Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading   doi:

Antidiarrheal effect of fractions from stem bark of *Thespesia populnea* in rodents: Possible antimotility and antisecretory mechanisms

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ARTICLE INFO

ABSTRACT

Objective: To evaluate antidiarrheal activity of the fractions of aqueous extract from stem barks of *Thespesia populnea* (Malvaceae). **Methods:** From the aqueous extract three fractions namely ethyl acetate fraction (EAF), methanolic fraction (MF) and residue fraction (RF) were made and studied for antidiarrheal activity. Antidiarrheal activity of the fractions were evaluated in castor oil induced diarrhea, prostaglandin E_2 (PG−E_2) induced diarrhea and charcoal meal test as *in vivo* models and the most potent fraction was further evaluated with *in vitro* models to determine the possible antimotility effect. **Results:** In castor oil induced diarrhea model, the RF (10, 25, 50 and 100 mg/kg, po.) and MF (100 mg/kg, po.) has significantly reduced the cumulative wet faecal mass, where as the EAF have not shown any significant antidiarrheal activity, RF was found to be more potent than MF. Based on these results and percentage yield, only RF was evaluated in PG−E_2 induced enteropooling and charcoal meal test. RF (10, 25 and 50 mg/kg) had shown significant inhibition of PG−E_2 induced secretions (antisecretory) and decreased the movement of charcoal in charcoal meal test indicating its antimotility activity. Furthermore, RF has showed significant inhibition of acetylcholine, histamine and $BaCl_2$ induced contractions on rat colon, guinea pig ileum and rabbit jejunum with EC_{50} values of 241.7, 303.1 and 286.1 μ g/mL, respectively indicating the antimotility effect of RF. The phytochemical analysis of RF showed presence of gums and mucilages and the possible mechanism may be the combination inhibition of elevated prostaglandin biosynthesis and reduced propulsive movement of the intestine. **Conclusions:** RF possesses good antidiarrheal activity comparing with other two fractions and the possible mechanism thought to be associated with combination of antisecretory and antimolity.

1. Introduction

Diarrhea, an important health problem worldwide, especially in developing countries, accounts for more than 5−8 million deaths in infants and children under 5 years of age, each year[1]. In recent years there has been a great interest in herbal remedies for the treatment of a number of ailments. In view of this, the World Health Organization (WHO) had initiated diarrhea disease control program to study traditional medical practices and other related aspects[2].

Thespesia populnea (T. populnea)(Malvaceae) is a

large tree found in the tropical regions and in coastal forests of India. Gossypol was found to be the major component of *T. populnea* producing anti−inflammatory and antifertility effects in rats as well as in human beings[3]. Four naturally occurring quinines, *viz*. thespone, mansonone−D, mansonone−H, and thespesone have also been extracted from heartwood of *T. populnea*[4], Apart from this *T. populnea* is been scientifically proved for useful medicinal properties such as antifertility, antibacterial, antinociceptive and anti−inflammatory[3], treatment of alzheimer's disorder[5], memory enhancing activity[6] antioxidant and hepatoprotective activity[7], anti−psoriatic activity[8], anti−steroidogenic activity[3], diuretic activity[9] and wound healing activity[10].

Traditionally the grounded bark was reported to be used in the treatment of skin diseases, dysentery and haemorrhoids[10]. Since the plants possesses antimicrobial property and it has been traditionally used for treatment of

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diarrhea, in our earlier work we have investigated various stem bark extracts of *T. populnea* for antidiarrheal activity. It was observed that aqueous extract of *T. populnea* possessed good antidiarrheal activity[11]. To further explore the active principle and possible mechanism; the aqueous extract was fractioned and the fractions were investigated for their antidiarrheal activity in both *in vivo* and *in vitro* models.

2. Materials and methods

2.1. Chemicals and drugs

All the solvents used for the extraction process are of laboratory grade purchased from SD Fine Chemicals, Mumbai. Castor oil (Medinova Chemicals. Bangalore, India), Deactivated Charcoal (New India Chemical Enterprises. Kochi, India), PG–E2 (Zydus Alidac. Ahmedabad, India), Atropine (SD Fine Chemicals. Mumbai, India), loperamide (Torrent Pharmaceuticals, Ahmedabad, India), neostigmine (Piramel Healthcare, Mumbai), acetylcholine chloride (Sigma–Aldrich Chemicals Pvt Limited, Bangalore), histamine dihydochloride (Sigma–Aldrich Chemicals Pvt Limited, Bangalore) and $BaCl_2$ (Ranboxy, Delhi) were used for the study.

2.2. Animals

Wistar albino rats (150–200 g), Swiss albino mice (18–22 g), guinea pig (300–450 g) and New Zealand white rabbits (600–800 g) were purchased from Bioneeds, Nelamangala, Tumkur. They were maintained in the animal house of PES College of Pharmacy, Bangalore for experimental purpose. All the animals were acclimatized for seven days under standard husbandry conditions, *i.e.* room temperature of (25 ± 1)℃; relative humidity 45%–55% and a 12:12 h light/ dark cycle. The animals had free access to standard rat pellet (Pranav Agro Industries Ltd, Bangalore, India), with water supplied *ad libitum* under strict hygienic conditions.

Each experimental group had separate set of animals and care was taken to ensure that animals used for one response were not employed elsewhere. Animals were habituated to laboratory conditions for 48 hours prior to experimental protocol to minimize if any of non–specific stress.

The approval of the Institutional Animal Ethical Committee (IAEC) of PES College of Pharmacy, Bangalore (Karnataka) was taken prior to the experiments. All the protocols and the experiments were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.3. Preparation of aqueous extract and fractions

The bark of the plant was collected in the month of May – June 2007 and authentified by Dr. Sreenath KP, Reader and Taxonomist, Department of Botany, Bangalore University. A sample specimen was deposited, bearing voucher number TP–Coll.no.Ⅰ. The shade dried plant material was powdered, the coarse powder was subjected to successive extraction with petroleum ether and ethanol (95%) in soxhlet apparatus and the marc obtained after ethanolic extraction was macerated with distilled water to obtain an aqueous extract. All the extracts were concentrated by vacuum

distillation and spray dried to obtain the final extracts. The percentage yield of petroleum ether, ethanolic and aqueous extracts were found to be 0.45%, 5.33% and 2.50%, respectively.

25 g of spray dried aqueous extract was successively macerated with ethyl acetate and methanol in the solvent: solute ratio of 4:1 for 72 hours with frequent shaking. The ethyl acetate (EAF) and methanolic fractions (MF) were filtered and evaporated to dryness, using vacuum rotary evaporator. The residue left after maceration with ethyl acetate and methanol was dried at 40℃ and termed as residue fraction (RF). The yield of EAF, MF and RF were 6%, 34% and 59%, respectively.

2.4. Antidiarrheal activity

2.4.1. Castor oil induced diarrhea in vivo[11]
Wistar rats of either sex weighing 150–200 g were used. All the animals were screened initially by giving 1 mL/100 g of castor oil and only those showing diarrhea were selected for the final experiment. The pre–tested rats were divided into various groups containing six animals in each group. They were fasted 24 h before the test with free access to water. The test animals of respective groups were orally treated with vehicle, loperamide, EAF, MF and RF. One hour after treatment, each animal received castor oil (1 mL/100g, po.). Each rat was then housed separately in cage over clean filter paper. Then diarrhea episodes were observed for a period of 4 h. During this period, cumulative wet faecal mass were recorded. Antidiarrheal activity was determined in terms of percentage reduction in cumulative wet faecal mass with respect to vehicle treated group.

2.4.2. PG–E2 induced diarrhea[11]
Wistar rats (150–200 g) of either sex were deprived of food and water for 18 h prior to the experiment. The animals were treated with vehicle or loperamide or RF, one hour followed by vehicle or loperamide or RF administration, all the animals were administered with PG–E2 (100 μg/kg in 2% v/v tween 80, po.) except normal control group. Thirty minutes after PG–E2, all the animals were sacrificed. The whole length of the intestine from the pylorus to the caecum was dissected out and its contents were collected and measured.

2.4.3. Charcoal meal test[11]
Swiss albino mice (18–22 g) of either sex consisting of 6 animals in each group were used. Mice were fasted for 4 h before commencing the experiment with free access to water. 1h after vehicle/atropine/RF/neostigmin (1 μg/kg, sc. in normal saline)/neostigmine (1 μg/kg,s.c. in normal saline)+RF(10, 25, 50, 100 mg/kg,p.o.) treatment, 1 mL of charcoal meal (3% deactivated charcoal in 2% aqueous tween 80) was administered by oral route to all the animals. After 60 min of charcoal meal treatment, each animal was sacrificed and distance moved by the charcoal from the pylorus to caecum was measured and expressed as percentage distance travelled by the charcoal meal in ratio to the total intestinal length. Percentage inhibition was calculated using the equation.

% Inhibition $= (Dc - Dt) / Dc \times 100$

Where, Dc: Distance travelled by the control, Dt: Distance travelled by the test.

2.4.4. In vitro models[12]
Wistar albino rats (150–200 g), guinea pig (300–450 g)

and New Zealand white rabbits (600–800 g) fasted for 24 h with free access to water, after 24h fasting they were sacrificed and colon (Wistar rat), Ileum (Guinea pig), and jejunum (New Zealand white rabbit) were isolated and suspended under a constant tension of 1 g in 15 mL organ baths containing Tyrode solution at 37 ℃ and the tissue was allowed to stabilize for a period of 30–40 min with changes of medium at every 5min. once the tissue is stabilized the dose response curve (DRC) for the agonists (Acetylcholine, histamine and BaCl$_2$) were recorded and based on the DRC, a submaximal dose of the agonist was selected to see the effect of RF on agonist response. RF was dissolved in 0.5% dimethyl sulphoxide and distilled water. various concentrations of RF (10 μg/mL to 1 200 μg/mL of the bath fluid) was incubated with the tissue for 1 min, after 1 min incubation with RF (Various concentrations) the agonist was added and the contractile response of the agonist was recorded in presence of RF by maintaining constant bath volume of 15 mL.

The submaximal response of the agonist was taken as 100% response and the percentage inhibition offered by the RF at various concentrations were calculated by using the equation.

% Inhibition = (Vc−Vt) / Vc×100

Where, Vc: Response of the agonist alone, Vt: Response of the agonist in presence of RF.

Using the percentage inhibition data of RF, EC$_{50}$ value (μg/mL) was calculated using Graphpad prism software version 5.0.

2.5. Statistical analysis

Values are expressed as mean±SEM from 6 animals. Statistical differences in mean were analyzed using one way ANOVA (analysis of variance) followed by Tukey's multiple comparison test. Probability values of 0.05 (*P*<0.05) or less were considered statistically significant.

3. Results

In castor oil induced diarrhea model, MF only at higher dose level (100 mg/kg) and RF at 10, 25, 50 and 100 mg/kg, po. showed significant and dose dependent reduction in number of faecal drops and cumulative faecal mass. However, the EAF had not offered significant antidiarrheal effect in any of the administered doses (Table 1). RF has showed more promising activity than EAF and MF.

In castor oil induced diarrhea model the RF was found to be more potent then EAF and MF; further, the percentage yield of RF was more than EAF and MF, hence RF may have all the active constituents that are responsible for the antidiarrheal effect. Hence in further studies only RF was assessed for antidiarrheal effect.

Administration of PG−E$_2$ (100 μg/mL) significantly increased the intestinal secretions as compared to normal rats. Pretreatment with RF (10, 25 and 50 mg/kg, po.) had significantly reduced the PG−E$_2$ induced intestinal secretions (Table 2). It was observed that RF (50 mg/kg) showed greater activity than the standard drug loperamide (3 mg/kg, po.)

In the charcoal meal test, RF (10, 25 and 50 mg/kg, p.o.) retarded the intestinal transit of charcoal meal in a dose−dependent manner as compared with the control group and the results were statistically significant. The RF (10, 25 & 50 mg/kg, p.o.) has also inhibited the promotility effect

of neostigmine (1 μg/kg, ip.) when given along with it (*P* <0.005 to *P*<0.001), confirming its antimotility effect through muscuranic receptors. And the higher dose of RF (50 mg/kg, p.o.) possessed better activity compare to atropine (Table 3).

Furthermore, the effect of RF on acetylcholine, histamine and BaCl$_2$ induced contrations of rat colon, guinea pig ileum and rabbit jejunum was studied respectively, in *in vitro* tissue experiments to explore the mechanism behind its antimotility effect. In *in vitro* studies the RF has shown significant inhibition of acetylcholine, histamine and BaCl$_2$ induced contractions of rat colon (Figure 1), guinea pig ileum (Figure 2) and rabbit jejunum (Figure 3) (Table 4), with EC$_{50}$ values of 241.7, 303.1 and 286.1 μg/mL, respectively, indicating the antimotility effect of RF. Furthermore, the RF was found to be non−specific blocker and by considering the percentage inhibition and EC$_{50}$ values, it can be concluded that the RF is predominantly acting through the muscuranic receptors.
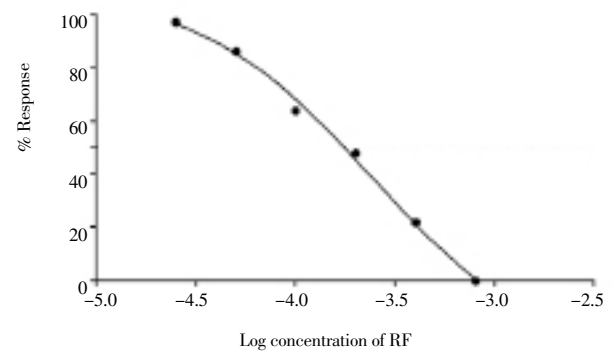
Figure 1. Effect of RF on acetylcholine induced contractions in rat colon.
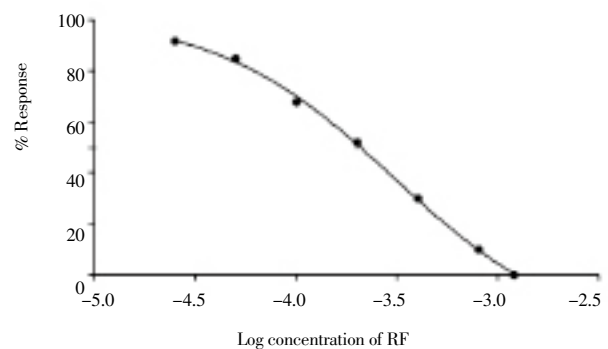
Figure 2. Effect of RF on histamine induced contractions in guinea pig ileum.
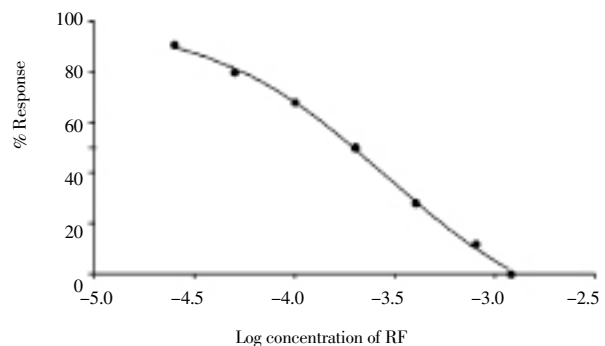
Figure 3. Effect of RF on BaCl$_2$ induced contractions in rabbit jejunum.

Table 1

Effect of EAF, MF and RF on castor oil induced diarrhea in rats.

Treatment	Dose	Number of defaecations(counts/4 h)	Cummulative wet faecal mass (g)	Percentage of inhibition(%)
Control	1 mL/100 g	6.14±0.28	7.65±0.55	–
Loperamide	3 mg/kg	1.71± 0.31	1.35±0.21**	82.35**
EAF	10 mg/kg	5.21±0.23	7.45±0.62	2.61
EAF	25 mg/kg	5.01± 0.37	6.92±0.52	9.54
EAF	50 mg/kg	4.86±0.15	6.45±0.32	15.68
EAF	100 mg/kg	4.18±0.28	6.05±0.18	20.91*
MF	10 mg/kg	5.76±0.43	7.15±0.52	6.53
MF	25 mg/kg	5.28±0.31	6.74±0.20	11.89
MF	50 mg/kg	4.14±0.28	6.19±0.43	19.08
MF	100 mg/kg	3.85±0.25*	5.29±0.21**	30.45**
RF	10 mg/kg	4.43±0.22	5.32±0.25**	30.58**
RF	25 mg/kg	3.71±0.20*	4.15±0.31**	45.75**
RF	50 mg/kg	2.57±0.22**	2.85±0.15**	62.74**
RF	100 mg/kg	1.28±0.20**	1.98±0.11**	74.11**

*: $P < 0.05$,**: $P < 0.01$ using one–way ANOVA followed by Dunnett's test.

Table 2

Effect of RF on PG–E$_2$ induced diarrhea in rats.

Treatment	Dose	Volume of intestinal fluid (mL)	Percentage of inhibition(%)
Naïve	1 mL/ 100 g	0.21±0.11	–
Prostagladin–E$_2$– (200 μ g/kg, po.)	1 mL/ 100 g	2.85±0.07	–
Lopermide	3 mg/kg	0.78±0.09**	72.63**
RF	10 mg/kg	1.45±0.08**	49.12*
RF	25 mg/kg	0.88±0.09**	69.01**
RF	50 mg/kg	0.55±0.06**	80.70 **

**: $P < 0.001$, *: $P < 0.01$ as compared with PG–E$_2$ alone treated group.

Table 3

Effect of RF on intestinal motility in charcoal meal test in mice.

Treatment	Mean % movement of charcoal (cm)	Percentage of inhibition (%)
Naïve	85.91±1.89	–
Atropine (3 mg/kg)	23.97±1.84**	72.09**
RF (10 mg/kg)	68.34±3.34*	20.45*
RF (25 mg/kg)	51.27±2.91**	48.32 **
RF (50 mg/kg)	35.44±1.75**	75.74**
Neostigmine (5 μ g/kg)	92.30±6.25	–
Neostigmine (5 μ g/kg)+Atropine (3 mg/kg)	27.76±2.36 ##	69.92 ##
Neostigmine (5 μ g/kg)+RF(10 mg/kg)	72.45±6.34 #	21.50 #
Neostigmine (5 μ g/kg)+RF(25 mg/kg)	56.67±5.76 ##	43.60 ##
Neostigmine (5 μ g/kg)+RF(50 mg/kg)	41.33±3.96 ##	71.24 ##

**: $P < 0.001$, * $P < 0.05$ vs. control group, ##: $P < 0.001$, #: $P < 0.05$ vs. Neostigmine per se treated group.

Table 4

Effect of RF on acetylcholine, histamine and BaCl$_2$ induced contractions on various tissue preparations (%).

Group	Inhibition		
	Acetylcholine	Histamine	BaCl$_2$
RF 25 μ g/mL	3.22	8.68	9.00
RF 50 μ g/mL	14.41*	15.71	20.00 Ψ
RF 100 μ g/mL	36.24**	32.64 ΨΨ	32.46 ΨΨ
RF 200 μ g/mL	52.85***	48.89 ΨΨΨ	50.62 ΨΨΨ
RF 400 μ g/mL	78.32***	70.09 ΨΨΨ	72.81 ΨΨΨ
RF 800 μ g/mL	100.00***	90.23 ΨΨΨ	88.25 ΨΨΨ
RF 1 200 μ g/mL	–	100.00 ΨΨΨ	100.00 ΨΨΨ

***: $P < 0.001$, **: $P < 0.01$ *: $P < 0.05$ vs. acetyl choline control (0% inhibition), ΨΨΨ: $P < 0.001$, ΨΨ: $P < 0.01$ Ψ: $P < 0.05$ vs. histamine control (0% inhibition), ΨΨΨ: $P < 0.001$, ΨΨ: $P < 0.01$, Ψ: $P < 0.05$ vs. BaCl$_2$ control (0% inhibition).

4. Discussion

The use of herbal drugs in the treatment of diarrheal diseases is a common practice in many countries of Asia, including India. A number of medicinal plants have been reported to be effective against diarrhea and dysentery, as they are used in traditional herbal practice[13].

In our earlier study we proved the ethnobotanical medicinal use of stem bark of *T. populnea* for its antidiarrheal activity[11]. In process of isolation of the active constituent from the aqueous extract we are trying to eliminate the other constituents (except antidiarrheal constituents) present in the aqueous extract. For this purpose we made three fractions namely EAF, MF and RF. The present study was undertaken to identify the active fraction responsible for antidiarrheal activity and also to explore the possible mechanism behind its antidiarrheal effect.

To determine the antidiarrheal fraction, we need to have a non–specific diarrhea model which can identify both antimotility and antisecretory agents. It is well known that the active component (ricinoleic acid) of castor oil irritates the small intestine leading to increased secretion of fluid and electrolytes and speed intestinal transit[13]. Hence for initial identification of active fractions, castor oil induced diarrhea model was selected. In our present study we observed that RF (10, 25, 50 & 100 mg/kg, po.) and MF only at high dose (100 mg/kg, po.) produced good antidiarrheal activity, whereas EAF could not produce the antidiarrheal effect. This may be due to the presence of small amount of active constituents in EAF. Hence EAF was discarded for further experiment. MF and RF produced better antidiarrheal activity. Considering the percentage yield and potency of RF than MF, it would be worthwhile to further explore RF for isolation of the antidiarrheal constituents.

Ricinoleic acid markedly increases the $PG-E_2$ in portal venous and gut lumen and also causes an increase in secretion of water and electrolytes into the small intestine[14]. Ricinoleic acid also produces irritation and inflammation of the intestinal mucosa, leading to release of prostaglandins, which stimulate fluid secretion and irritation produced by the ricinoleic acid leads to increased propulsive movement[15]. Based on our study it seems that the antidiarrheal effect of RF may be due to the inhibition of prostaglandin biosynthesis or by decreasing the peristaltic movement.

In order to differentiate whether the RF has predominant action on prostaglandin or on intestinal motility, further antidiarrheal tests were carried out using $PG-E_2$ induced enterpooling and charcoal meal test. It was observed that, the RF inhibited both $PG-E_2$ induced intestinal secretions and gastrointestinal motility (determined by charcoal meal test).

Acetylcholine is the endogenous neurotransmitter at cholinergic synapses in the central and peripheral nervous system. The stimulation of vagal input to the gastrointestinal tract increases tone, amplitude of contraction and secretory activity of the stomach and intestine[16]. Since such responses are inconsistently seen with administered acetylcholine, possibly because of poor perfusion and rapid hydrolysis by plasma butryl cholinesterase, use of neostigmine was made in the present investigation. Neostigmine is an inhibitor of acetylcholinesterase and increases the amount of acetylcholine at the synapse and thus exerts a pro–kinetic effect, Atropine is a parasympatholytic drug, which acts by blocking the actions of acetylcholine at muscarinic receptors[17–20]. In charcoal meal test RF has shown significant antimotility effect compare to control group and RF also showed significant antimotility effect when given along with neostigmine, a prokinetic drug. The anti–motility action of RF thus appears to be due to blockade of muscarinic receptor. Futhermore, the RF (50 mg/kg) could produce about 75.4% inhibition in motility which is more than atropine (72.1%).

However, In $PG-E_2$ induced diarrhea model, administration of prostaglandins causes secretion of fluid and electrolyte and the by increases the fluidity and bulk of the intestine, which leads to secretory diarrhea. Loperamide is used as standard drug to inhibit $PG-E_2$ induced diarrhea, loperamide is an opioid–receptor agonist and acts on the μ–opioid–receptors in the myenteric plexus of large intestine. It works by decreasing the activity of myenteric plexus and like morphine decreases the tone of the longitudinal smooth muscles but increases the tone of circular smooth muscles (*eg*., anal sphincter) of the intestinal wall. This action increases the amount of time taken by the substances to pass through the intestine, allowing more water to be absorbed out of the fecal matter[21]. The RF (50 mg/kg) showed significant dose dependent inhibition of $PG-E_2$ induced diarrhea, at 50 mg/kg, po. the RF had offered about 81% reduction in $PG-E_2$ induced enter pooling which is greater than the standard drug loperamide (3 mg/kg).

Furthermore, in *in vitro* studies the absence of contractile activity of the drug itself on various smooth muscle preparations shows the lack of agonistic activity on muscaranic and histaminergic receptors. The results obtained in *in vitro* studies of RF on various isolated smooth muscle preparations showed inhibition of acetylcholine, histamine and barium chloride–induced contractions in rat colon, guinea pig ileum and rabbit jejunum respectively.

Binding of ach to muscarinic receptors or histamine to H_1 receptor in smooth muscles results in opening of receptor operated channels, thereby allowing sodium influx, which causes a depolarization of the cell membrane. This depolarization opens voltage dependent calcium channels and calcium ions enter the cell to induce the release of calcium from the sarcoplasmic reticulum. The cytosolic calcium thus binds to calmodulin, which results in contraction[11].

Since acetylcholine, histamine and $BaCl_2$ have different modes of action, the antagonism elicited by RF indicates that it might be acting at a common step in the contraction mechanism elicited by these agonists. The antagonism offered by the RF was concentration dependent. The EC_{50} values and percentage inhibition offered by the RF

against acetylcholine, histamine and BaCl$_2$ on various tissue preparations showed that it is having slightly more predominant antagonism towards acetylcholine than histamine and BaCl$_2$. Therefore, the antimotility effect of RF may be involves non–specific antagonism.

These results suggest that RF possessed good antidiarrheal activity which may due to the inhibition of prostaglandin biosynthesis and decreasing the propulsive movement of the intestine. However, in the process of isolation of the component responsible for antidiarrheal activity, the phytochemical analysis of the RF showed presence of gums and mucilages. Hence the gums and mucilages present in the RF are may be responsible for its antidiarrheal activity and possible mechanism may be thought to associated with combination of antisecretory and antimotility effect. Further works are initiated to isolate and characterize the antidiarrheal constituents from RF.

The findings from the above research suggest that, among the three from aqueous extract of stem bark of *T. populnea* (EAF, ME and RF), the RF possesses better antidiarrheal activity compare to other two fractions and the mechanism behind its activity may be associated with the inhibition of prostaglandin biosynthesis and decreasing the propulsive movement of the intestine. However, RF possesses both antisecretory and antimotility effects, hence may be helpful in the treatment of diarrhea.

Conflict of interest statement

We declare that we have no conflict of interest.

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