

Studies on *Dalbergia sissoo* (Roxb.) leaves: Possible mechanism(s) of action in infectious diarrhoea

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ABSTRACT

Objective: Several medicinal plants have been evaluated for their antidiarrhoeal activity. Most studies evaluated their effect on intestinal motility and antimicrobial activity and, therefore, did not take into account the pathogenesis of infectious diarrhoea. Features of infectious diarrhoea like abdominal pain, cramps, inflammation, and passage of blood/mucus in the stools are the combined effect of one or more virulence factors of the infecting organism. The effect of medicinal plants on the microbial virulent features can serve as marker(s) for testing their efficacy. In this study, we evaluated the effect of a decoction of dried leaves of *Dalbergia sissoo* on aspects of pathogenicity, that is, colonisation to intestinal epithelial cells and production/action of enterotoxins. This was done to define its possible mechanism(s) of action in infectious diarrhoea.

Materials and Methods: Antibacterial, antiprotozoal, and antiviral activities of the plant decoction were checked by agar dilution method, tube dilution method, and neutral red uptake assay, respectively. Cholera toxin (CT) and *Escherichia coli* labile toxin (LT) were assayed by ganglioside monosialic acid receptor ELISA. Suckling mouse assay was used to assess *E. coli* stable toxin (ST). As a measure of colonisation, the effect against adherence of *E. coli* and invasion of *E. coli* and *Shigella flexneri* to HEp-2 cells were studied.

Results: The decoction had no antibacterial, antiprotozoal, and antiviral activity. It reduced the production and the binding of CT and bacterial adherence and invasion.

Conclusion: This study showed that *D. sissoo* is antidiarrhoeal as it affects bacterial virulence. However, it has no antimicrobial activity.

KEY WORDS: Gastrointestinal infection, Indian rosewood, plant antimicrobial.

Introduction

Infectious diarrhoea is the most common infectious disease worldwide.^[1] Gastrointestinal infections kill 1.8 million people globally each year, mainly children in developing countries.^[2] Acute, watery, bloody diarrhoea may be due to a variety of pathogens- bacterial (e.g., *Escherichia coli*, *Vibrio cholerae*, *Shigella flexneri*, and *Campylobacter jejuni*), protozoal (e.g., *Giardia lamblia*, *Entamoeba histolytica* and *Cryptosporidium parvum*) and viral (e.g. rotavirus, astrovirus, and adenovirus) agents. These organisms disrupt intestinal functions and cause diarrhoea through several mechanisms. These include microbial attachment to the intestinal epithelium and localised effacement, production of toxin(s), and penetration and invasion of intestinal epithelial cells that result in alteration of absorption due to the rearrangement in cytoskeletal structure.^[1]

Dalbergia sissoo Roxb. (Fabaceae), known as Indian Rosewood, is reported to be useful in many conditions including

fever, ulcers, digestive disorders, and skin diseases.^{[3],[4]} It is also known to be effective against diarrhoea and dysentery.^{[3],[5]} Furthermore, this plant had the highest frequency of quote (5.2%) in an ethnobotanical survey carried out by us (unpublished observation). To the best of our knowledge, no experimental evidence is available on its antidiarrhoeal activity.

This work was, therefore, undertaken to assess the antidiarrhoeal activity of the dried leaves of *D. sissoo* on antimicrobial (antibacterial, antiprotozoal, and antiviral) activity and bacterial virulence parameters, such as, colonisation, production and action of toxins.

Materials and Methods

The study design

The institutional ethical committee (IEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) approved this study. The clearance from IEC was obtained in December, 2002.

Cell cultures, media, and reagents

The human laryngeal epithelial cell line, HEp-2, and the embryonic monkey kidney cell line, MA104, were obtained from National Centre for Cell Sciences, Pune, India. The cell lines were maintained in Dulbecco's modified eagle medium (DMEM) and minimal essential medium (MEM), respectively, supplemented with 5% fetal calf serum (FCS) in 60 mm diameter tissue culture dishes (Tarsons Pvt. Ltd, Kolkata) at 37°C in a 5% CO₂ atmosphere. The cells were maintained in logarithmic growth by passage every 3-4 days.

The bacterial growth media and MEM were purchased from Himedia laboratory, Mumbai, India. DMEM and FCS were procured from GibcoBRL, UK. The constituents of the Diamond's medium for *G. lamblia* and the antibiotics (penicillin, streptomycin, gentamicin, and metronidazole) were procured from local manufacturer. Trypan blue, neutral red, polymyxin B sulphate, anticholera toxin, and bovine serum albumin were purchased from Sigma, USA, and swine anti-rabbit immunoglobulin (Ig) was obtained from Dako, Denmark.

The 96-well ELISA plates were purchased from Nunclon, Denmark, and the ELISA plate reader was purchased from Labsystems, Finland.

Preparation of plant extract

D. sissoo leaves were collected from Parinche valley, Pune district, Maharashtra in March 2004 and a voucher specimen deposited at the Botanical Survey of India, Pune, under herbarium number 124673. The decoction was prepared by boiling 1 g of the shade dried leaves in 16 ml of the distilled water till the volume was reduced to 4 ml. To replicate the conditions in field, fresh decoction was prepared every time. The decoction was centrifuged and filtered through a membrane of 0.22 µm pore size before use. The yield of the decoction obtained was 16.7% ± 0.02% (w/w) with respect to the starting material. For each experiment, 1%, 5%, and 10% (v/v) concentrations of the decoction in appropriate media were used.

Phytochemical analysis

Qualitative phytochemical analysis was carried out using standard procedures^[6] to determine the presence of carbohydrates, glycosides, proteins, amino acids, phytosterols, saponins, flavonoids, alkaloids, and tannins.

Microorganisms used

E. coli B170, *E. coli* B 831-2, *E. coli* TX1 (all obtained from Centre for Disease Control, Atlanta, USA), *E. coli* E134 (kindly provided by Dr. J. Nataro, Veterans Affairs Medical Centre, Maryland, USA), *V. cholerae* El Tor (kindly provided by Dr. S. Calderwood, Massachusetts General Hospital, Boston, USA), *S. flexneri* M90T (kindly provided by Dr. P. Sansonetti, Institut Pasteur, France), *G. lamblia* P1 (kindly provided by Dr. P. Das, National Institute of Cholera and Enteric Diseases, Kolkata, India), and rotavirus SA-11 (kindly provided by Dr. S. Kelkar, National Institute of Virology, Pune, India) were used as representative organisms.

Antimicrobial activity

The protocol followed for assaying the antibacterial activity of the decoction was the agar dilution method.^[7] A log phase culture of each bacterium grown in nutrient broth was plated

onto nutrient agar (NA) without (control) and with different dilutions of the decoction and incubated at 37°C for 18-20 h. Thereafter, the viability of individual strain was graded on a scale of 0 (no growth) to +4 (control) depending on the extent of the growth. Gentamicin (100 µg/ml) was used as the antibiotic control.

The antiprotozoal activity was assayed by incubating a 24 h culture of *G. lamblia* trophozoites without (control) and with different dilutions of the decoction for 24 h. The number of viable trophozoites was counted in a haemocytometer with trypan blue.^[8] The anti-giardial drug metronidazole (100 µg/ml) was used as the positive control.

The antiviral activity was determined by assaying the entry and the subsequent survival of rotavirus in MA-104 cells by the neutral red uptake assay.^[9] Briefly, a 72 h culture of MA-104 cells was infected with rotavirus and incubated without (control) and with different dilutions of the decoction for 90 min. The culture was further incubated for 72 h after removal of the decoction and the unabsorbed virus. Thereafter, the cells were incubated with neutral red dye for 30 min. The intracellular dye was released with 1:1 solution of 100 mM acetic acid and ethanol. The released dye was measured at 540 nm (reference 630 nm) in an ELISA plate reader.

Effect on toxins

The *E. coli* labile toxin (LT), an enterotoxin, was obtained from *E. coli* B831-2 by lysing the bacterial cells with polymyxin B sulphate.^{[10], [11]} Cholera toxin (CT), an exotoxin, was obtained as a culture supernatant of *V. cholerae*. LT and CT were assayed by a modification of the ganglioside monosialic acid enzyme linked immunosorbent assay (GM1-ELISA).^[11] Briefly, the toxins were added to ELISA plates pre-coated with the receptor GM1. Anticholera toxin and peroxidase labeled swine antirabbit Ig were used as primary and secondary antibodies, respectively. The colour developed was read at 492 nm in an ELISA reader.

The assays were based on two protocols:

- i) *Preincubation*: Bacterial strains were grown without (control) and with different dilutions of the decoction in casein hydrolysate yeast extract broth (CAYE), and the LT/CT produced by the respective bacterial strains was assayed using the GM1-ELISA.
- ii) *Competitive*: To detect if the plant extract competes for binding with GM1, the GM1-ELISA was done wherein the toxin was assayed without (control) and with different dilutions of the decoction.

Stable toxin (ST), which is an exotoxin, obtained as a culture supernatant of *E. coli* TX1 was assayed by the method originally described by Gianella.^[12] Briefly, the toxin was inoculated intra-gastrically into 2-3 day-old Swiss albino suckling mice. Following an incubation of 3 h at room temperature, the pups were sacrificed and the ratio of gut weight to that of the remaining carcass weight was calculated. Ratio of ≥ 0.083 was considered as positive.

The assays were based on two protocols:

- i) *Preincubation*: ST obtained as supernatant of the bacterial culture grown without (control) and with different dilutions of the decoction in CAYE was assayed by intra-gastric inoculation of suckling mice.

- ii) *Competitive*: ST was intra-gastrically inoculated in the suckling mice without (control) and with different dilutions of the decoction.

Effect on colonisation to HEp-2 cell line

The effect of the decoction on the adherence of *E. coli* strain B170 to epithelial cells was assayed by a method described earlier.^[13] Briefly, a 48 h culture of HEp-2 cells was infected with a log phase culture (5×10^7 cells/ml) of *E. coli* B170 grown in brain heart infusion broth (BHI) and incubated for 3 h. Non-adherent bacteria were washed off and the microcolony formation was observed by toluidine blue staining (0.1% w/v). HEp-2 cells having ≥ 5 adherent *E. coli* cells were counted.

The effect of the decoction on invasion by *E. coli* E134 and *S. flexneri* was studied by a method described by Vesikari et al.^[14] Briefly, a 48 h culture of HEp-2 cells grown in a 24-well tissue culture plate was infected with log phase culture (10^8 cells/ml) of the bacterial grown in BHI and incubated for 2 h. The culture was further incubated with gentamicin (100 μ g/ml) for 3 h to kill the uninvaded bacteria. The epithelial cells were then lysed by cold shock, and the released bacteria were counted by plating on NA.

The assays were based on two protocols:

- Preincubation*: HEp-2 cells were incubated without (control) and with different dilutions of the decoction in DMEM for 18–24 h prior to incubation, with the respective bacterial strain.
- Competitive*: Bacterial strain and HEp-2 cells were simultaneously incubated in DMEM without (control) and with different dilutions of the decoction.

Statistical analysis

Each assay was performed three times and the results were expressed as their mean \pm SD. The differences in the mean values of the treated groups were analysed by analysis of variance (ANOVA). Furthermore, the significance of the difference between the means of the test and the control observations was established by Dunnett's post-test. Statistical analyses were performed using the programme Prism 4.0 (GraphPad, Inc.). $P \leq 0.05$ was considered to be statistically significant

Table 1.

Effect of a decoction of *Dalbergia sissoo* leaves on *E. coli* labile toxin and cholera toxin.

<i>D. sissoo</i> extract		% Reduction in optical density			
		Cholera toxin assay (<i>V. cholerae</i>)		Labile toxin assay (<i>E. coli</i> B-831-2)	
		Competitive ¹	Pre- incubation ²	Competitive ¹	Pre- incubation ²
1% Decoction		8.16 \pm 6.42	3.41 \pm 13.05	11.70 \pm 2.21	-30.39 \pm 20.52 [†]
5% Decoction		8.01 \pm 5.17	22.32 \pm 12.07*	22.45 \pm 6.61**	-58.26 \pm 34.77** [†]
10% Decoction		16.99 \pm 8.60**	28.73 \pm 12.09**	28.21 \pm 4.71**	-104.98 \pm 17.73*** [†]
One-way	F	4.075	6.581	26.27	12.28
ANOVA	df	2,6	2,6	2,6	2,6
	P	0.0498	0.0149	0.0002	0.0023

Values are mean \pm SD (from three individual experiments), of percentage reduction in optical density compared with respective controls (100%).¹Incubation of the toxin on to GM1 receptor carried out in presence of the decoction. ²Toxin produced by bacteria grown in the presence of decoction. * $P < 0.05$; ** $P < 0.01$ by Dunnett's post-test. [†] The negative values indicate that there was an increase in the production of labile toxin.

Results

Phytochemistry

The decoction contained carbohydrates, proteins, flavonoids, and tannins. Phytosterols, glycosides, saponins, and alkaloids were absent.

Antimicrobial activity

The decoction exhibited no antibacterial activity. Similarly, there was no effect on the viability of *G. lamblia* trophozoites or on the entry of rotavirus into MA-104 cells.

Effect on toxins

The decoction inhibited the production of CT [Table 1], while it increased the production of LT. Binding of both LT and CT to the GM1 receptor was reduced. [Table 1] Neither the production nor the action of ST was affected.

Effect on colonisation

The adherence of *E. coli* B170 to the epithelial cells was reduced when the HEp-2 cells were incubated with the decoction prior to infection. [Table 2] The adherence was also reduced when the cells were incubated with the decoction, simultaneously with the infection. Similarly, the decoction significantly reduced the invasion [Table 2] of both *E. coli* E134 and *S. flexneri* to the epithelial cells in both the protocols.

Discussion

According to the World Health Report 2004, diarrhoea is the cause of 3.3% of all deaths.^[2] The past decade has witnessed several attempts towards the management of diarrhoea. These include improved formulations of oral rehydration solution (ORS) and the development of a feasible vaccine. Although ORS has contributed to reduction in diarrhoeal mortality rates, it is often less efficient in high stool output state. In addition, response to vaccines in developing countries is not encouraging.^[15] With the threat of drug resistance, a definite niche exists for the development of an alternative approach to treat infectious diarrhoea. Medicinal plants can fill this niche. This study was an attempt to explore the anti-diarrhoeal activity of crude decoction of *D. sissoo* leaves.

Table 2

Effect of a decoction of *Dalbergia sissoo* leaves on bacterial colonisation to HEp-2 cells.

<i>Dalbergia sissoo</i> extract	% Reduction in colonization ^a					
	Adherence assay (<i>E. coli</i> B170)		Invasion assay (<i>E. coli</i> E134)		Invasion assay (<i>S. flexneri</i>)	
	Competitive ¹	Pre-incubation ²	Competitive ¹	Pre-incubation ²	Competitive ¹	Pre-incubation ²
1% Decoction	4.68±0.97	14.88±3.18**	26.29±22.53	43.95±13.98**	84.39±8.87**	62.97±13.84**
5% Decoction	20.51±1.93	100±0**	57.84±2.1**	75.50±19.27**	88.87±4.08**	79.52±19.97**
10% Decoction	74.62±22.03**	100±0**	62.74±16.52**	96.22±4.94**	79.23±1.23**	97.16±3.45**
One-way F	28.74	3435	13.15	35.61	221.3	35.68
ANOVA df	2,6	2,6	2,6	2,6	2,6	2,6
P	0.0001	<0.0001	0.0018	<0.0001	<0.0001	<0.0001

^a Values represent mean ± SD (from three individual experiments), of percentage reduction in colonisation compared with respective controls (100%). ¹Infection of HEp-2 cells carried out in presence of the decoction. ² HEp-2 cells incubated with the decoction prior to the infection. **P* < 0.05; ***P* < 0.01 by Dunnett's post-test.

The decoction did not have antibacterial activity against the strains tested nor did it have anti-giardial or antirotaviral activity. It was observed that although the decoction did not arrest the growth of *V. cholerae*, it prevented the production of CT indicating that the reduction in the production of CT was metabolic and not due to reduction in bacterial counts. There was a two-fold increase in the production of LT in the presence of the decoction, but its binding to the receptor was reduced. It is known that LT and CT are closely related structurally, functionally, biologically, and immunogenically.^[16] Therefore, the reduction in binding of LT and CT to GM1 receptor implies that the decoction may contain chemical(s) that either bind(s) directly to the receptor or to the common antigenic moiety of the toxins.

The decoction also affected colonisation. It inhibited the adherence of *E. coli* B170 and invasion by *E. coli* E134 and *S. flexneri*. The decrease in colonisation was observed in both the protocols suggesting that *D. sissoo* modifies/affects receptors on HEp-2 cells in a way that restricts bacterial attachment and entry. This is especially true because the decoction did not affect the morphology of the HEp-2 cells and, as mentioned earlier, had no antibacterial activity.

The findings of the biological assays are indicative of the selective antidiarrhoeal action of *D. sissoo* leaves. The results suggest that the leaves may not be active against diarrhoea induced by LT and ST or those caused by protozoa and virus. However, it appears to be most efficacious against cholera and diarrhoeal episodes caused by enteropathogenic and enteroinvasive bacterial strains.

To conclude, this study besides describing the possible mechanisms of antidiarrhoeal action of *D. sissoo* leaves also highlights the necessity of including multiple parameters for judging the efficacy of medicinal plants. Assaying bacterial virulent features as a marker for demonstrating the antidiarrhoeal efficacy of a plant, has been previously reported by us using two indigenous plants viz. *Cyperus rotundus*^[17] and *Holarrhena antidysenterica*.^[18] This is especially important in the absence of antimicrobial activity, which in most of the studies reported earlier^[19-22] has been considered the marker for antidiarrhoeal activity.

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The **Motan Devi Dandiya Prize** in **Pharmacy** has been awarded to **Dr. R.B. Umamaheshwari** for her work on “Receptor-mediated targeting of lipobeads bearing acetohydroxamic acid for eradication of *Helicobacter pylori*” carried out at Department of Pharmaceutical Sciences, Dr HS Gaur University, Sagar. The certificate of merit in this category has been recommended for **Dr. Sushama Talegaonkar**, Lecturer, Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, New Delhi.

The **Chandra Kanta Dandiya Prize** in **Pharmacology** has been awarded to **Mr Jayesh B Majithiya** of Pharmacy Department, Faculty of Technology and Engineering, MS University, Baroda for his work on “Effect of pioglitazone on L-NAME and streptozotocin induced hypertension and diabetic rats and time dependent changes in antioxidant enzymes and vascular reactivity of aorta in streptozotocin induced diabetic rats treated with curcumin”. The certificate of Merit in this category has been recommended for **Mr. Vijai Pal Singh**, Research Associate, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh.

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