

Evaluation of *In Vitro* Anti-Oxidant, Anti-Nociceptive and Anti-Inflammatory properties of *Desmodium gangeticum* (L.) in Experimental Animal Models

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ABSTRACT

The study was undertaken to evaluate the antioxidant activity of ethanolic extract of *Desmodium gangeticum* and compared with ascorbic acid (Standard) and the analgesic and anti-inflammatory activities in animal models. The extract has an anti-inflammatory effect demonstrated by its inhibitory effects on Carrageenan induced paw edema. *Desmodium gangeticum* leaves were studied indifferent models of inflammation in rats after oral administration at doses of 50, 100 and 200mg/kg. *Desmodium gangeticum* leaves significantly inhibited carrageenin, paw edema. It also produced significant increase in the hot plate reaction time in mice indicating analgesic effect. Anti-oxidant activity of ethanolic extracts of *Desmodium gangeticum* leaves was studied for its free radical scavenging property in different *in vitro* methods as 1, 1 diphenyl-2-picrylhydrazyl, nitric oxide, reducing power assay and hydrogen peroxide radical method. Different concentrations of ethanolic extract of DGE were prepared and evaluated by standard methods. The IC₅₀ values of ethanolic extract of DGE were compared with ascorbic acid (Standard) and it was noted that, the extract showed significant concentration dependent free radical scavenging property in all the methods. However this study therefore, supports its use in indigenous system of medicine both as analgesic and anti-inflammatory agent and calls for further investigations to elucidate its mechanism of action.

Keywords: *Desmodium gangeticum*; Anti-inflammatory; Analgesic; indigenous system of medicine

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INTRODUCTION

Desmodium gangeticum (L.) DC. (family: Fabaceae) Commonly known as

Shalparni is a small shrub of tropical region, which has been used in Indian system of

medicine as a bitter tonic, febrifuge, digestive, anti-catarrhal, antiemetic; in inflammatory conditions of chest and various other inflammatory conditions¹. Though the roots the plant is one of the ingredients of popular Ayurvedic drug – Dashmoola, a potent rejuvenating formulation used in Ayurveda. It is one of the constituents of ‘Dashmoola’ which is used traditionally for treatment of a number of diseases like jaundice, rheumatism, puerperal fever, paralysis, edema, filaria and post-natal care to avoid secondary complications. It is also used as a dietary supplement². Aqueous extract of the plant root has also been shown to have hypocholesterolemic and antioxidant effects in isoproterenol induced myocardial infraction³. The aqueous extracts of this species reported to have severe anti-writhing activity and moderate central nervous system (CNS) depressant activity⁴. Total alkaloids of this species showed smooth muscle stimulant, anticholinesterase, CNS stimulant and depressant responses⁵. The extracts of *Desmodium gangeticum* reported to have severe visceralleishmaniasis activity⁶.

MATERIALS AND METHODS

Plant Material

Fresh leaves of *Desmodium gangeticum* were collected from their natural habitats in and around Dehradun. Shade dried at room temperature (25⁰-35⁰ C) for 20-25 days. The plants were authenticated by comparison with the herbarium and voucher specimen was lodged in the departmental herbarium of Botanical Research survey of India Dehradun (Voucher Spaceman BSD112718).

Preparation of extract

The dried powdered leaves (1 kg) were extracted with 90% ethanol in a soxhlet extractor respectively. The plant material was

separated by filtration and ethanolic extract was concentrated (by Rotavapour, Büchi, Switzerland) and lyophilized to preserve it. The residue was obtained 18.2 gm. in ethanol. Dilutions of the extract were made in 2% gum acacia for the various studies.

Preliminary Phytochemical Investigation

Preliminary phytochemical screening of extracts was carried out to know the different constituents present in it as per the standard procedures Trease and Evans (1983)⁷. The extracts were tested for alkaloids, sterols, glycosides, phenolic compounds, flavonoids, carbohydrates, saponins and fats.

Isolation of Phytoconstituents

Isolation of phytoconstituent was done by column chromatography. The lower end of the column was plugged with glass wool. Hexane was poured in to the glass wool to release the air bubbles, which might be tapped with the flat end of the packaging rod. The column was clamped in the vertical position. It was filled up to three fourth the length of the column after mixing the adsorbent (silica gel) in to a slurry with the solvent and pouring the mixture in the glass tube. The stopper at the lower end was opened and the solvent was run off until the level falls to about 1 cm above the adsorbent. After the adsorbent settled, a filter paper disc was placed. Dissolved about 5 gm. of ethyl acetate extract in solvent and concentrated it to 10 ml volume and added at the top of the column by mean of pipette. Care was taken to ensure that it does not adhere to the wall of the column. The solvent was run off and sample was settled on the top of the column.

Animals

For anti-inflammatory activity Male Wistar rats (200–300 g), kept in the Animal House at the College of pharmacy, GIS IPS, Dehradun (U.K.) was used. After procuring

the animals were acclimatized for 10 days under standard husbandry conditions, room temperature ($27^{\circ} \pm 3^{\circ}\text{C}$), relative humidity ($65 \pm 10\%$) and 12 hours light / dark cycle. They were allowed free access to standard dry pellet diet and water *ad libitum* under strict hygienic conditions. All the described procedure were reviewed and approved by the Institutional Animal Ethical Committee (Reg. No: 1145/A/2007/CPCSEA).

Reagents and chemicals

Nitro blue tetrazolium, Riboflavin, Meta phosphoric acid and all the solvents used in the study were of analytical grade and were procured from S D Fine Chemicals Limited, Mumbai, India. 1, 1 diphenyl-2-picrylhydrazyl, 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), Vitamin C, thiobarbituric acid, malondialdehyde and other chemicals were obtained from Sigma Chemical Company (St. Louis, MO U.S.A). Carrageenan and Indomethacin were purchased from Hi-Media Laboratories, Mumbai, India. Water represents the double distilled water; standard orogastric cannula was used for the oral administration.

Pharmacological Evaluation

Anti-inflammatory study

The anti-inflammatory effects of the extracts were assessed using the carrageenan and Acetic acid-induced writhing test models in rats. The animals used for each of the tests were divided into five groups with each group containing five rats. The control group (A) and the reference group (E) received normal saline (10 ml/kg) and indomethacin (5 mg/kg) respectively. While the test groups (DG 1, DG 2 and DG 3) were treated with 50, 100 and 200 mg/kg of the extract, respectively. Saline, extract and indomethacin were all administered orally. Inflammation was produced by sub-plantar injection of 0.1 ml of

1% suspension of carrageenan with 2% gam acacia in normal saline, in the right hind paw of the rats, one hour after oral administration of the drugs (BDH; Winter., *et al* 1962). The standard reference group was treated orally with 100 mg/kg body weight of indomethacin aqueous solution while the control group was given normal saline. Measurement of paw size was carried out by wrapping a piece of cotton thread round the paw and the length of the thread corresponding to the paw circumference was determined using a meter rule (Hess and Miloning., 1972; Olajide., *et al* 2000). Measurement was done immediately before and 1–5 h following carrageenan injection. The inhibitory activity was calculated according to the following formula⁸.

Percentage inhibition =

$$\frac{(C_t - C_0)_{\text{control}} - (C_t - C_0)_{\text{treated}}}{(C_t - C_0)_{\text{control}}} \times 100$$

Where C_t = paw circumference at time t , C_0 = paw circumference before carrageenan injection and $C_t - C_0 = o$ edema.

Analgesic activity

Hot plate test

The analgesic tests were by the hot plate test and the formalin-induced paw licking test. The hot plate latency assay was based on the method of Eddy⁹. The extracts, saline and indomethacin were given to the animals orally after a 12 h fast. All the animals in each group were placed on a hot plate (maintained at $55 \pm 0.5^{\circ}\text{C}$). Reaction time was taken as the interval between the instant the animal reaches the hot plate till the moment the animal licks its forepaws or jumps out. Measurements were carried out 5 min before and 30, 90 and 150 minutes after oral administration of *Desmodium gangeticum* leaves crude suspension (500 mg/kg body weight). The control group was

given 1 ml normal saline while the standard reference group was treated with 1 ml (100 mg/kg body weight) aqueous solution of aspirin. Each result was calculated as the means of three readings.

% potential =

$$\frac{\text{Drug latency (Test)} - \text{Base line latency (Control)}}{\text{Base line latency (Control)}} \times 100$$

Formalin-induced paw licking tests

The formalin-induced paw licking was studied in rats using the method of Hunskaar and Hole¹⁰. In this method, 100 μ l of 3% formalin was injected into the subcutaneous tissue on the planter surface of the left hind paw of rats 1 h after oral administration of the extracts, normal saline or indomethacin. The rats in groups DG 1, DG 2 and DG 3 were given oral doses of the extracts 50, 100 and 200 mg/kg respectively 1 h before formalin injection. The rats in groups E and A were given oral doses of indomethacin (5 mg/kg) and an equivalent amount of normal saline (10 ml/kg), respectively 1 h before the injection. The time spent on licking the injected paw by each rat was observed as soon (0–5 min, post-injection) as the formalin was injected and later (late phase 20–30 min, post-injection). The mean of the time spent on licking the injected paw in each group was determined.

In Vitro Antioxidant Activity

DPPH Method

DPPH scavenging activity was measured by the spectrophotometric method of Facino RM¹¹. Stock solution of DPPH (1.5 mg/ml in ethanol) was prepared such that 75 μ l of it in 3 ml of ethanol gave an initial absorbance of 0.973. Decrease in the absorbance in presence of sample extract at different concentration (10-125 μ g/ml) was

noted after 15 min. IC₅₀ was calculated from % inhibition.

Protocol for DPPH Free Radical Scavenging Activity

Preparation of stock solution of test sample: 100 mg of extract was dissolved in 100 ml of ethanol to get 1000 μ g/ml solution.

- Dilution of test solution: 100, 200, 300, 400 and 500 μ g/ml solution of test were prepared from stock solution.
- Preparation of DPPH solution: 15 mg of DPPH was dissolved in 10 ml of ethanol. The resulting solution was covered with aluminum foil to protect from light.
- Estimation of DPPH scavenging activity: 75 μ l of DPPH solution was taken and the final volume was adjusted to 3 ml with ethanol, absorbance was taken immediately at 517 nm for control reading. 75 μ l of DPPH and 50 μ l of the test sample of different concentration were put in a series of volumetric flasks and final volume was adjusted to 3 ml with ethanol.
- Absorbance at zero time was taken in UV-Visible at 517 nm for each concentration. Final decrease in absorbance of DPPH with sample of different concentration was measured after 15 minute at 517 nm.

Percentage inhibitions of DPPH radical by test compound were determined by the following formula.

$$\% \text{ Reduction} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

Calculation of IC₅₀ value using graphical method.

Reducing Power¹²

The reducing power of the ethanolic extract of *Ceasalpinia bonduc* was determined according to the method of Oyaizu.

Accurately weighed 10 mg of the extract in 1 ml of distilled water were mixed in to the mixture of 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. Following incubation, 2.5 ml of 10% trichloro acetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1 %) and the absorbance was measured at 517 nm. Increased absorbance of the reaction mixture indicated the increased reducing power.

Assay for Nitric Oxide Scavenging Activity¹³

The method was followed for the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of the extract dissolved in ethanol and incubated at room temperature for 2½ h. The same reaction without the sample but equivalent amount of ethanol served as control. After incubation period, 0.5 ml of Griess reagent was added. Absorbance of the chromophore formed was measured at 546 nm. Ascorbic acid was used as positive control.

The procedure is based on the principle that, Sodium nitro prusside solution spontaneously generates nitric oxides, which reacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxides compete with oxygen leading to reduce production of nitrite ions.

Assay for Superoxide Radical Scavenging Activity¹⁴

The assay for superoxide radical scavenging activity was performed as per standard procedure. The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA and 0.1 mg/ml of NBT (nitro blue tetrazolium), all added in chronological sequence. Reaction

was started by illuminating the reaction mixture containing different concentrations of the sample extract for 90 sec and then measuring the absorbance at 590nm. Ascorbic acid was taken as the positive standard.

Statistical analysis

Results are expressed as mean ± S.E.M. statistical evaluations were made using Statistical significance was determined using the Student's *t*-test. Values with *P* < 0.05 compared with control were considered significant. Data are represented as mean ± S.E.M.

RESULTS

The study of the acute anti-inflammatory tests, the result show that oral pretreatment of animals with *Desmodium gangeticum* extract (50–200 mg/kg body weight) and indomethacin (5 mg/kg) significantly (*P* < 0.05) inhibited carrageenan-induced paw oedema (Table 1).

In the analgesic studies, the results from hot plate test show that at 30 min the oral doses of *Desmodium gangeticum* and indomethacin increases animal reaction time to from 6.7 ± 0.4 to 8.5 ± 0.7s likewise, at 60 and 90 min the reaction time were significantly increased compared to the control (Table 2). The formalin induced paw licking test, oral doses of *Desmodium gangeticum* extract and indomethacin decreased the time spent on licking (licking time) from 110.3 ± 8.4 to 52.6 ± 3.0 in the early phase after formalin injection. Similarly in the late phase after injection licking time was reduced from 65.9 ± 10.6 to 35.2 ± 2.8 (Table 3).

The free radical scavenging activity of the extract was examined in various in vitro models like, DPPH, NO, OH and lipid peroxidation was compared with the standards/ ascorbic acid. It was observed that ethanolic extract of *Desmodium gangeticum* leaves had higher activity than that of leaves.

At a concentration of 0.5 mg/ml, the scavenging activity of ethanol extract of *Desmodium gangeticum* leaves reached 53.89%. Though the DPPH radical scavenging abilities of the extract were less than those of ascorbic acid (98%) and Nitric Oxide and Super Oxide shows % Inhibition 87.21 and 92.31 at 1000µg/ml, the study showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants (Table 4 and 5).

DISCUSSION

Carrageenan-induced paw oedema shows that paw oedema was markedly inhibited by the oral administration of the extracts this is because carrageenan-induced paw oedema is an acute model of inflammation and has been reported to be active in detecting orally active anti-inflammatory drugs¹⁵.

The analgesic properties of the extract were studied using two important laboratory models. These include the hot plate latency method and the formalin-induced paw licking method. The hot plate method is sensitive to strong analgesic¹⁶.

Excessive generation of reactive oxygen species (ROS) leads to a variety of pathological processes such as inflammation, diabetes, hepatic damage and cancer¹⁷. The extract having the potentiality to scavenge the free radical contains flavonoids. Flavonoids have been reported to have anti-inflammatory, antiarthritic activity¹⁸.

In the analgesic studies, the results from hot plate test show that the oral doses of *Desmodium gangeticum* and indomethacin increases animal reaction time were significantly increased compared to the control. The ability of the decoction of *Desmodium gangeticum* in analgesic activity is may be due to the involvement of prostaglandins and other mediators in a

different order of magnitudes. Oral doses of *Desmodium gangeticum* extract and indomethacin decreased the time spent on licking (licking time). Additional investigations are required to find the biochemical way of pain modulation in order to describe the role of the aerial and root decoctions of *Desmodium gangeticum* and its interaction with the sensory nerves. Thus, the results of present study prove the claims of *Desmodium gangeticum* leaves mentioned in the Indian system of medicine.

CONCLUSION

The present investigation has shown that the leaf and stem extract of *Desmodium gangeticum* have active phytochemicals. The ethanolic leaves extract fractions showed strong antioxidant and anti-inflammatory properties were confirmed in the ethanol extract fractions. These activities may be due to strong occurrence of polyphenolic compounds such as flavonoids, tannins, terpenoids, phenols and Saponins. The antioxidant activity and anti-inflammatory activity was comparable with standard ascorbic acid, Indomethacin and aspirin. These findings provide scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of an analgesic, antioxidant and anti-inflammatory agent from *Desmodium gangeticum* plant. This medicinal plant by *in vitro* results appear as interesting and promising and may be effective as potential sources of novel analgesic, antioxidant and anti-inflammatory drugs.

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Table 1. Effect of *Desmodium gangeticum* leaves extract on carrageenan-induced paw oedema in rats

Groups	Dose orally (mg/kg)	Initial paw size (cm)	Paw oedema ^a (mm)		Inhibition (%)	
			3h	5h	3h	5h
a. Control (saline)	--	2.2 ± 0.2	7.6 ± 0.7	8.9 ± 0.6	--	--
b. DG1	50	1.9 ± 0.7	2.4 ± 0.8***	1.6 ± 0.3***	68.42	82.02
c. DG2	100	2.0 ± 0.1	2.2 ± 0.6***	0.5 ± 0.2***	71.05	94.38
d. DG3	200	1.8 ± 0.6	2.4 ± 0.7***	0.2 ± 0.3***	68.42	97.75
e. Indomethacin	5	1.8 ± 0.0	2.8 ± 0.9*	0.6 ± 0.5***	63.15	93.25

^aEach value is the mean ±S.E.M of five rats.

**P* < 0.05 compared with control.

****P* < 0.001 compared with Student's *t*-test.

Table 2. Effect of *Desmodium gangeticum* leaves extract on hot plate test in rats

Groups	Dose orally (mg/kg)	Reaction Time ^a (S)		
		30	60	90
a. Control (saline)	--	5.6±0.8	3.8 ± 0.6	2.9 ± 0.2
b. DG1	50	6.7 ± 0.4	9.1 ± 0.3***	10.2 ± 0.5***
c. DG2	100	7.1 ± 0.8	9.1 ± 0.4***	10.4 ± 1.0***
d. DG3	200	6.6 ± 0.3	9.2 ± 0.8***	10.4 ± 0.4***
e. Indomethacin	5	8.5 ± 0.7*	9.0± 0.8***	9.3 ± 0.7***

^aEach value is the mean ±S.E.M of five rats.

**P* < 0.05 compared with control.

****P* < 0.001 compared with Student's *t*-test.

Table 3. Effect of *Desmodium gangeticum* leaves extract on formalin-induced paw licking in rats

Groups	Dose orally (mg/kg)	Licking Time (S) ^a	
		Early Phase	Late Phase
a. Control (saline)	--	110.3 ± 8.4	65.9 ± 10.6
b. DG1	50	78.6 ± 9.1	59.1 ± 7.3
c. DG2	100	56.9 ± 8.6*	42.1 ± 3.4*
d. DG3	200	52.6 ± 3.0***	35.2 ± 2.8***
e. Indomethacin	5	74.6 ± 4.7*	47.0 ± 3.8***

^aEach value is the mean ± S.E.M of five rats.

**P* < 0.05 compared with control.

****P* < 0.001 compared with Student's *t*-test.

Table 4. In vitro antioxidant activity of ethanolic extract of *Desmodium gangeticum* leaves via DPPH Method –

DPPH Absorbance (control): 1.8144

S N.	Conc. of Sample (g/ml)	Absorbance at 517nm	%Inhibition
1	0.1	0.4538	25.01%
2	0.2	0.6953	38.32%
3	0.3	0.8144	44.88%
4	0.4	0.9216	50.79%
5	0.5	0.97794	53.89%

Table 5. In vitro antioxidant activity of ethanolic extract of *Desmodium gangeticum* leaves

Group	Conc ($\mu\text{g/ml}$)	% Inhibition	
		Nitric oxide	Super oxide
Ethanolic extract of <i>Desmodium gangeticum</i> leaves	250	70.39 \pm 4.84	67.51 \pm 2.78
	500	77.55 \pm 3.45	79.41 \pm 3.65
	750	82.28 \pm 5.23	87.66 \pm 3.51
	1000	87.21 \pm 3.11	92.31 \pm 2.63
Ascorbic acid	100	81.27 \pm 3.82	94.44 \pm 4.71
	150	78.63 \pm 4.62	51.62 \pm 3.52
	200	74.41 \pm 4.43	30.61 \pm 2.31
	250	65.52 \pm 2.76	21.42 \pm 1.62

Result expressed as mean \pm SEM from six observation **P<0.01

Each values is the mean \pm S.E.M and Data were analyzed by one way ANOVA