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Original Article

Antidiarrhoeal investigation of *Apium leptophyllum* (Pers.) by modulation of Na^+K^+ATPase, nitrous oxide and intestinal transit in rats

Himanshu Bhusan Sahoo [a,*], *Rakesh Sagar* [b], *Anjan Kumar* [c], *Amrita Bhaiji* [c], *Subrat Kumar Bhattamishra* [d]

[a] Department of Pharmacology and Experimental Biology, Vedica College of Pharmacy, RKDF University, India
[b] Department of Pharmacognosy and Phytochemistry, Sri Satya Sai Institute of Pharmaceutical Sciences, RKDF University, India
[c] Department of Pharmaceutical Chemistry, Roland Institute of Pharmaceutical Sciences, India
[d] Department of Life Sciences, School of Pharmacy, International Medical University, Malaysia

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ABSTRACT

Background: *Apium leptophyllum* (Pers.) is an annual herb with traditional appreciation for various pharmacological properties; however, the scientific information on this herb is insufficient. The aim of the present investigation was undertaken to evaluate flavonoidal fraction of *A. leptophyllum* fruit (FFALF) against diarrhoea on albino rats.

Methods: The antidiarrhoeal study was conducted by castor oil induce diarrhoea, prostaglandin E_2 (PGE_2) induced enteropooling and intestinal transit by charcoal meal test. The rats were divided into five groups (six/group). Group I served as control and received orally 2% acacia suspension; Group II served as standard and received orally loperamide (3 mg/kg) or atropine sulphate (5 mg/kg); Group III, IV and V served as test groups and received the FFALF at doses of 5, 10 and 20 mg/kg orally, respectively.

Results: In castor oil-induced diarrhoeal model, the FFALF significantly ($p < 0.001$) reduced the frequency of diarrhoea, defecation and weight of faeces as well as increased the sodium–potassium ATPase (Na^+K^+ATPase) activity and decreased nitric oxide (NO) content in the small intestine. In prostaglandin induced enteropooling model, it significantly ($p < 0.01$) and dose dependently slowed the intestinal fluid accumulation by decreasing the masses and volumes of intestinal fluid where as in charcoal meal test, it decreased charcoal meal transit in gastrointestinal tract as compared with control.

Conclusions: The study reveals that the FFALF possess anti-diarrhoeal properties mediated through inhibition of hyper secretion and gastrointestinal motility which support the traditional use of the plant.

* *Corresponding author*. Department of Pharmacology and Experimental Biology, Vedica College of Pharmacy, RKDF University, Bhopal, 462033, Madhyapradesh, India. Tel.: +91 9439893903.
E-mail address: bhusan.himanshu@yahoo.co.in (H.B. Sahoo).
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At a glance commentary

Scientific background on the subject

Diarrhoea is a very common health issue affecting all age groups as well as all races. In this antidiarrhoeal study, we used different diarrhoeal inducer (i.e. castor oil, prostaglandin E_2 and charcoal meal) to find out different antidiarrhoeal mechanisms of tested drug in animals. Our test substance was able to meet all demands against all diarrhoeal situations.

What this study adds to the field

The Sodium–potassium ATPase (Na^+K^+ATPase) and nitric oxide (NO) is important key factor for antidiarrhoeal study because both have direct or indirect involvement in reabsorption of intestinal fluid and electrolytes. In this study, the estimation of Na^+K^+ATPase and NO from intestinal homogenate was additional studied along with measurement of charcoal meal transit time and intestinal content.

Diarrhoea is an alteration of bowel movement characterized by an increase of volume, fluidity, frequency and passage of loose or watery stool with abdominal pain and bowel sounds. These symptoms of diarrhoea are due to bacteria, viruses or parasites which is transmitted by water, food, utensils, hands and flies [1]. The enteropathogenic microorganisms are responsible for gastrointestinal infection viz. *Escherichia coli, Campylobacter jejuni, Vibrio cholera, Giardia intestinalis, Cryptosporidium parvum, Entamoeba histolytica, Cyclospora cayetanensis, Staphylococcus aureus, Salmonella typhi, Candida albicans,* Shigella, Clostridium, Rota or Adeno viruses etc. [2] Besides this, other causative factors are alcohol, irritable bowel syndrome, bile salts, hormones, secretory tumours and intoxication [3]. The diarrhoea causative microbes exhibit the influx of water and ions to the intestinal lumen by stimulating intestinal motility and poor absorption of intestinal fluid which results as dehydration of body [4]. The intestinal fluid is also depends upon the intestinal enzyme i.e. Sodium–potassium ATPase (Na^+K^+ATPase), which have direct effect on reabsorption of sodium and fluid in the intestine. Also, intestinal nitric oxide (NO) is a potent activator of intestinal secretion and has been considered as a regulator of basal intestinal water transport.

Since ancient times, diarrhoea has been recognized as one of the most important health issue world widely, particularly affecting people of all ages that results in electrolyte loss, dehydration, shock, and sometimes death [5]. According to World Health Organization (WHO), it affects globally about 2.2 million people annually, majority of whom are infants and children below the age of 5 years. Several options employed in the management of the diseases include oral agents such as Diphenoxylate, Loperamide, Diloxanide furoate, Racecadotril, Atropine sulphate, antibiotics and oral rehydration therapy (ORT). With over a decade of the practice and promotion of ORT, diarrhoea is still the second among the causes of child death. In addition, these management options which often fail during high stool output state are also associated with undesirable side effects such as bronchospasm, headache, convulsion, abdominal cramp, vomiting, constipation, hallucination etc. In order to overcome these menace of anti-diarrhoeal drugs in world market, WHO has introduced a programme, which encourages towards traditional herbal medicines [6].

Apium leptophyllum Pers. (Family-Umbelliferae), ayurvedically known as Ajamoda and is distributed in India, Sri Lanka, Pakistan, South America, Queensland and some tropical areas. In India, it is cultivated in Andhra Pradesh, Gujarat, Madhya Pradesh, Jammu-Kashmir and Karnataka [7]. Traditionally, the fruit is widely used as an antinephritic, antirheumatic, carminative and is also beneficial for prevention of tumour, anorexia, vomiting, colic pain and mitch [8]. The volatile oil from the leaves possesses antimicrobial activity and radical scavenging activity [9]. Earlier scientific investigation showed that the fruits of this plant possesses antioxidant, chemopreventive and antimutagenic activity in mice [10,11]. Phytochemically, it contains volatile oils, coumarin derivatives, terpene hydrocarbons, phenolics and alkaloids, and is a rich source of flavonoids [8,10]. This plant is used in the treatment of diarrhoea and stomachaches due to its strong antifungal, antibacterial and anti-inflammatory property [12,13]. *A. leptophyllum* fruit has been traditionally acclaimed to be used in the management of diarrhoea without any scientific study that has validated or reputed this claim. Therefore, in the present study, the traditionally acclaimed use of *A. leptophyllum* fruit in the management of diarrhoea was substantiated with scientific evidence using chemically-induced diarrhoea models on rat.

Materials and methods

Chemicals

Gum acacia, castor oil, charcoal meal (SD Fine chemicals, Mumbai); Loperamide (Micro labs, Bangalore), Ethanol (Merck, Mumbai); PGE_2 (Astrazeneca, Bangalore) and Ketamine, Atropine Sulphate (Hi Media, Mumbai) were procured. All other reagents and chemicals were of analytical grade.

Plant materials

The fruits of *A. leptophyllum* were collected from local region of Bhopal district, Madhya Pradesh, India. Further taxonomic identification and authentification was conducted at Department of Botany, Jiwaji University, Madhya Pradesh, India and the voucher specimen (F/HERB/2010/3405) was deposited in the herbarium for future reference.

Extraction and isolation of flavonoidal fraction from A. leptophyllum *fruit (FFALF)*

The collected fruits were cleaned, washed with distilled water and dried in shade for 4–6 days. The dried fruits were powdered by using blender and then passed through 40 mesh size. The powdered material (250 gm) was initially defatted with petroleum ether and then exhaustly extracted with 80% methanol into the soxhlet assembly for 48 h. The extract was separated by filtration through whatman No.1 paper, concentrated on vacuum evaporator. The extract (Yield-16.4% w/w) was filled in plastic bottle and stored at 4 °C until used.

The crude methanolic extract (10 gm) was subjected to column chromatography and the collected fractions were subjected to shinoda test, followed by TLC. The fractions showing positive response for flavonoid were pooled together and considered as total flavonoid fraction [10]. The total flavonoid fraction was concentrated and subjected to further studies.

Experimental animals

Adult *albino* rats (200–250 g) of either sex were procured from the animal house of Vedica College of Pharmacy, Bhopal, India. The study protocol was approved from the Institutional Animal Ethics Committee (IAEC) and CPCSEA guidelines were adhered during the maintenance and experiment. All animals were maintained under standard husbandry conditions with food and water *ad libitum*.

Evaluation of in-vivo antidiarrhoeal activity

Castor oil induced diarrhoea model

Healthy male *wistar* rats (150–200 gm) were fasted overnight and divided into five groups ($n = 6$). Group I served as control (2% acacia suspension, orally), group II served as standard (Loperamide 3 mg/kg/p.o.) and group III, IV and V served as test groups (received the graded dose of FFALF i.e. 5, 10 and 20 mg/kg body weight respectively). After 60 min of administration, the animals of each group received 1 mL of castor oil orally. The frequency of diarrhoea, wt of faecal material and delay of defecation time was noted up to 4 h in the transparent metabolic cages with pre weighed plastic dishes placed at the base. Weight of plastic dish before and after defecation was noted and compared to control [14].

At the end of the 4 h, the animals were sacrificed and the small intestine was removed. The supernatant was collected from intestinal homogenate and used for the determination of concentrations of Na^+K^+ ATPase and nitric oxide. For estimation of Na^+K^+ ATPase, 20 μL of intestinal supernatant was added with 400 μL NaCl, 40 mM KCl, 60 mM of Tris (pH 7.4), 20 μL $MgCl_2 \cdot 6H_2O$, 20 μL EDTA and 240 μL distilled water. Then, the mixture was allowed to incubate at 37 °C for 5 min and again reincubated at 37 °C for 30 min after addition of 100 μL of 8 mM ATP. Furthermore, 200 μL of sodium dodecyl sulphate (5%) and 2000 μL of reagent C (mixture of ammonium molybdate/sulphuric acid solution [reagent A] and 9%ascorbic acid [reagent B] in ratio 4: 1 v/v) were added. The mixture was left undisturbed for 30 min at room temperature for colour development. The blank was constituted in the same manner except that the small intestine supernatant was replaced with 20 μL of distilled water. The absorbance of the test solution was read against that of the blank at 820 nm. The concentration of Na^+K^+ ATPase was extrapolated from the calibration curve of phosphate [15].

For estimation of nitric oxide, 0.5 mL of the intestinal supernatant was added with 2 mL $ZnSO_4$ and 2.5 mL NaOH. The solution was mixed thoroughly, adjusted to a pH of 7.3, incubated for 10 min, and centrifuged at 504 × g for 10 min. The blank was constituted in a similar manner like the test except that 0.5 mL of supernatant was replaced by distilled water. Furthermore, 1 mL of glycine-NaOH buffer, 2 mL of deproteinized solution and freshly activated cadmium granules was added to the test sample and blank. After 60 min, 2.0 mL each of test and blank was added to tubes containing 2.5 mL EDTA, 3.0 mL HCl and 0.3 mL fuchsin acid solution, mixed thoroughly and incubated for 2 min. Then, 0.2 mL resorcinol and 3.0 mL NH_4OH were added. The absorbance of the test solution was read against the blank at 436 nm and the concentration of serum nitrite was extrapolated from the calibration curve of nitrite [16].

Charcoal meal induced intestinal transit/motility

Healthy *wistar* rats were treated as described earlier except that Atropine sulphate (5 mg/kg IM) was used as a standard drug. One hour later after drug treatments, each of these animals were given 1 mL of charcoal meal (3% charcoal suspension in 5% suspension of acacia) by oral route to induce diarrhoea. All animals were sacrificed after 30 min; the stomach and small intestine were removed and extended on a clean glass surface. The length of the intestine as well as distance travelled by the charcoal meal through the intestine was measured. The percentage of gastrointestinal motility was computed as the ratio of distance moved by the charcoal meal to the length of the small intestine [17].

Prostaglandin E_2 (PGE_2) induced enteropooling model

Healthy *wistar* rats were fasted overnight and divided into five groups; each group carries six animals. Group I served as negative control (2% acacia suspension orally); group II served as positive control (loperamide 3 mg/kg orally as suspension) and group III, IV and V served as test groups; received FFALF of 5, 10 and 20 mg/kg P.O. respectively. After 30 min of administration, PGE_2 was administered to each of the rats in all the groups. After 2 h of administration of PGE_2, each rat was sacrificed by administering excessive dose of ketamine and the small intestine was removed after tying the end with thread and weighed. The intestinal contents were collected by milking into a graduated tube and their volume was measured by measuring cylinder. The volumes of the intestinal contents were noted and used to compute the percentage of inhibition of intestinal content [18].

Statistical analysis

The values were expressed as Mean ± SEM of six animals. For statistical analysis of the data, group means were compared by one-way Analysis of variance followed by Dunnett's t'test. Probability values with $p < 0.01$ and 0.001 were considered as significant. It was carried out with graph pad in Stat 3 software.

Results

Effect of FFALF on castor oil induced diarrhoea model in rats

In the castor oil induced diarrhoeal experiment, the FFALF significantly ($p < 0.05$ and $p < 0.01$) and dose-dependently decreased the frequency of diarrhoea. In addition, the water content of the faeces and the total wt of faeces decreased significantly ($p < 0.001$) and the defecation time of FFALF treated groups were almost delayed as compared with control ($p < 0.01$) as depicted in Table 1. These reductions exhibited better by FFALF at 20 mg/kg as compared to the other dose levels of FFALF and also closer resemblance to the reference drug as loperamide. The inhibition of defecation time increased in a dose-dependent manner with the most remarkable inhibition occurred in all dose ranges of FFALF as compared with control. Furthermore, the activity of Na^+K^+–ATPase in the small intestine increased significantly ($p < 0.01$) in a dose-dependent manner following the administration of the FFALF. Also, the FFALF treated groups significantly ($p < 0.01$) and dose-dependently decreased the concentration of nitric oxide as shown in Table 1.

Effect of FFALF on charcoal meal test in rats

All the doses of FFALF and atropine sulphate showed the decrease the propulsion of the charcoal meal as compared to control group. The distance travelled by the charcoal meal in the FFALF treated groups was found to be 35.74, 38.77 and 43.28% at the dose of 5, 10 and 20 mg/kg respectively; where as the standard showed 48.42% compared to control group. All these observations were significant ($p < 0.001$) reduction with dose dependency in intestinal transit as compared to control. The activity of FFALF at dose of 20 mg/kg on charcoal meal test was found to be closer resemblance when compared to atropine as shown in Table 2.

Effect of FFALF on prostaglandin E_2 induced enteropooling model in rats

The FFALF significantly ($p < 0.001$) and dose-dependently decreased the volumes of the intestinal fluid in the prostaglandin E_2 induced enteropooling in wistar rats. The FFALF treated groups inhibited the intestinal content by 10.47%–62.82% in respect to negative control group. All the doses of the FFALF showed the decrease of intestinal propulsive movement as compared with control as shown in Table 3.

Discussion

The diarrhoea causative agents were acting by numerous mechanisms viz. abnormal electrolyte secretion or absorption, increased luminal osmolality, changes in mucosal morphology and permeability and disorder of motor activity. In experimental animals, castor oil induces diarrhoea due to its active metabolite i.e. ricinoleic acid which causes stimulation of the peristaltic movement of small intestine and reduction/inhibition of Na^+K^+ATPase activity. These consequently lead to changes in the electrolyte permeability of the intestinal mucosa, hyper secretion of the intestinal contents, and a slowdown of the transport time in the intestine [19,20]. Furthermore, ricinoleic acid serves as diarrhoeic agent which acting on nitric oxide/prostaglandin pathway. Nitric oxide elicits diarrhoea by increasing the net secretion of intestinal electrolytes, where as prostaglandin stimulate fluid secretion and inhibiting sodium absorption [21]. Therefore, the decreased frequency of diarrhoea, weight of wet faeces and water content of faeces by the FFALF not only is suggestive of

Table 1 – Effect of flavonoidal fraction of *Apium leptophyllum* fruit (FFALF) on castor oil induced diarrhoea in rats.

Treatment	Control	Loperamide (3 mg/kg)	FFALF		
			(5 mg/kg)	(10 mg/kg)	(20 mg/kg)
Mean frequency of diarrhoea	3.5 ± 0.01	0.6 ± 0.30***	2.1 ± 0.50*	1.6 ± 0.50**	1.0 ± 0.70**
Mean wt of faecal drops	10.9 ± 0.48	2.7 ± 0.15***	7.3 ± 0.69***	5.9 ± 0.54***	4.7 ± 0.15***
Mean wt of faeces after 4 h	1.85 ± 0.37	0.27 ± 0.19***	1.23 ± 0.14***	0.76 ± 0.73***	0.41 ± 0.25***
Delay in defecation time (Min.)	36.14 ± 2.71	191.13 ± 4.29**	97.32 ± 2.54**	144.32 ± 5.78**	183.23 ± 6.13**
Percentage of Inhibition	0	82.85	40.00	54.28	71.42
Intestinal Na^+K^+ATPase concentration	842.31 ± 11.58	1092.05 ± 17.94**	952.69 ± 09.57**	1019.23 ± 08.74**	1074.19 ± 14.19**
Intestinal nitric oxide concentration	153.48 ± 6.35	97.58 ± 8.57**	68.59 ± 5.91**	76.26 ± 4.57**	89.27 ± 7.21**

The values were expressed as Mean ± SEM ($n = 6$); significant at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control.

Table 2 – Effect of flavonoidal fraction of *Apium leptophyllum* fruit (FFALF) on charcoal meal induced peristalsis in rats.

Treatment	Control	Atropine SO_4 (5 mg/kg)	FFALF		
			(5 mg/kg)	(10 mg/kg)	(20 mg/kg)
Mean length of intestine (cm)	45.23 ± 1.34	44.25 ± 1.53	43.81 ± 1.02	45.65 ± 1.15	46.18 ± 1.24
Mean distance travelled by charcoal meal (cm)	38.12 ± 1.68	22.82 ± 1.37***	28.15 ± 1.81***	27.95 ± 1.76***	26.19 ± 1.02***
Mean % movement of charcoal	84.28 ± 1.36	51.57 ± 1.61***	64.25 ± 2.18***	61.22 ± 1.28***	56.71 ± 2.46***
% Inhibition	15.71	48.42	35.74	38.77	43.28

The values were expressed as Mean ± SEM ($n = 6$); significant at ***$p < 0.001$ vs control.

Table 3 – Effect of flavonoidal fraction of *Apium leptophyllum* fruit (FFALF) on PGE_2 induced enteropooling in rats.

Treatment	Negative Control	Atropine SO_4 (5 mg/kg)	FFALF		
			(5 mg/kg)	(10 mg/kg)	(20 mg/kg)
Mean volume of intestinal fluid (ml)	1.91 ± 0.07	0.48 ± 0.07***	1.71 ± 0.09***	1.09 ± 0.03***	0.71 ± 0.55***
% inhibition of intestinal content	0	74.86	10.47	42.93	62.82

The values were expressed as Mean ± SEM ($n = 6$); significant at $^{***}p < 0.001$ vs control.

antidiarrhoeal action but also might explain the rationale for its sustained use in folk medicine as an antidiarrhoeal agent in the animals. The antidiarrhoeal activity of FFALF was supported by increasing the defecation time. Na^+K^+ATPase have an important role in the absorption of sodium and fluid in the intestine of the animals. Thus, inhibition of this intestinal enzyme may contribute to intestinal fluid accumulation which consequently inhibits the frequency of diarrhoea [22]. The dose-dependent increase in Na^+K^+ATPase by FFALF suggests that the accumulation of fluids in the intestine might have been impaired and this consistently emphasizes the antidiarrhoeal activity of FFALF. Again, the reduction in the levels of intestinal nitric oxide by FFALF might be an indication that the net secretion of the electrolytes was not enhanced. It may therefore be proposed that the mechanism of action of FFALF may be due to enhancement of fluid and electrolyte absorption through the gastrointestinal tract and enhance the activity of Na^+K^+ATPase through its *de novo* synthesis or might have an influence on the NO/prostaglandin pathway. These inhibitions may be due to presence of flavonoids and terpenoids, by inhibiting the release of autacoids and prostaglandins in intestinal cells [23,24].

Earlier studies have shown that the anti-dysenteric and anti-diarrhoeal properties of medicinal plants were due to the presence of tannins, saponins, flavonoids, sterols or triterpenes and reducing sugars [25–28]. Flavonoids might be responsible for the antidiarrhoeal activity by inhibiting intestinal motility and hydroelectrolytic secretion [29], while saponins also exhibit antidiarrhoeal activity by inhibiting the release of histamine [30]. Steroids are useful for the treatment of diarrhoea and also may enhance intestinal absorption of Na^+ and water [26]. Therefore, the presence of flavonoidal compounds in the FFALF may confer antidiarrhoeal activity on rats. Again, FFALF increased the re-absorption of water by decreasing the intestinal motility as well as intestinal transit in the charcoal meal test. The antidiarrhoeal effect of FFALF may be also due to an inhibition of muscle contraction, as observed by charcoal meal and consequently, in a reduction of intestinal propulsion. The inhibition of intestinal muscle contraction is due to presence of flavonoidal constituents [6]. The cumulative results of this study revealed that the FFALF possesses antidiarrhoeal activity by reducing the frequency of diarrhoeal stool, weight and volume of intestinal content and intestinal transit as compared to standards (Fig. 1). The FFALF exerted dose dependent antidiarrhoeal effect as comparable to control.

In summary, the FFALF showed markedly reduction in frequency of diarrhoea, the weight and volume of intestinal contents as well as intestinal transit. In addition, the mechanism of antidiarrhoeal effect may be due to reduction of

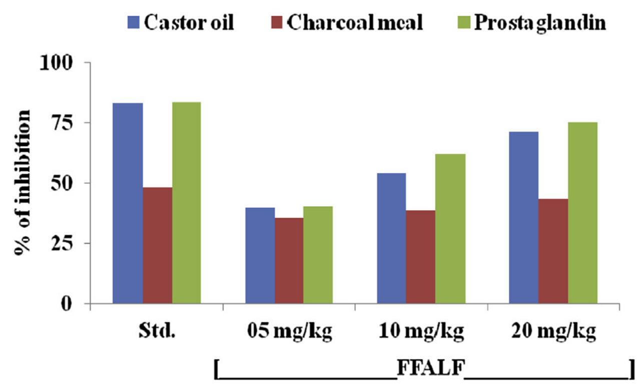

Fig. 1 – The percentage of inhibition of flavonoidal fraction of *Apium leptophyllum* fruit (FFALF) Vs Std. drug in various chemical induced diarrhoea models on rats.

gastrointestinal motility, inhibition of the synthesis of prostaglandin and intestinal muscle contraction. These above antidiarrhoeal mechanisms are possible due to presence of secondary metabolites i.e. flavonoids. Further research is needed to unravel the bioactive agent(s) and its (or their) actual mechanism of action as an antidiarrhoeal agent.

Conflicts of interest

The authors report no conflicts of interest and they alone are responsible for the content and writing of the paper.

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