMENTAL

Chemicals

Caffeic acid (99%), chlorogenic acid (95%), tetraethoxypropane (TEP), superoxide dismutase (SOD) and catalase (CAT) were obtained from Sigma, USA. Ferrous sulphate (FeSO_4), trichloroacetic acid (TCA), thiobarbituric acid (TBA), acetic acid, 2-nitrobenzoic acid (DTNB) and ethylene diamine tetracetic acid (EDTA) were obtained from Sd Fine Chemicals Ltd, India. All other reagents and solvents were of analytical grade.

Plant material

Aerial parts of *D. gangeticum* (L.) DC. were collected from Chitrakoot, Madhya Pradesh (India), during September of 2002. The plants were authenticated and the voucher specimen (code No. LWG 4502) was lodged in the departmental herbarium of the National Botanical Research Institute, Lucknow, India.

Extraction and isolation of bioactive phenolics

Aerial parts of *D. gangeticum* (1 kg) were oven dried (40–45 °C) and powdered coarsely. The dried powder was subjected to cold percolation in methanol for one week till exhaustive extraction. The extract was concentrated at reduced temperature (–5 °C) on a rotary evaporator (Büchi, USA) and then freeze-dried (Freezone® 4.5, Labconco, USA) under high vacuum (1.75×10^4 Pa) and at a temperature of -40 ± 2 °C. The residue obtained was dissolved in ethyl acetate and the resulting solution was repeatedly extracted with 5% hydrochloric acid until the extract showed only a weak reaction with Dragen-

borff's reagent. The acidic solution, after extraction with diethyl ether, was neutralized with 25% ammonia to pH 9 and exhaustively extracted with chloroform. The combined chloroform extract was washed twice with water, dried over sodium sulphate and evaporated to dryness to yield a crude base alkaloid extract (1.3 g). The whole process was repeated until the ethyl acetate soluble extract showed no reaction with ferric chloride. The ethyl acetate fraction was later dried to obtain the phenolic fraction (2.3 g) (10). Both the alkaloid and phenolic fractions were taken for *in vitro* antioxidant screening. The alkaloid fraction showed only 43.4% antioxidant activity.

The phenolic fraction was diluted with 30% aqueous methanol and passed under vacuum through a conditioned octadecyl silane (C18) micro-bore column. The column was conditioned using 10 mL methanol followed by 10 mL water. Vacuum manifold processor was used for solid phase extraction. The elutes containing the mixture of phenolics were then subjected to preparative HPLC using Waters (USA) PrepLC system 4000 series with a symmetry prep C18, 7 μm (19 x 300 mm i.d.) column with a flow rate of 19 mL min^{-1} . Water/phosphoric acid (100:0.3, V/V) as solvent A and acetonitrile/water/phosphoric acid (80:20:0.3, V/V) as solvent B were used for gradient elution in 0–5 min of 88–85% A, 5–30 min 85–70% A, 30–35 min 70–50% A and 30–40 min with 50–30% A and isocratic till 45 min with 30% A.

Animals

Male Sprague-Dawley 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*. All studies were conducted after obtaining prior approval from the institutional ethical committee in accordance with the National Institute of Health »Guide for the Care and Use of Laboratory Animals«.

Experimental design

The rats were divided into seven groups of six animals each. The first group served as the control that received only the vehicle (gum acacia). Arthritis was induced to other groups with 0.1 mL of Complete Freund's Adjuvant (CFA) by intra-dermal injection in the right hind paw. The second group animals received gum acacia only while the third group was administered the standard drug (indomethacin, 0.5 mg kg^{-1}). Group 4 received the total extract (100 mg kg^{-1}), group 5 received the phenolic fraction (50 mg kg^{-1}) while groups 6 and 7 received caffeic and chlorogenic acid (10 mg kg^{-1}), respectively, as emulsion in gum acacia with water daily for a period of 6 weeks by oral route (11). The hind paw swelling was recorded every week using a plethysmograph. The animals were sacrificed by cervical dislocation on the 42nd day. The liver was rapidly removed and washed with ice-cold saline. The tissues were cut into small pieces and homogenized using Tris buffer (0.01 mol L^{-1} , pH 7.4) to give 10% homogenate. Protein estimation was carried out according to Lowry *et al.* (12).

Antioxidant activity

Levels of lipid peroxides were estimated using the standard method of Okhawa *et al.* (13) with minor modification. TBARS was estimated by a reaction with thiobarbituric acid in the presence of butylated hydroxy toluene and by measuring the absorbance at 535 nm of the pink coloured chromogen formed against the reagent blank. Total reduced glutathione was measured by the DTNB assay (14). Super oxide dismutase activity was assayed by the method of Kakkar *et al.* (15) whereby inhibition of NADPH dependent chromogen formation by enzyme dismutase was measured. Catalase activity was assayed in cytosolic fractions according to the method of Cohen *et al.* (16). Estimations were made in rat liver homogenates.

Statistical analysis

Values were represented as mean \pm SEM and data were analyzed by paired-*t*-test using SPSS software for the Windows 11.0 package.

RESULTS AND DISCUSSION

Two of the compounds isolated by preparative HPLC were identified through co-chromatography (after spiking with standards) and spectral data. Retention time was 7.18 and 7.76 min for caffeic and chlorogenic acid, respectively. Both caffeic and chlorogenic acid are being reported for the first time from *Desmodium gangeticum*. Quantification was performed by calibration; the amount of caffeic acid (yield) in the flavonoid fraction and total plant was found to be 21.33 and 0.09%, respectively, while chlorogenic acid was found to be 22.66 and 0.12%, respectively. The total extract, along with phenolic fraction, and the isolated compounds were further tested for the *in vivo* antioxidant activity in arthritic rats.

The lipid peroxide activity significantly increased in the tissues of the arthritis-induced group animals compared to the control. The results showed that the phenolic fraction of *D. gangeticum* (group 5) exhibits significant inhibition ($p < 0.001$) of ferrous sulphate induced lipid peroxidation in liver (1.47 ± 0.04 nmol mg protein⁻¹) compared to the disease control group (Table I). A significant increase in glutathione content was observed in the tissues of the phenolic fraction treated group compared to the disease-induced control animals. The increase in glutathione in the liver of phenolic fraction treated group was remarkable compared to the control and indomethacin-treated groups (groups 2 and 3) (Table I). Catalase activity was also significantly ($p < 0.001$) increased in the phenolic fraction treated group compared to the disease control group (Table I). Reduced SOD activity was observed in the disease-induced group, whereas a significant ($p < 0.001$) increase in the SOD activity was found in the drug treated groups, with a maximum for the phenolic fraction (1.25 ± 0.27 U mg protein⁻¹), restoring the value close to the normal control (Table I).

Fractionation of the total plant extract followed by further purification through solid phase extraction followed by preparative HPLC yielded two very potent antioxidant compound, caffeic acid and chlorogenic acid, which showed potent activity against lipid

Table I. GSH, SOD and CAT levels and their effect on ferrous sulphate induced lipid peroxidation by *D. gangeticum*^a

Group ^b	TBARS (nmol mg protein ⁻¹)	GSH (μ g mg protein ⁻¹)	SOD (U mg protein ⁻¹)	Catalase activity (μ g mg protein ⁻¹)
1	1.46 \pm 0.07 ^c	3.50 \pm 0.28 ^c	1.24 \pm 0.29 ^c	25.2 \pm 1.80 ^c
2	2.27 \pm 0.08	1.87 \pm 0.16	0.58 \pm 0.07	11.99 \pm 3.45
3	1.56 \pm 0.04 ^c	3.15 \pm 0.09 ^e	1.08 \pm 0.12 ^c	24.29 \pm 2.76 ^c
4	1.72 \pm 0.11 ^d	3.01 \pm 0.09 ^d	0.66 \pm 0.11	18.31 \pm 2.17
5	1.47 \pm 0.04 ^c	3.49 \pm 0.15 ^c	1.25 \pm 0.27 ^c	25.23 \pm 6.92 ^c
6	1.54 \pm 0.07 ^c	3.42 \pm 0.13 ^c	1.16 \pm 0.11 ^c	24.32 \pm 2.97 ^c
7	1.56 \pm 0.04 ^c	3.31 \pm 0.17 ^c	1.09 \pm 0.27 ^c	24.96 \pm 2.79 ^c

^a The values represent the mean \pm SEM for six rats per group.

^b Group 1 (control), group 2 (arthritic-induced control), group 3 (arthritic-induced + indomethacin, 0.5 mg kg⁻¹), group 4 (arthritic-induced + total extract, 100 mg kg⁻¹), group 5 (arthritic-induced + phenolic fraction, 50 mg kg⁻¹), groups 6 and 7 (arthritic-induced + caffeic acid and chlorogenic acid, 10 mg kg⁻¹ respectively). Significant difference compared to group 2: ^c $p < 0.001$, ^d $p < 0.05$, ^e $p < 0.01$.

peroxidation significant SOD and catalase activity ($p < 0.001$), along with inhibition of lipid peroxidation. The potent activities of these compounds reported for the first time from this plant, from the most active phenolic fractions, prove that these compounds may be partly responsible for the antioxidant activity of the total extract and largely for the therapeutic efficacy of the plant.

Isolation of caffeic acid and chlorogenic acid from this plant is reported for the first time in this study. Several plant phenolics like caffeic acid can inhibit LDL oxidation *in vitro*; also flavonoids are often powerful inhibitors of lipid peroxidation; caffeic acid has been reported to possess a number of pharmacological attributes including antitumor activity (17) and immunomodulating activity (18). Caffeic acid, a phenolic related to the derivatives of hydroxycinnamic acids, is the principal component possessing antioxidant activity (19). Chlorogenic acid, a phenolic compound found ubiquitously in plants, is an *in vitro* antioxidant and metal chelator (20).

Arthritic rats showed soft tissue swelling around their ankle-joints during the acute phase of arthritis which was considered to be oedema of the particular tissues. The swelling was found to be increasing in the initial phase of inflammation and then remained constant for 2 weeks. An appreciable increase in paw volume was observed in group 2 compared to group 1. A significant reduction in paw-volume was observed in both total extract and phenolic fraction treated rats compared to the disease-induced group (Table II). This was observed in the third week of drug treatment. A moderate reduction in paw-volume was also observed in both caffeic and chlorogenic acid treated rats which could be one of the reasons for the activity of the total extract. Paw swelling is a major factor in evaluating the degree of inflammation and therapeutic efficacy of the drugs. In phenolic fraction and indomethacin-treated groups, reduction in paw-swelling from the third week onwards may be due to their immunological protection.

Table II. Effect of *D. gangeticum* on rat adjuvant induced arthritis

Group	PAW swelling (%) ^a
1	–
2	162.6 ± 6.2
3	82.3 ± 6.4 ^b
4	130.3 ± 4.7
5	88.3 ± 5.9 ^b
6	96.2 ± 3.7 ^c
7	102.5 ± 6.7 ^c

^a The values represent the mean ± SEM for six rats per group. Significant difference compared to control group 2: ^b $p < 0.001$, ^c $p < 0.01$.

The extent of lipid peroxidation is measured through malondialdehyde activity (MDA), a pro-oxidant factor that determines the oxidative damage. In the present study, MDA content of the liver of arthritis-induced animals (group 2) was found to be significantly increased compared to the normal group. This indicates that the tissues were subjected to increased oxidative stress while a statistically significant reduction of lipid peroxide activity was observed in drug treated groups. Thus, the potent inhibition of lipid peroxidation as showed by the phenolic fraction and total extract may be due to the presence of chlorogenic acid. Lipid hydroperoxide can be decomposed to produce alkoxy and peroxy radicals (LO[•] and LOO[•]), which eventually lead to numerous carbonyl products such as malondialdehyde that are responsible for DNA damage, generation of cancer and aging related diseases (21).

Low concentration of glutathione has been implicated in rheumatoid arthritis. In the present study, glutathione content was found to be lower in the disease-induced group while it was elevated in sample and standard groups. Phenolic fraction inhibited oxidation of reduced glutathione and depletion of GSH induced by FeSO₄ (Table I). GSH is a nonenzymic mode of defence against free radicals. *In vitro*, GSH can react with OH[•], hypochlorous acid (HOCl), peroxylnitrite, carbon centered radicals and singlet oxygen yielding thiyl radicals (GS[•]), which in turn can generate superoxide radicals. Hence, SOD might cooperate with GSH in helping remove free radicals *in vivo* (22).

CONCLUSIONS

The results indicate that *D. gangeticum* possesses a strong antioxidant activity, which might be responsible for its anti-arthritic activity. Caffeic acid and chlorogenic acid could be useful as antioxidants in experimental animal models and might be responsible for the antioxidant activity of the plant. It is likely that the antioxidant activity of *D. gangeticum* is a result of synergistic activities of its polyphenolic compounds.

Acknowledgements. – We are thankful to the Council of Scientific and Industrial Research (CSIR) for providing a research fellowship for the second author (MV).

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S A Ž E T A K

Antioksidativno djelovanje biljke *Desmodium gangeticum* i njezinih fenola u artritičnim štakora

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Ispitivan je mogući mehanizam protuupalnog djelovanja alkoholnog ekstrakta biljke *Desmodium gangeticum* na artritičnim štakorima. Nakon frakcioniranja, najjače djelovanje pokazale su fenolne frakcije. Ekstrakcijom i preparativnom HPLC kromatografijom iz fenolne frakcije izolirana su, prvi put iz te biljke, dva spoja sa snažnim antioksidativnim djelovanjem, kavena i klorogena kiselina. Biološki antioksidativni obrambeni sustav koji se sastoji od superoksidismutaze, glutationa i katalaze značajno je porastao u odnosu na kontrolnu skupinu, nakon davanja ekstrakta *D. gangeticum* (100 mg kg⁻¹) i njegovih fenola (50 mg kg⁻¹) artritičnim štakorima. Istovremeno se smanjio sadržaj peroksida u lipidima. To ukazuje da ekstrakt djeluje antioksidativno u stanju upale.

Keywords: *Desmodium gangeticum* (*Fabaceae*), antioksidativno djelovanje, artritis, fenoli, kavena kiselina, klorogena kiselina

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