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Studies on *Pongamia pinnata* (L.) Pierre leaves: understanding the mechanism(s) of action in infectious diarrhea*

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Abstract: While data are available on the effect of medicinal plants on intestinal motility and their antibacterial action, there is a paucity of information on their mode of action on various aspects of diarrheal pathogenicity, namely colonization to intestinal epithelial cells and production/action of enterotoxins. Crude decoction of dried leaves of *Pongamia pinnata* was evaluated for its antimicrobial (antibacterial, antigiardial and antirotaviral) effect; and its effect on production and action of enterotoxins (cholera toxin, CT; *Escherichia coli* labile toxin, LT; and *E. coli* stable toxin, ST); and adherence of enteropathogenic *E. coli* and invasion of enteroinvasive *E. coli* and *Shigella flexneri* to epithelial cells. The decoction had no antibacterial, antigiardial and antirotaviral activity, but reduced production of CT and bacterial invasion to epithelial cells. The observed results indicated that the crude decoction of *P. pinnata* has selective antidiarrheal action with efficacy against cholera and enteroinvasive bacterial strains causing bloody diarrheal episodes.

Key words: *Pongamia pinnata*, Medicinal plants, Diarrhea, Antidiarrheal agent, Enterotoxin, Colonization
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INTRODUCTION

Diarrhea occurs worldwide and causes 3.2% of all deaths (WHO, 2004). It is the second largest cause of years of productive life lost due to premature mortality and disability (Murray and Lopez, 1997a). Even though economic development and progress in health care delivery are expected to catalyze substantial improvements in infectious disease related morbidity and mortality by the year 2020, it is predicted that diarrhea will remain a leading health problem (Murray and Lopez, 1997b). Diarrhea is most commonly caused by gastrointestinal infections, which kill around 1.8 million people globally each year, mostly children in developing countries (WHO, 2004). The main cause of death from diarrhea is dehydration, which results from loss of electrolytes in diarrheal stools.

During the past decade there have been some major improvements regarding the treatment of infectious diarrhea. Oral rehydration therapy has contributed greatly to the reduction of diarrheal mortality rates in children and the elderly. However, the diarrheal attack rate has remained unchanged and this treatment often fails in the high stool output state. Symptomatic therapy with antimotility agents is restricted to non-dehydrated patients without features of systemic infection. These are not indicated in infants and patients with febrile bloody diarrhea. Moreover, there is an increasing threat of drug resistance, side effects of treatment with antibiotics, supra infection

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when normal flora is eradicated with antimicrobial agents and the possibility of induction of disease producing bacteriophages by antibiotics (Dham, 2003). Vaccines have been considered as the most feasible approach to diarrheal management. Various attempts for developing vaccines against diarrhea causing organisms have been made (Cohen *et al*., 2000; Ghose, 1996; Klee *et al*., 1997; Martines *et al*., 1993). However, the response to such vaccines in developing countries has not been encouraging (Lagos *et al*., 1999). Considerable technical barriers need to be overcome before continuing clinical evaluation of prospective vaccine candidates. Thus an important niche exists for development of cost effective alternative approaches and medicinal plants may serve to fulfill this niche.

Despite the phenomenal growth and success of modern medicine, plant remedies have gained immense popularity over the recent years as cures for treatment of several ailments. With emphasis being on scientific validation, a number of plants have been chemically and biologically evaluated for their acclaimed properties.

A number of medicinal plant preparations have been recognized for treating diarrhea, although their mode of action in elimination of organisms causing diarrheal diseases is not well understood. While data are available on the effect of various plants on intestinal motility in experimental models and antibacterial action (Akah *et al*., 1999; Kambu *et al*., 1990; Miranda *et al*., 1993; Tona *et al*., 1999), there is paucity of information on the mode of action of medicinal plants on various aspects of diarrheal pathogenicity.

The pathogenesis of infectious diarrhea has been extensively studied. It is caused by a variety of enteric pathogens including bacteria such as enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), *Salmonella*, *Shigella flexneri*, *Vibrio cholerae*, and *Campylobacter jejuni*; viruses such as rotavirus, astrovirus, adenovirus, and calicivirus; and protozoa such as *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium parvum* (Martines *et al*., 1993; Bhattacharya, 2000; Guerrant and Bobak, 1991). Mechanisms by which these organisms disrupt intestinal function to cause malabsorption or diarrhea are microbial attachment and localized effacement of the epithelium, production of toxin(s) and direct epithelial cell invasion (Guerrant *et al*., 1999).

Adherence is a means of colonizing the appropriate ecological niche. It enables the organism to resist being swept away by mucosal secretions and also helps the organism in subsequent proliferation and colonization of the gut. Adherence can be followed by toxin production or invasion (Ashkenazi and Pickering, 1989). Furthermore, bacterial adherence may increase the efficiency of these toxins particularly in the intestinal tract, where proteolytic enzymes may inhibit the long-term effect of toxins.

Toxigenic diarrhea is caused by enterotoxins secreted by bacteria (e.g., *E. coli* stable toxin), some of which act directly on a transmembrane signaling peptide such as membrane bound particulate guanylate cyclase. Toxins such as *E. coli* haemolysin, listeriolysin, and Streptolysin O alter membrane permeability, while others enzymatically alter specific intra cellular targets (Ashkenazi and Pickering, 1989).

Invasive diarrhea is caused by damage to intestinal epithelial cells due to invasion and penetration by the organism. Pathogens like *Salmonella* sp., *Shigella* sp., *Listeria* sp., EIEC, and *Yersinia* sp. (Kagnoff, 2003) infect deeper layers of intestinal mucosa, and spread systemically while *C. parvum*, and *Chlamydia trachomatis* (Kagnoff, 2003) are minimally invasive. These pathogens invade the intestinal epithelium, where they reside and replicate.

Leaves of *Pongamia pinnata* (L.) Pierre (synonym, *P. glabra* vent) has been known as a remedy for diarrhea (Nadkarni, 1954; Warrier *et al*., 1993). Shoba and Thomas (2001) reported on the effectiveness of *P. pinnata* in controlling castor oil induced diarrhea. However, there is no information on its effect on infectious forms of diarrhea. Hence we selected *P. pinnata* for studying its effect on various parameters such as adherence to and invasion of intestinal epithelium and production and action of toxins towards understanding its possible mechanism(s) of action in controlling infectious diarrhea.

MATERIALS AND METHODS

Plant materials

Fresh leaves of *P. pinnata* were collected from

Parinche Valley (near Pune, Maharashtra, India) in November, 2003. The plant was authenticated by Dr. P. Tetali, Naoroji Godrej Centre for Plant Research. A voucher specimen has been deposited at the Botanical Survey of India (Western Circle, Pune, India), under herbarium No. 124677. The leaves were shade dried, hand crushed and stored at 4 °C. All experiments were performed with the same dried material within six months from the date of collection.

Preparation of extract

The decoction was prepared by boiling 1 g of the shade dried leaves in 16 ml double distilled water till the volume reduced to 4 ml as described in Ayurvedic text (Thakkur, 1976). To replicate field conditions, studies were carried out with only the hot water decoction that was freshly prepared every time. The decoction was centrifuged and filtered through a 0.22 μm pore size membrane before use. The yield of the decoction thus obtained was 8.15%±0.42% (w/w) of the starting material. For each experiment, 1%, 5%, and 10% (v/v) concentrations of the decoction in appropriate medium were used.

Cell culture and media

The human laryngeal cell line, HEp-2, and the embryonic monkey kidney cell line, MA-104, were obtained from the National Centre for Cell Sciences, Pune, India. The cell lines were maintained in Dulbecco's modified eagle's medium, DMEM (GibcoBRL, UK) and minimal essential medium, MEM (Himedia, Mumbai, India) respectively, supplemented with 5% fetal calf serum (GibcoBRL, UK), in 60 mm-diameter tissue culture dishes (Tarsons Pvt. Ltd., Kolkata, India). The cells were maintained in logarithmic growth by sub-culturing every 3~4 d.

Antibacterial activity

The antibacterial activity was determined against six different bacterial strains viz. *E. coli* B170, *E. coli* B831-2, *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* M9OT (kindly provided by Dr. P. Sansonetti, Institut Pasteur, France), by minimum inhibitory concentration plate method (Cruickshank *et al*., 1975). Log phase cultures (10^6 cells/ml) were plated onto nutrient agar containing the decoction and the growth was graded on a scale of 0 (no growth) to 4+ (control). Gentamycin (100 μg/ml) was used as the antibiotic control.

Antigiardial activity

A 24 h culture of *Giardia lamblia* P1 trophozoites (kindly provided by Dr. P. Das, National Institute of Cholera and Enteric Diseases, Kolkata, India) was incubated with the decoction in Diamond's TYI-SS medium (constituents procured from local Indian manufacturers), supplemented with bovine serum (Sigma, USA). The number of viable trophozoites after 24 h was counted in a haemocytometer using the vital stain, trypan blue (HiMedia, Mumbai) (Trowell, 1965). Metronidazole (100 μg/ml) was used as an antibiotic control.

Antirotaviral activity

The entry and subsequent survival of rotavirus (kindly provided by Dr. S. Kelkar, National Institute of Virology, Pune, India) in MA-104 cells were assayed by the neutral red uptake assay (Parish and Mullbacher, 1983). Briefly, MA-104 cells were grown in 96-well tissue culture plates for 72 h. The cells were then infected with rotavirus in the presence of trypsin (0.4 μg/ml) for 90 min without and with different concentrations of the decoction. The rotavirus was previously treated with trypsin (7 μg/ml) at 37 °C for 30 min. After infection, the decoction and the unadsorbed virus were removed and the culture was further incubated for 72 h. Thereafter, the cells were incubated with 0.03% neutral red dye (Sigma, USA) for 30 min. The intracellular dye was released with 1:1 (v/v) solution of 100 mmol/L acetic acid and ethanol. The released dye was measured at 540 nm (reference 630 nm) in an ELISA plate reader (Thermo Electron Corp., Finland) as an indicator of cell viability.

Effect on adherence

The effect on the adherence of *E. coli* strain B170 to epithelial cells was assayed by a method described by Cravioto *et al*.(1979). 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 and incubated for 3 h. Non-adherent bacteria were washed off and

microcolony formation was observed by toluidine blue staining (0.1%, w/v). HEp-2 cells having ≥5 adherent *E. coli* cells were counted.

The adherence assay was performed using two different protocols. The HEp-2 cells were incubated with different concentrations of the decoction either before infection (pre-incubation) or simultaneously with the infection (competition).

Effect on invasiveness

The effect on invasion of *E. coli* E134 and *S. flexneri* was based on a method described by Vesikari *et al*.(1982). 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 bacteria and incubated for 2 h. The culture was further incubated with gentamycin (100 μg/ml) for 3 h. The epithelial cells were then lysed by cold shock and the released bacteria were counted by plating on nutrient agar.

The invasive assay was performed using two different protocols. The HEp-2 cells were incubated with different concentrations of the decoction either before infection (pre-incubation) or simultaneously with the infection (competition).

Effect on labile toxin (LT) and cholera toxin (CT)

An endotoxin, LT, was obtained from *E. coli* B831-2 by lysing the bacterial cells with polymyxin B sulphate (Sigma, USA). CT was obtained as culture supernatant of *V. cholerae*, as it is an exotoxin. Both toxins were assayed by the ganglioside monosialic acid-enzyme linked immunosorbent assay (GM1-ELISA) (Svennerholm and Wilkund, 1983). Briefly, the toxins were added to ELISA plates pre-coated with 1.5 μmol/ml of GM1 (Sigma, USA). Anti-cholera toxin (Sigma, USA) and peroxidase labelled swine anti-rabbit immunoglobulin (Dako, Denmark) were used as primary and secondary antibodies respectively. Orthophenylene diamine was used as the substrate. The color developed was read at 492 nm in an ELISA plate reader.

To study the effect of the decoction on the action of LT and CT, the toxins were assayed with different concentrations of the decoction. To study its effect on their production, the respective bacteria were grown with different concentrations of the decoction and the toxins thus obtained were assayed.

Effect on stable toxin (ST)

An exotoxin, ST, was obtained as a culture supernatant of *E. coli* TX1. It was assayed by the method originally described by Gianella (1976). Briefly, the toxin was inoculated intra-gastrically into 2~3 d old Swiss White suckling mice. Following 3 h incubation at room temperature, the suckling mice were sacrificed and the ratio of gut weight to that of the remaining carcass weight was calculated. Ratio ≥0.083 was considered as positive for ST.

To study the effect of the decoction on the action of ST, the toxin was inoculated intra-gastrically with different concentrations of the decoction. To study its effect on the production of ST, the bacterium was grown with different concentrations of the decoction and the toxin thus obtained was inoculated intra-gastrically.

The institutional ethical committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) cleared the use of animals in the study. The Foundation for Medical Research (FMR) is registered with CPCSEA (registration No. 424/01/a/CPCSEA, June 20th, 2001).

Phytochemical analysis

Qualitative phytochemical analyses were carried out with the decoction in order to determine the presence of carbohydrates, glycosides, proteins, amino acids, phytosterols, saponins, flavonoids, alkaloids and tannins (Kokate *et al*., 1990). The leaves, flowers, seeds and stem bark of *P. pinnata* are known to have karanjin (Malik *et al*., 1977; Meera *et al*., 2003), a furanoflavanoid, which is toxic (Prabhu *et al*., 2002). The levels of this compound in water decoction and acetone extract of the leaves of *P. pinnata* were detected with high performance liquid chromatography (HPLC) at 254 nm with acetonitrile:water (60:40, v/v) as mobile phase at a flow rate of 1 ml/min in a 12.5 cm Lichrosphere RP-18 column (Shimadzu CLASS-VP V6.10).

Statistical analysis

Each assay was performed three times and the results were expressed as their mean±standard deviation. The differences in the mean values amongst the treatment groups were analyzed by analysis of variance (ANOVA). Further, the significance of the difference between the means of the test and the control

observations were established by Dunnett's post-test. Statistical analyses were performed using the software Prism 4.0 (GraphPad, Inc.). $P \leq 0.05$ was considered to be statistically significant. The statistical analysis was not applied on the results of the antibacterial and the suckling mouse assays.

RESULTS

Antimicrobial activity

As shown in Table 1, the decoction did not inhibit the growth of any of the six bacterial strains tested. Similarly, the viability of the *G. lamblia* trophozoites (Fig.1) and rotavirus (Fig.2) was also not affected by the decoction.

Effect on colonization to HEp-2 cell line

No competitive inhibition of the adherence of *E. coli* B170 to the epithelial cells was observed when the HEp-2 cells were incubated with the decoction simultaneously with infection. Similarly, no effect of the decoction was observed when the HEp-2 cells were incubated with the decoction prior to infection (Table 2). This indicates that the decoction did not have any effect on the epithelial cells that could lead to a decrease in adherence. However, the decoction significantly reduced the invasion by both *E. coli* E134 and *S. flexneri* of the epithelial cells in both the protocols (Table 2). Since the reduction was observed in both protocols, it appears that decoction prevents invasion by modification of the host cells.

Effect on LT and CT

There was significant reduction in the production of CT when *V. cholerae* was grown in the presence of the decoction (Table 3) even though the bacteria were not killed. Unlike CT, the decoction did not inhibit the production of LT by *E. coli* B831-2. The decoction had no effect on the action of CT once the

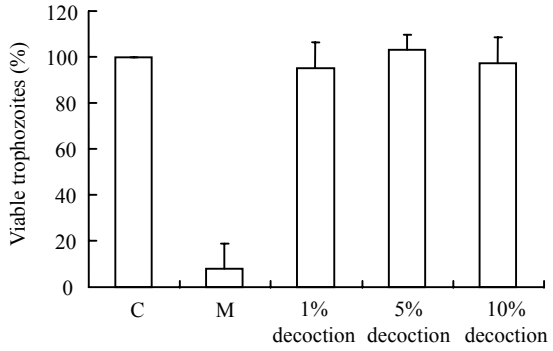


Fig.1 Antigiardial activity of decoction of *Pongamia pinnata* leaves
The bars represent mean±standard deviation (from three individual experiments) of percentage reduction in viability of trophozoites as compared to respective value of controls (100%); C: Trophozoites incubated without the decoction was used as a control; M: Metronidazole (100 μg/ml) was used as a positive control

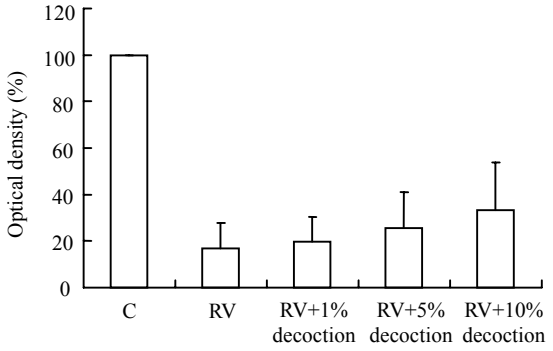


Fig.2 Antirotaviral activity of decoction of *Pongamia pinnata* leaves
The bars represent mean±standard deviation (from three individual experiments) of percentage optical density as compared to respective value of controls (100%); C: Uninfected MA-104 cells incubated without the decoction was used as a control; RV: Rotavirus infected MA-104 cells

Table 1 Antibacterial activity of decoction of *Pongamia pinnata* leaves

Bacteria	Growth[a]				
	Control[b]	Gentamycin (100 μg/ml)	1% decoction	5% decoction	10% decoction
E. coli B170	4+	0	4+	4+	4+
E. coli B831-2	4+	0	4+	4+	4+
E. coli E134	4+	0	4+	4+	4+
E. coli TX1	4+	0	4+	4+	4+
S. flexneri	4+	0	4+	4+	4+
V. cholerae	4+	0	4+	4+	4+

[a] Growth of bacteria graded on a scale of 0 (no growth) to 4+ (control); [b] Bacteria grown on nutrient agar without the decoction

toxin was produced, as there was no competitive inhibition of the binding of toxin to its receptor GM1 in presence of the decoction. Similarly, the action of LT was also not inhibited by the decoction once the toxin was produced. On the contrary, an increase in binding of LT to GM1 was observed.

Effect on ST

The decoction was neither able to inhibit the production of ST when *E. coli* TX1 was grown in the presence of the decoction nor could it control the excessive intestinal secretion due to the binding of ST as was evident from the ratio of the gut weight to that of the carcass weight in the suckling mouse assay (Table 4).

Phytochemical analysis

Carbohydrates, proteins, saponins, flavonoids and tannins were detected in the decoction. The HPLC profiles of the water decoction and the acetone extract of the leaves of *P. pinnata* showed presence of negligible (0.367%) amount of karanjin in the water extract (Fig.3a) as compared to 14.749% in the acetone extract (Fig.3b). A similar form of the water decoction is a common form of administration used in popular medicine.

DISCUSSION

The leaves of *P. pinnata*, also known as Indian

Table 2 Effect of decoction of *Pongamia pinnata* leaves on bacterial colonization to HEp-2 cells

Parameter		Organism	Reduction in colonization[a] (%)			ANOVA[b]		
Assay	Protocol		1% decoction	5% decoction	10% decoction	*F*	*df*	*P*
Adherence	Competitive[1]	*E. coli* B170	−5.88±2.03$^{\#}$	−7.23±4.31$^{\#}$	21.70±16.30*	7.607	3, 8	0.0099
	Pre-incubation[2]	*E.coli* B170	−0.87±1.83$^{\#}$	−2.73±4.50$^{\#}$	6.22±3.63	4.901	3, 8	0.0321
Invasion	Competitive[1]	*E. coli* E134	56.94±28.4*	83.78±13.38**	73.96±19.57**	12.370	3, 8	0.0023
		S. flexneri	20.02±11.08**	16.71±28.04**	64.79±26.88**	133.000	3, 8	0.0237
	Pre-incubation[2]	*E. coli* E134	78.15±3.74	81.82±12.62	98.33±0.96*	5.528	3, 8	<0.0001
		S. flexneri	43.79±3.12**	65.76±3.49**	43.13±4.65**	209.100	3, 8	<0.0001

[a] Values represent mean±standard deviation (from three individual experiments) of percentage reduction in colonization as compared to respective value of controls (100%); [b] Analysis of variance. [1] HEp-2 cells incubated with the decoction simultaneously with the infection; [2] HEp-2 cells incubated with the decoction prior to the infection. * $P<0.05$, ** $P<0.01$ by Dunnett's post-test. $^{\#}$ The negative values indicate that there is an increase in adherence

Table 3 Effect of decoction of *Pongamia pinnata* leaves on *E. coli* labile toxin and cholera toxin

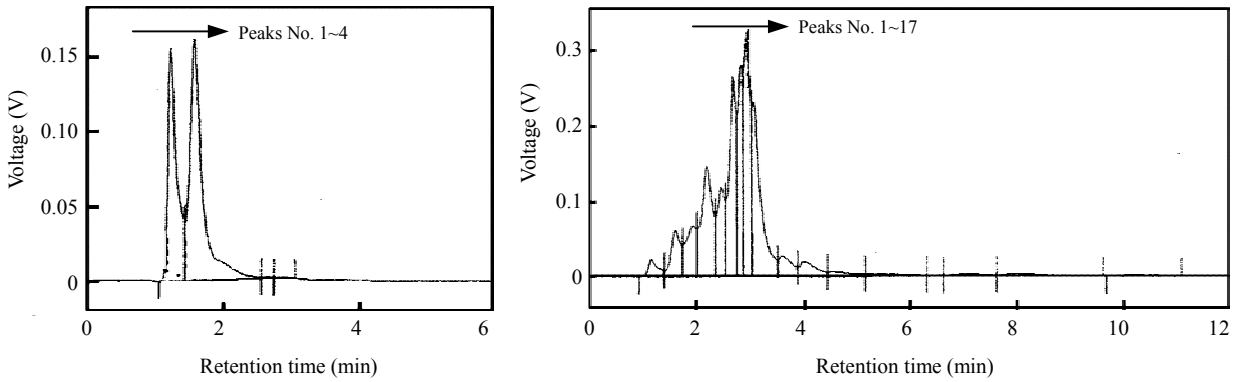
Parameter		Organism	Reduction in optical density[a] (%)			ANOVA[b]		
Assay	Protocol		1% decoction	5% decoction	10% decoction	*F*	*df*	*P*
Cholera toxin	Action[1]	*V. cholerae*	7.41±2.69	11.26±6.49*	7.69±3.11	5.483	3 ,8	0.0242
	Production[2]	*V. cholerae*	15.32±13.05	39.04±14.02*	23.09±5.21	5.298	3, 8	0.0264
Labile toxin	Action[1]	*E. coli* B 831-2	−31.94±11.46$^{*\#}$	−58.24±12.66$^{**\#}$	−44.16±12.59$^{**\#}$	16.450	3, 8	0.0009
	Production[2]	*E. coli* B 831-2	10.82±15.94	−11.73±21.74$^{\#}$	5.19±6.17	1.450	3, 8	0.2991

[a] Values represent mean±standard deviation (from three individual experiments) of percentage reduction in optical density as compared to respective value of controls (100%); [b] Analysis of variance. [1] Toxin incubated onto GM1 receptor with the decoction; [2] Toxin produced by bacteria grown in presence of the decoction. * $P<0.05$, ** $P<0.01$ by Dunnett's post-test. $^{\#}$ The negative values indicate that there is an increase in the action/production of labile toxin

Table 4 Effect of decoction of *Pongamia pinnata* leaves on *E. coli* stable toxin

Protocol	Gut weight/carcass weight[a]				
	CT control[b]	ST control[c]	ST+1% decoction	ST+5% decoction	ST+10% decoction
Action[1]	0.058±0.006	0.109±0.010	0.111±0.003	0.101±0.010	0.097±0.010
Production[2]	0.054±0.001	0.109±0.015	0.114±0.009	0.109±0.002	0.110±0.008

[a] Values represent mean±standard deviation (from three individual experiments) of ratio of gut weight to that of the carcass weight; [b] Cholera toxin without the decoction was used as negative control; [c] Stable toxin without the decoction was used as positive control. [1] Intra-gastric inoculation of the toxin carried out with the decoction; [2] Toxin produced by bacteria grown in presence of the decoction. All the readings observed are in the positive range (≥0.085) except CT control



Detector A-1 (254 nm)

Peak No.	Retention time (min)	Area (mV·min)	Area (%)	Height (mV)	Height (%)	Theoretical plates
(a)						
1	1.233	1462603	39.548	156412	48.817	524.03
2	1.600	2212725	59.831	161789	50.495	457.48
Karanjin→3	2.675	13556	0.367	1442	0.450	648.40
4	2.925	9404	0.254	765	0.239	89.69
Totals		3698288	100.000	320408	100.000	
(b)						
1	1.158	285993	1.754	23189	1.477	262.78
2	1.617	759710	4.659	61378	3.911	215.19
3	1.942	930300	5.706	66936	4.265	37.52
4	2.208	2114870	12.971	146250	9.318	398.91
5	2.483	1119520	6.866	118185	7.530	262.53
Karanjin→6	2.708	2404873	14.749	259085	16.507	675.86
7	2.850	1664648	10.209	280128	17.848	211.83
8	2.950	2773810	17.012	315490	20.101	475.23
9	3.083	2616074	16.045	231795	14.769	152.37
10	3.617	449405	2.756	27176	1.732	379.67
11	4.017	427193	2.620	19911	1.269	436.09
12	4.542	201337	1.235	6569	0.419	170.28
13	5.375	172986	1.061	3292	0.210	40.57
14	6.417	30113	0.185	1612	0.103	0.00
15	7.075	129236	0.793	2930	0.187	380.05
16	7.967	161057	0.988	3466	0.221	379.39
17	10.158	63859	0.392	2107	0.134	3881.30
Totals		16304984	100.000	1569499	100.000	

Fig.3 HPLC profiles of water decoction (a) and acetone extract (b) of *Pongamia pinnata* leaves

Beech, are known to be digestive, laxative and anti-helminthic, and are good for flatulence, dyspepsia, diarrhea, leprosy, and gonorrhea and cough (Warrier *et al*., 1993). The hot water decoction of the leaves is good for rheumatalgia and for cleaning ulcers and wounds (Warrier *et al*., 1993). On the basis of this utilization profile it is suggested that *P. pinnata* may have antimicrobial as well as anti-inflammatory action.

In this study, we examined the antidiarrheal properties of hot water decoction of dried leaves of *P. pinnata* against various virulence parameters of infectious diarrhea. The decoction of *P. pinnata* leaves did not have antibacterial, antigiardial and antirotaviral activity. Though it did not arrest the growth of *V. cholerae*, it prevented the production of CT, thereby indicating that the decrease in production of the toxin was due to its effect on bacterial metabolism and not

due to reduction in bacterial counts. The binding of LT and CT to the GM1 receptor was not inhibited. On the contrary an increase in the binding of LT was noted. It was interesting to see that in spite of CT and LT being structurally, functionally, biologically, and immunologically closely related (Ganguly and Kaur, 1996), there was a difference in the binding of these toxin moieties to the receptor in presence of the decoction.

The decoction did not affect adherence of *E. coli* B170, while it was observed that the invasion of both *E. coli* E 134 and *S. flexneri* was significantly reduced. Interestingly, the reduction in the invasion of the bacteria to the epithelial cells, and not the adherence, indicates that the decoction affects bacterial entry by acting at a post-adherence stage.

Another interesting observation of the biological results is the effectiveness of a 5% dilution of the decoction over the 10% dilution in certain assays as indicated by the reduction in invasion of *S. flexneri* in the pre-incubation protocol (Table 2) and suppression of production and action of CT (Table 3). This probably indicates the synergistic combination of active compound(s) being attained at a slightly lower concentration.

Literature shows presence of flavonoids, especially furanoflavonoids, quercetin, amino acids, fatty acids and triterpenoids in *P. pinnata* (Satyavati *et al*., 1987; Sharma *et al*., 2001). However, none of the known chemical constituents from the plant has been attributed with antidiarrheal activity. Moreover the chemical composition of the water decoction of *P. pinnata* leaves has not been studied in detail. In our study, the decoction showed presence of carbohydrates, proteins, saponins, tannins and flavonoids. Tannins, flavonoids and saponins in general have been reported to have several pharmacological activities including antidiarrheal activity (Agbor *et al*., 2004; Galvez *et al*., 1991; 1993a; 1993b; Lutterodt *et al*., 1999; Miranda *et al*., 1993; Mukherjee *et al*., 1998; Oben *et al*., 2006; Longanga Otshudi *et al*., 2000; Venkatesan *et al*., 2005). The antidiarrheal activity of these groups of compounds has been attributed to antimotility and antisecretory effects (Di Carlo *et al*., 1993; Galvez *et al*., 1993a; Oben *et al*., 2006; Rao *et al*., 1997) and antimicrobial action (Lutterodt *et al*., 1999; Miranda *et al*., 1993; Tona *et al*., 1999). Thus, the tannins, flavonoids and saponins present in the decoction may be responsible for the observed activity in the present study. Nevertheless it must be stressed that the decoction did not show any antimicrobial activity. This is probably due to the difference in the individual constituents of the aqueous extract. Most of the studies reporting antimicrobial activity have used crude organic extracts or active principles.

In the absence of antimicrobial activity, especially in infectious diarrhea, it is necessary that the study of antidiarrheal activity of a plant should consider the disease pathogenesis. Assaying bacterial virulent features as a marker for demonstrating the antidiarrheal efficacy of a plant had been previously reported by us using indigenous plants namely *Cyperus rotundus* (Daswani *et al*., 2001), *Holarrhena antidysenterica* (Daswani *et al*., 2002) and *Dalbergia sissoo* (Brijesh *et al*., 2006).

The study was restricted to the crude extract since it is our belief that the biological activity could be due to the synergistic activity of a combination of two or more active components as discussed above.

CONCLUSION

The findings of the biological assays are indicative of the selective antidiarrheal action of *P. pinnata* leaves. The results suggest that it is not active against toxin induced diarrhea or those caused by protozoa and virus. Amongst bacterial diarrhea, it appears to be most efficacious against cholera and enteroinvasive bacterial strains causing bloody diarrheal episodes. These results support their traditional use as an antidiarrheal therapy.

The study also highlights the necessity of including multiple parameters for assessing the medicinal properties of plants. The approach can be replicated for studying other medicinal plants for their effectiveness against infectious forms of diarrhea, especially those that have no antimicrobial or antimotility activities.

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