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

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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.
Diarrhoea is a very common health issue affecting all age groups as well as all races. In this anti diarrhoeal study, we used different diarrhoeal inducer (i.e. castor oil, prostaglandin E2 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.

Materials and methods

Chemicals

Gum acacia, castor oil, charcoal meal (SD Fine chemicals, Mumbai); Loperamide (Micro labs, Bangalore), Ethanol (Merck, Mumbai); PGE2 (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 authentication 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 in reabsorption of intestinal fluid and electrolytes. In this study because both have direct or indirect involvement

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.
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 MgCl2-6H2O, 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 ZnSO4 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 NH4OH 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 E2 (PGE2) 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, PGE2 was administered to each of the rats in all the groups. After 2 h of administration of PGE2, 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 diarrheal experiment, the FFALF significantly ($p < 0.05$ and $p < 0.01$) and dose-dependently decreased the frequency of diarrheah. In addition, the water content of the faeces and the total wt of faeces decreased significantly ($p < 0.001$) and the defection 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 defection 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 atropline 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) | FFALF (10 mg/kg) | FFALF (20 mg/kg) |
|------------------------------------|------------------|----------------------|----------------|----------------|----------------|
| Mean frequency of diarrheah        | 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 defection 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 conc. | 842.31 ± 11.58   | 1092.05 ± 17.94**    | 952.69 ± 09.57**| 1019.23 ± 08.74**| 1074.19 ± 14.19**|
| Intestinal nitric oxide conc.      | 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) | FFALF (10 mg/kg) | FFALF (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.
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 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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