ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Pharmacological Evaluation of Ethyl Acetate Fraction of Leaves of *Canthium coromandelicum* (Burm.f) Alston: with Special Reference to Its Anti-diarrhoeal Activity on Albino Rats.

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ABSTRACT

Canthium coromandelicum (Rubiaceae) is one of traditional medicinal herb used for treatment of diarrhoea. However, no scientific data has been made available in support of the claimed ethnomedicinal reported species for the treatment of diarrhoea. To evaluate the antidiarrhoeal activity of the ethyl acetate fraction of methanolic extract of leaves *Canthium coromandelicum* (EA-MECC) and justifies its use in traditional medicine for the treatment of diarrhoea. The antidiarrhoeal activity of EA-MECC was evaluated for castor oil induced diarrhoea along with gastrointestinal motility and enteropooling in albino rats. The fraction was given to the animals orally at the doses of 100 mg/kg and 200 mg/kg body weight. Loperamide was used as a standard drug as antidiarrhoeal agent. The oral administration of EA-MECC (100 and 200 mg/kg b.w.) showed a significant (p<0.05) dose dependent inhibitory activity against the castor oil induced diarrhoea, gastrointestinal motility in charcoal meal test and enteropooling in albino rats. The severity of diarrhoea was reduced to 54.21% and 74.94% at the dose level of 100 and 200 mg/kg body weight respectively; whereas 75.99% inhibition was found for standard drug Loperamide (5 mg/kg). Castor oil-induced GIT motility was significantly (p<0.05) reduced to 42.79% and 65.05%; whereas castor oil-induced enteropooling was found to be reduced to 55.79% and 67.39% at 100 and 200 mg/kg b.w. dose of EA-MECC, respectively. The present study signified that the EA-MECC possessed a potential antidiarrhoeal activity; which may be due to its background chemical constituents. This study also scientifically validates the facts in conformity with the traditional claim of anti-diarrheal properties of the *C. coromandelicum*.

Keywords: *Canthium coromandelicum*, Antidiarrhoeal, Loperamide, Castor oil, Intestinal motility, Enteropooling.

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ISSN: 0975-8585

INTRODUCTION

The gastrointestinal diseases commonly exhibit symptoms of constipation and diarrhoea affecting more than 70% of the population worldwide [1]. In developing countries like India, diarrhoeal disease constitutes a leading cause of mortality and morbidity especially in human beings. It causes the death of millions of people each year. Particularly children are more prone towards this disease which acts as the second leading cause of death of children age group below five years [2]. Diarrhoea is one of the GIT disorder characterized by an increase in frequency and change in stool consistency in the processes of defecation [3]. GIT disorders are the symptoms of many other diseases like diabetes mellitus, inflammatory bowel disease (crohn's disease and ulcerative colitis) and colon cancer etc [4-7]. Majority of people in developing countries use traditional system of medicines for various diseases, including diarrhoea to avoid adverse effects of synthetic drugs. World Health Organisation (WHO) has encouraged studies for treatment and prevention of diarrhoeal diseases depending on traditional medical practices to reduce mortality rate in these countries. In ancient times, plant kingdom played an important role for enriching natural drug sources. Interestingly it became necessary to search new species from the flora having antidiarrhoeal properties that could be used against different types of diarrhoeal diseases. A list of medicinal plants with antidiarrhoeal activity used by traditional healers is reviewed.

A large proportion of the population in many developing countries relies on traditional herbal practioners to meet their primary health care needs. Amidst wide range of availability of modern i.e. synthetic medicines, herbal medicines more appropriately the herbal drugs or herbals often retain their popularity for their intense historical and cultural values. These herbals and their isolated compounds i.e. the bio-active principles, have demonstrated huge spectra of biological activities. Therapeutic data on such herbals are much comprehensive from the medico folk lore literatures of many regions as recorded from time to time. In view of the increasing demand of these herbal drugs, the issues regarding their safety, efficacy and quality maintenance in industrialized and developing countries as well are cropped up. Several studies have evaluated the effectiveness of some traditional medicines in treatment of diarrhoea, in different continents [8-10].

Canthium coromandelicum (Syn.*C. parviflorum*) of Family: Rubiaceae is a bushy thorny herb, native of India, found mainly in coast of the coramandel region of India. The plant is popularly recorded under the local name ie in Odisha state "Tutidi saga" (in Odia). Different parts of this plant like leaf, bark, stem, fruits, root and even whole plant have been used to cure various diseases by tribal people. Traditionally roots are used for snake bite when taken along with milk in some villages in Shimoga district of Karnataka [11]. The leaves and fruits are edible, astringent and effective against cough and indigestion [12]. The leaves were used for wound healing and diuretic activity in animals [13] and gastrointestinal disorders like diarrhoea and constipation [14]. The root and leaves were used as diuretic, Kapha (sputum), diarrhoea, strangury, fever, leucorrhoea and intestinal worm in children [15]. The bark is made into paste along with turmeric and lime and applied on the forehead to cure headache [16]. The whole plant is used against Diabetes by major tribal groups in South TamilNadu, controls high blood pressure; reduce unwanted fats in the body and as a blood purifier [17]. As there is lack of scientific report has been published on the evaluation of diarrhoea about this plant, the present investigation was done to carry out its antidiarrhoeal activity.

MATERIALS AND METHODS

Plant materials

Leaves of *C. coromandelicum* were collected from the campus of College of Pharmaceutical sciences (CPS) Mohuda village, Brahmapur, Ganjam and Odisha during the month of June-August 2012. The plant materials had been identified by Prof. S. K. Dash, Taxonomist, Department of Biosciences, CPS, Brahmapur, Ganjam, and Odisha and authenticated further too from taxonomy department of Botanical Survey of India (BSI), Kolkata. The voucher specimen (CNH/76/2012/Tech.II/899) was retained in the Department of Pharm. Science & Technology, BIT-Mesra, Ranchi, Jharkhand (India) for future reference.

Preparation of plant extracts

The collected leaf materials were washed to clean the adhered debris next shad dried at room temperature under shade keeping away from sunlight. The dried leaves were macerated to coarse powder

using tissue blender. About 1kg of air dried powder was successively extracted through hot extraction process by using Soxhlet apparatus using chronologically with 6L of solvents in increasing order of polarity index viz., petroleum ether (40-60 grade), and chloroform, methanol and water for 72 hours. Next, the extracts were filtered and the filtrates were dried using rotaevaporator to get dried crude fractional extracts.

Qualitative preliminary phytochemical studies

The different leaf extracts of *C. coromandelicum* were tested for the major class of secondary metabolites like steroids, alkaloids, glycosides, saponins, flavonoids, phenolic compounds, tannins, triterpenoids, carbohydrates, proteins and fats etc. Phytochemical screening of the extracts was carried out according to the standard methods with some modifications [18].

Use of animals with ethical clearance

Albino rats of either sex (180-200 g) were procured from the animal house of Department of Pharmaceutical Sciences & Technology, BIT, Mesra, Ranchi, Jharkhand (India). The animals were housed in standard cages and allowed to acclimatize for 1 week before the commencement of the experimental study. Standard commercial chow (animal feed) and water were provided *ad libitium* for the animals. Housing conditions were maintained at 25 ± 2ºC at 12 h day/ night cycles. This study was given ethical clearance by the Institutional Animal Ethics committee, Department of Pharmaceutical Science & Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand (India). Approval No: BIT/PH/IAEC/23/2013 in September 2013 under CPCSEA guidelines.

Drugs and Chemicals

All the chemicals and solvents used in this study were of analytical grade. The marketed drug viz; Loperamide (Torrent Laboratory, Ahmedabad) and Castor oil (Dabur Pharma, India) were used in the study. The other Chemicals were procured from Central Drug House, Delhi.

Acute toxicity study (LD_{50} / LC_{50})

The acute toxicity was performed in albino mice, maintained under standard conditions. Overnight fasted animals were used as per the protocol. Fixed dose (OECD Guideline no. 423, Annexure 2d) method of CPCSEA was adopted for toxicity studies [19]. The tested extract was administered orally. The sign of mortality was observed at 2000 mg/kg in all the cases (OECD, 1997). Common side effects such as loss of weight and depression of treated groups of animals were recorded within the 7 days observation period.

Antidiarrhoeal activity

Castor oil induced diarrhea

Diarrhoea was inducted in the animal systems according to the method of Shoba *et al*., 2001; Uddin *et al*., 2005 and Nwafor and Bassey 2007[20-22]. All selected animals of either sex were fasted for 24 h; but, allowed free accesses to water prior to the commencement of experiment. They were randomly divided into four groups of six animals in each group. Group 1 (control) received normal saline (2 ml/kg) by, Group 2 (standard) treated with 5 mg/kg of loperamide, Group 3 and 4 were administered with 100 mg/kg and 200 mg/kg of EA fraction of MECC. All the treatments were done through oral route. After 1 hour of drug treatment, each animal received 2 ml castor oil. The animals were kept in separate metabolic cages with a plain sheet of butter paper placed on floor to collect their dropouts. They were then observed for the presence of characteristic diarrhoea dropping and the dry stools. The consistency of faecal matter and the frequency of defaecation were counted at 1 hr intervals for 4 hours. The total diarrhoeal faeces for the control group were considered to be 100%. The absence of stool was considered as protection from diarrhoea and the percentage protection was calculated as follows:

$$\% \text{ of diarrhoeal inhibition} = (T_0 - T_1/T_0) * 100$$

T_0 = number of wet faeces in Control group, T_1 = number of wet faeces in Test group.

The induction of diarrhea with castor oil results from the action of ricinoleic acid formed by hydrolysis of the oil. Ricinoleic acid produces changes in the transport of water and electrolytes resulting in a hypersecretory response. In addition to hypersecretion, ricinoleic acid sensitizes the intramural neurons of the gut, which enhances the animal's defecation in the biological system.

Gastrointestinal motility test

This experiment was designed using charcoal as a diet marker [23]. Animals of either sex were fasted for 18h but allowed free access to water prior to onset of the experiment. They were grouped into four groups at random containing six animals in each group. All groups were administered with 2 ml of castor oil to produce diarrhoea. One hour later, Group 1 (control) received normal saline (2 ml/kg); Groups 2 (standard) treated with 5 mg/kg of loperamide; Group 3 and 4 were administered with 100 mg/kg and 200 mg/kg of EA fraction of MECC. All the treatment was done through oral route. After one hour of drug administration, all animals received one ml of marker (10% charcoal suspension in 5% gum acacia) orally. Again after one hour, all the animals were sacrificed through cervical dislocation. The GIT was dissected out and the distance traveled by charcoal meal in intestine from the pylorus to caecum was measured and expressed as percentage (%) of distance covered [24].

Castor oil induced enteropooling

The castor oil induced enteropooling was carried out according to the method of Robert *et al.,* 1976 [25]. Animals of either sex divided into four groups of 6 animals in each were fasted for 18 hours prior to the experiment. Group 1 (control) received normal saline (2 ml/kg); Groups 2 (standard) treated with 5 mg/kg of loperamide; Group 3 and 4 were administered with 100 mg/kg and 200 mg/kg of EA fraction of MECC. All the treatments were done through oral route. After 1 hour of drug treatment, all groups received 2 ml castor oil to produce diarrhoea. After 1 hour of castor oil induction, the animals were sacrificed through cervical dislocation and small intestine from the pylorus to the caecum was isolated. There after the intestinal contents were weighed and volume measured through graduated tubes [26].

Statistical analysis

The experimental data were expressed as mean±SEM of six animals. The significance of difference between the control and treated groups was determined by using one-way analysis of variance (ANOVA) followed by Dunnet's t-test. $P<0.05$ level were considered as significant. The statistical analysis was carried out using Graph Pad Prism 5 software.

RESULTS

Phytochemical screening

Table 1: Preliminary Phytochemical analysis of different extracts of *C. coromandelicum*

	Pet. Ether	chloroform	Methanol	Aqueous
Alkaloids	-	-	++	-
Glycosides	-	-	++	-
Tannins	-	+	++	+
Saponins	-	-	+++	++
Flavonoids	-	-	+++	+
Phytosterols	+	-	+	-
Terpenoids	-	-	++	++
Phenols	-	-	++	+
Steroids	-	+	-	-
Carbohydrates	-	-	+	-
Proteins	-	-	-	-
Fats	+	-	-	-

. +++ Present significantly; ++ Present moderately; + Present slightly; - Absent

Preliminary phytochemical screening of different metabolites (steroids, alkaloids, phenolic groups, tannins, coumarins and anthraquinones etc) were tested in four different solvent leaf extracts of *C.*

coromandelicum. The methanolic extract revealed the presence of more numbers of phytoconstituents than other extracts. The results were recorded in Table 1.

Acute toxicity study

In acute toxicity study, the extract did not exhibit any mortality at the dose of 2000 mg/kg. Therefore 2000 mg/kg dose was chosen as LD_{50} (lethal dose 50) in accordance with Globally Harmonised Classification System (GHS) category 5 (safe dose), as per OECD guideline 423 (Annexure 2d). Common side effects mainly loss of body weight and depression of treated groups of animals were not observed within 7 days of treatment period. Thus, in the present study, only 100 mg/kg and 200 mg/kg body weight doses were selected for *in-vivo* studies.

Castor oil induced diarrhea

Oral pretreatment castor oil induced diarrhoea lasted for 24 hours in the control group. The EA fraction of MECC leaves exhibited pronounced anti diarrhoeal effect in a dose dependent manner as compared with control. Drug treatment prolonged the onset time of diarrhoeal defecation, 75.55 to 135.45 min, at the dose of 100 and 200 mg/kg, respectively. The effect is significant and more comparatively similar with the loperamide 5mg/kg (145 min). The EA fraction significantly (p< 0.05) inhibited both the frequency of defaecation as well as the wetness of stools of animal. The inhibition was found to be 54.21% and 74.94%, with the dose of 100 and 200 mg/kg b.w. respectively. The drug treatment reduced the weight of the stool and the frequency of defecation as well, compared to the control group. The standard drug loperamide (5 mg/kg) produced diarrhoeal inhibition of 75.99%. All the above results were shown in Table 2.

Table 2: Effect of EA-MECC on castor oil induced diarrhoea in albino rats.

Groups	Treatment	Total no. of faeces before diarrhoea	Total no. of diarrhoeal faeces	% inhibition of diarrhoeal faeces	Total wt. of stools (in gm)	% inhibition of total wt. of faeces
Control	Normal saline (2ml/kg) + Castor oil (2ml p.o)	5.833±0.603	29.66±1.612	---	8.542±0.245	--
Standard	Loperamide (5mg/kg) + Castor oil (2ml p.o)	6.500±0.43	8.33±0.425*	71.91	2.052±0.089*	75.99
Test-1	EA-MECC (100mg/kg) + Castor oil (2ml p.o)	6.00±0.706	14.0±1.069*	52.79	3.907±0.388*	54.21
Test-2	EA-MECC (200mg/kg) + Castor oil (2ml p.o)	6.167±0.795	9.33±0.718*	68.54	2.14±0.262*	74.94

The resultant values are expressed as Mean ± SEM (n=6). Probability value of *$P<0.05$ *when compared to control group.*

Gastrointestinal motility test

The gastrointestinal distance travelled by charcoal meal in the experimental animals was significantly (P<0.05) lessened in both the test groups as compared to control group. Distance travelled by the charcoal meal was reduced to 42.79% and 65.05% in treated groups. Loperamide (5 mg/kg) on the other hand, produced a marked decrease (71.78%) in the propulsion of charcoal meal through gastrointestinal tract. The results are shown in Table 3.

Castor oil induced enteropooling test

Castor oil showed a significant increase in the fluid volume of animals intestine in control group (Table-4).The EA-MECC inhibited castor oil induced enteropooling significantly (p<0.05) in animals by both the doses. The percentage of reduction of enteropooling was 55.79% and 67.39% w/w with the dose of 100 and 200 mg/kg, respectively in comparison with the control. The standard drug, loperamide (5 mg/kg), significantly (p<0.05) inhibited intestinal fluid accumulation 79.71% and the fraction was found almost potential compared to the standard (Loperamide).

ISSN: 0975-8585

Table 3: Effect of EA-MECC on charcoal induced GIT motility on albino rats.

Groups	Treatment	Total length of intestine (cm)	Distance traveled by charcoal meal (cm)	% inhibition
Control	Normal saline (2ml/kg) + Castor oil (2ml p.o)	94.33±2.929	89.16±3.347	--
Standard	Loperamide (5mg/kg) + Castor oil (2ml p.o)	93.5±2.848	25.167±3.384*	71.78
Test-1	EA-MECC (100mg/kg) + Castor oil (2ml p.o)	90.167±1.708	51.0±3.185*	42.79
Test-2	EA-MECC (200mg/kg) + Castor oil (2ml p.o)	96.667±2.562	31.167±2.88*	65.05

The resultant values are expressed as Mean ± SEM (n=6). Probability value of *$P<0.05$ *when compared to control group.*

Table 4: Effect of Effect of EA-MECC on castor oil induced enteropooling in albino rats.

Groups	Treatment	Mean weight of intestine before milking (gm)	Mean weight of intestine after milking (gm)	Volume of intestinal content (ml)	% inhibition
Control	Normal saline (2ml/kg) + Castor oil (2ml p.o)	5.4±0.202	4.017±0.201	1.383±0.048	---
Standard	Loperamide (5mg/kg) + Castor oil (2ml p.o)	5.683±0.186	5.35±0.247	0.283±0.031*	79.71
Test-1	EA fraction (100mg/kg) + Castor oil (2ml p.o)	5.75±0.237	6.533±0.307	0.617±0.06*	55.79
Test-2	EA fraction (200mg/kg) + Castor oil (2ml p.o)	5.667±0.296	6.117±0.326	0.45±0.077*	67.39

The resultant values are expressed as Mean ± SEM (n=6). Probability value of *$P<0.05$ *when compared to control group.*

DISCUSSION

The acute toxicity study revealed that the extracts of *Canthium coromandelicum* is nontoxic since there is no mortality at the dose level of 2000 mg/kg. Hence, in the present study, LD50 was found to be greater than 2000 mg/kg. As per in published reports it has suggested that ≥200 mg/kg b.w. of extracts for *in-vivo* and ≥ 200µg/ml of extract concentrations in *in-vitro* studies are likely to be artificial despite yielding reproducible effects. Such high concentrations may also trigger non-physiological effects resulting in ambiguity [27]. Hence, a dose of 100 and 200 mg/kg b.w. were chosen and supposed to be effective for initiated activity in the present experimental protocol.

In preliminary phytochemical study of leaves of *C. coromandelicum* showed the presence of alkaloids, glycosides, flavonoids, tannins, phenols and saponins. Plants synthesize various chemicals in their profiles as to how these phytoconstituents provides as chemical defense mechanisms for them against various harmful organisms and also provide protection to external environmental changes. The phytoconstituents include alkaloids, steroids, flavonoid, tannins and phenolics; have been used by human kind in various disorders for their antioxidants, antimicrobial, anti-inflammatory, immune-stimulant, anthelmentic, antiviral properties etc [28]. Flavonoids exhibit antidiarrhoeal activity obtained from some traditional medicinal plants [29, 30]. The antidiarrhoeal activity of flavonoids has been ascribed to their ability to inhibit intestinal motility and hydro electrolytic conditions [31]. Presence of flavonoids in the plant extracts are also reported to inhibit release of autocoids and prostaglandins, thereby may inhibit motility and secretion induced by castor oil [32]. Tannins present in antidiarrhoea plants denature proteins in the intestinal mucosa by forming protein tannates which make the intestinal mucosa more resistant to chemical alteration and reduce secretion [33]. Frei *et al*., 1998 [34] and Bruneton 1999[35] reported the tannins containing drugs are widely used for the treatment of diarrhoea and related disorders. Longanga *et al.,* [36] showed the antidiarrhoeal activities of many such medicinal plants were due to tannins, alkaloids, saponins, flavonoids, terpens and glycosides present in them. Similarly, various plants and plant constituents have scientifically been investigated for various gastro-

intestinal disorders including diarrhea. Sarin and Bafna 2012 [37] reviewed about anti-diarrheal herbal population and enlisted *Acacia catechu*, *Acorus calamus*, *Asparagus racemosus*, *Azadirachta indica*, *Aegle marmelos*, *Butea monosperma* etc., as potent anti-diarrheal species. Number of plants from Rubiaceae family have also been screened and reported for anti-diarrheal property namely *Pausinystalia macroceras, Paederia foetida*, *Morinda morindoides*, *Mitragyna diversifolia, Guettarda speciosa, Ixora cocciena*, *Morinda citrifolia* etc [23, 38-43]. *C. coromandelicum* belonging to family Rubiaceae is traditionally reported for diarrhea like gastro-intestinal complications [14]. Therefore, EA fraction of MECC (potent antioxidant fraction, data not shown), was evaluated for anti-diarrheal property in Wistar rats using various diarrheal models.

Diarrhoea results from altered motility and fluid accumulation within the intestinal tract, causing an excess loss of body fluid through faeces. In some cases the secretory components predominates, while some are characterized by hyper motility. Castor oil is a triglyceride characterized by a high content of the hydroxylated unsaturated fatty acid, the ricinoleic acid [44] and 90% of ricinoleate content of castor oil is mainly responsible for diarrhoea production [45]. Oral administration of castor oil releases ricinoleic acid in the intestinal lumen by lipase enzyme, and considerable amounts of ricinoleic acid absorbed in the intestine [46, 47]. Ricinoleic acid acts as a local irritant to GIT mucosa resulting in increased intestinal motility [48]. Ricinoleic acid in small intestine increases the peristaltic movement which alters the Na+ and Cl- permeability in the intestinal mucosa [30]. On the other hand, ricinoleic acid also increases the secretion of endogenous prostaglandin in the GIT mucosa [49], which augments the ricinoleic acid -associated changes in the bowel and stimulates the diarrhea [50]. Recent study shows that the laxative effect of ricinoleic acid present in castor oil is due to the induction of contracting intestinal smooth muscles which are mediated by the activation of EP3 receptors on intestinal smooth muscle [51]. Many antidiarrhoeal agents act by reducing the gastrointestinal motility and/or the secretions from the GIT.

The result of present study show that EA-MECC produced significantly (P<0.05) reduction in frequency of diarrhoea and severity of this action produced by castor oil. It is also noted that this fraction significantly (P<0.05) inhibited intestinal fluid accumulation and the volume of intestinal content in a dose dependent manner, compared to the control. The experimental findings reported by Afroz *et al.*, 2006; Jebunnessa *et al*., 2009; Karimulla *et al*., 2011of anti-diarrheal property also corroborated with the same doses [39, 40, 43]. The anti-diarrheal effect of EA-MECC was found to be comparable to standard loperamide, which is reported to slow down transit in the intestine, reduce the colon flow rate and consequently have any effect on colonic motility [52, 53]. Loperamide is an opioid derivative which has been sown to slow intestinal motility by acting on μ receptors on neurons in the sub mucosal neural plexus of the intestinal wall and its antimuscarinic activity in gastrointestinal tract [48, 54-55].

CONCLUSION

The present study scientifically justifies the traditional claim of antidiarrhoeal property of *C. coromandelicum* due to the presence of major phytoconstituents like flavonoids, tannins, alkaloids, glycosides, phenols and saponins. Further pharmacological evaluations are under taken to provide more precise elucidation of mechanism of isolated constituent involved in this study. The isolated compound may serve as useful prototypes of antidiarrhoeal drugs of natural origin possessing desired pharmacological activities lacking of untoward effects.

ACKNOWLEDGEMENTS

The authors would like to acknowledge AICTE (QIP), Govt. of India for financial support, Botanical Survey of India, Kolkata for authentification of plant specimen & Department of Pharmaceutical Science & Technology, Birla Institute of Technology, Mesra, Ranchi, India for providing the research facilities to carry out the study.

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