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ORIGINAL ARTICLE

Antidiarrheal Potential of *Eriosema chinense* Vogel. against Enteropathogenic *Escherichia coli*-Induced Infectious Diarrhea

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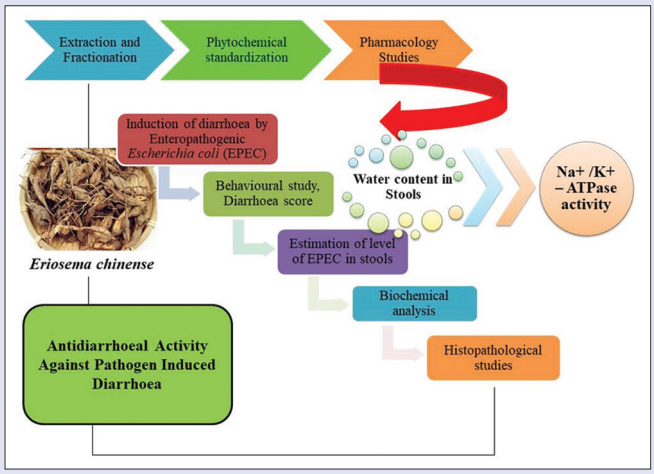
ABSTRACT

Background: The plant *Eriosema chinense* Vogel (Fabaceae) is mainly found in the Eastern Himalayan regions of India and China, and its roots are used traditionally by the tribal people of Meghalaya (India) in treatment of diarrhea. **Objective:** The objective of the study was to evaluate the potential of roots from *E. chinense* against enteropathogenic *Escherichia coli* (EPEC)-induced infectious diarrhea. **Materials and Methods:** Ethanolic extract of *E. chinense* (EEC) roots and its chloroform fraction (CEC) were standardized with eriosematin E using high-performance liquid chromatography. The efficacy of EEC (100 and 200 mg/kg, p.o.) and CEC (50 and 100 mg/kg, p.o.) was evaluated against EPEC-induced infectious diarrhea, where behavioral parameters at the 6th and 24th h followed by determination of water content and density of EPEC in stools along with blood parameters examination. Further, the colonic and small intestinal tissues were subjected to biochemical analysis, antioxidant evaluation, determination of ion concentration, Na^+/K^+-ATPase activity, and histopathology. **Results:** The results demonstrated a significant antidiarrheal potential of EEC and CEC at both dose levels; however, EEC at 200 and CEC at 100 mg/kg p.o. were found to be more effective, which also reduced EPEC density in stools and also its water content. The treatment also demonstrated a significant restoration of altered antioxidant and electrolyte status and reactivated Na^+/K^+-ATPase and prevented epithelial tissue damage. **Conclusion:** The effect may be attributed to an inhibition in intestinal secretion, nitric oxide production, and reactivation of Na^+/K^+-ATPase.

Key words: Diarrhea score, enteropathogenic *Escherichia coli*, *Eriosema chinense*, eriosematin E, Na+/K+-ATPase, nitric oxide

SUMMARY

- The roots of the plant *Eriosema chinense* Vogel (Fabaceae) is rationally used by the tribal people of North East India, especially in Meghalaya in treatment of infectious diarrhea which remains to be one of the major problems in developing countries like India. The objective of the present study was to evaluate the potential of roots from *E. chinense* against enteropathogenic *Escherichia coli* (EPEC)-induced diarrhea. The results demonstrated a significant antidiarrheal potential of ethanolic extract and its bioactive chloroform fraction and reduced the EPEC density in stools along with its water content. The treatment also demonstrated a significant restoration of altered antioxidant and electrolyte status. They also reactivated Na+/K+-ATPase activity and prevented epithelial tissue damage from EPEC. The effect may be attributed to an inhibition in intestinal secretion, nitric oxide production, and reactivation of Na+/K+-ATPase.



Abbreviations used: ATP: Adenosine triphosphate, CAT: Catalase, CEC: Chloroform fraction from ethanolic extract of *E. chinense*, CFU: Colony-forming unit, CMC: Carboxymethyl cellulose, EEC: Ethanolic extract of *E. chinense*, EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid, EPEC: Enteropathogenic *Escherichia coli*, Hb: Hemoglobin, Ht: Hematocrit, KCl: Potassium chloride, LPO: Lipid peroxidation, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean corpuscular volume, MgCl2: Magnesium chloride, MIC: Minimum inhibitory concentration, MTCC: Microbial Type Culture Collection, NaCl: Sodium chloride, NO: Nitric oxide, PCs: Platelet cells, RBCs: Red blood cells, SDS: Sodium dodecyl sulfate, SOD: Superoxide dismutase, WBCs: White blood cells.

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INTRODUCTION

Diarrhea may be defined as a disorder including increases in volume or fluidity of stools, changes in consistency, and increased frequency of defecation. Most recent estimates showed that, among 1 billion episodes of diarrhea every year in children younger than 5 years, the number of deaths reported is around 5–6 million.[1] Thus, diarrhea remains to be one of the major problems of developing nations like India, both for morbidity and mortality, which may be attributed to malnutrition, inadequacy of safe drinking water, and hygiene.[2] Pathogenic *Escherichia coli* and *Vibrio cholerae* are considered to be the most common culprits of diarrhea accounting for about 2%–5% in developed and 14%–17% in developing countries. The other important

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causative organisms include *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., and *Yersinia* spp.[2]

Indigenous system of medicines from Indian origin has recommended the use of number of medicinal plants that have been reported to have potential antidiarrheal activity and has resulted in scientific exploration of several plants such as *Aegle marmelos* L. (Rutaceae), *Bombax ceiba* L. (Bombacaceae), *Eclipta prostrata* L. (Asteraceae), *Hemidesmus indicus* Br. (Asclepiadaceae), *Jatropha curcas* L. (Euphorbiaceae), *Mangifera indica* L. (Anacardiaceae), *Tridax procumbens* L. (Asteraceae), and *Zingiber officinale* Rose. (Zingiberaceae).[3] The plant *Eriosema chinense* Vogel (Fabaceae) is mainly found in the Eastern Himalayan regions of India and China and is also distributed in countries such as Thailand, Myanmar, and Australia. The tribal people of Meghalaya (India) traditionally use the roots of the plant in treatment of diarrhea.[1,4,5] Phytochemistry conducted on the roots of the plant has revealed the presence of khonklonginols A-H, lupinifolin, lupinifolinol, dehydrolupinifolinol, flemichin D, eriosemaone A, eriosemaone E, and yangambin. Studies have also reported the cytotoxic and antimycobacterial potential of the roots.[6] Recently, we have successfully evaluated the antidiarrheal activity of alcoholic root extract and its bioactive fraction, lupinifolin, and eriosematin E from the roots of the plant *Eriosema chinense* against non-infectious (chemical induced) diarrhea.[1,5] Further, eriosematin E, a major biomarker from the plant, has been also reported for its potency against infectious diarrhea.[7] However, there are no scientific reports available on the efficacy of its extract against infectious diarrhea. Therefore, the present investigation has been designed to evaluate the efficiency of the extracts and its bioactive fractions against pathogen (infectious)-induced diarrhea. Thus, the study may act as a contributing factor in achieving the goal of the World Health Organization in minimizing the death rate from infectious diarrhea.

MATERIALS AND METHODS

Plant material, its extraction, and fractionation

The roots of the plant *E. chinense* were collected from Jowai area, Jaintia Hills district of Meghalaya (India) in May–June 2016 and authenticated from Botanical Survey of India, Shillong, India. The voucher specimen (COG/EC/14) of the plant has been deposited in the Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India. The roots (500 g) of the plant were shade dried, grounded to coarse powder, and then were extracted using ethanol (1.5 l) following Soxhlet method until the whole powder was completely exhausted. The extract so obtained was then concentrated under reduced pressure in a Rota evaporator (BUCHI India Pvt. Ltd, Mumbai, India) and evaporated to brown color extract (yield: 13.6% w/w) which was kept in a desiccator until use. The extract was then subjected to fractionation using column chromatography taking silica gel as a stationary phase, and different fractions such as hexane (2.75% w/w), chloroform (24.32% w/w), and ethyl acetate (10.14% w/w) were obtained. Further, based on the previous reports[5] and obtained percentage yield of the fractions, the parent extract along with bioactive chloroform fraction was selected for future studies.

Phytochemical standardization

The alcoholic extract of *E. chinense* (EEC) roots and its chloroform fraction (CEC) were standardized using eriosematin E as a marker compound with the help of high-performance liquid chromatography (HPLC), where separation was carried out with a Cosmosil C_{18} column (150 mm × 4.6 mm, 5-µm particle). A stock solution of sample (5 mg/ml) and eriosematin E (0.5 mg/ml) was prepared in methanol. The mobile phase consisted of a gradient mixture prepared from 0.5% glacial acetic acid (component A) and acetonitrile (component B), starting with 20%–25% B for 0–10 min, then 25%–30% B for 10–20 min, 30%–35% B for 20–30 min, 35%–50% B for 30–50 min, 50%–60% B for 50–60 min, and 60%–80% B for 60–80 min. The flow rate was kept at 1.0 mL/min, with an injection volume of 10 µL. The data were collected at wavelength 279 nm while the peaks were identified by comparing its retention time with that of standard.

Experimental animals

Healthy Wistar rats of either sex weighing between 150 and 200 g were obtained from the Central Animal House (Reg. No.: 92/1999/CPCSEA, dated: April 28, 1999) of the Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India. The animals were kept in standard conditions, i.e., 12-h light and dark cycle with an ambient temperature of 25°C ± 1°C and relative humidity of 45%–55%. Rats were fed with commercially available rat feed and water *ad libitum* and were allowed to acclimatize for 7 days to the environment before commencement of the protocol. All experimental protocols were conducted after the Central Animal Ethical Committee's approval (Letter No.: IAEC/UDPS/2017/43, dated August 14, 2017) and were conducted in accordance with accepted standard guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

Induction of diarrhea

Diarrhea was induced in the rats using suspension of enteropathogenic *Escherichia coli* (EPEC; MTCC 724) procured from Microbial Type Culture Collection (MTCC), Chandigarh, India, as described previously.[7,8] After 7 days of acclimatization, the rats were fasted for 6 h and randomly assigned into two groups, including the normal group and the diarrheal model group. The normal group rats were administered 0.02 ml/g BW sterile water by gavage, while the diarrheal model group rats were given 1 ml of the prepared EPEC suspensions (3.29×10^9 colony-forming unit/ml) once. The animals were then kept under observation for any symptom of diarrhea, which initiated after 40–50 min of EPEC administration.

Grouping of animals

After confirmation of diarrhea to the rats, they were divided into seven groups. Group 1 consisted of normal control rats treated with normal saline (1 ml/kg, p.o.); Group 2 was served as EPEC control group administered with normal saline; Group 3 and 4 animals consisted of diarrheal-induced group treated with EEC at 100 and 200 mg/kg p.o. suspended in 0.5% carboxymethyl cellulose (CMC); and Group 5 and 6 animals consisted of diarrheal-induced group administered with CEC at 50 and 100 mg/kg p.o. suspended in 0.5% CMC, while Group 7 included diarrheal-induced group treated with standard drug norfloxacin (Cipla India Pvt. Ltd., Mumbai, India) at 5.7 mg/kg p.o. The EEC, CEC, and standard drug were administered 1 h after administration of EPEC.

Behavioral evaluations

Rats were shifted individually to cages containing plastic sheets at the base and were kept under observation for up to 6 h initially and then for up to 24 h. The observation was made after 2, 4, 6, and 24 h, and various behavioral parameters were evaluated as described previously.[7,8]

Estimation of water content of stool

Stool water content was measured at the 6th and 24th h after treatment by weighing the stool weight initially and after drying it at 37°C in incubator for 48 h. The differences between initial wet weights and dry weights were used to calculate the percentage of water in the stools.[1]

Estimation of level of enteropathogenic *Escherichia coli* in stools

The enumeration of EPEC in feces was determined at the 2nd, 4th, 6th and 24th h following the induction of diarrhea. For this purpose, 0.5 g of feces was homogenized in 4.5 ml of sterile saline; serial dilutions were made, and 500 µl of each dilution was spread over *Salmonella–Shigella* agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India) plate. After incubation for 24 h at 37°C, the number of CFU was determined.[7,8]

Blood cell count

The blood was collected from the retro-orbital plexus of eyes of each animal, 24 h after the treatment, and sufficient quantity of blood was used for counting the hemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs), platelet cells (PCs), hematocrit, mean corpuscular volume, mean corpuscular hemoglobin (MCH), and MCH concentration using the standard procedure.[8]

Biochemical analysis and determination of ion concentration

The rats were sacrificed using intraperitoneal administration of thiopental sodium (65 mg/kg) after 24 h of treatment, and the colonic portion of the rats was dissected out removed and rinsed with Tyrode's solution. The tissue was homogenized with phosphate buffer and centrifuged, and the supernatant was used for nitric oxide (NO) determination using Griess reagent.[9] Total carbohydrate in the tissues was estimated using ferricyanide method following the method proposed by Yemm and Willis.[10] To check any cellular proliferative activity, total DNA and total protein content were estimated following the standard procedure as previously described.[11,12] The tissues were also subjected to antioxidant evaluation such as lipid peroxidation (LPO),[13] superoxide dismutase (SOD),[14] and catalase (CAT).[15]

The concentration of Cl^-, Na^+, K^+, and Ca^{2+} in the tissue homogenate of the treated animals was also determined using Nulyte Electrolyte Analyzer (Tech Medisystems, Chandigarh, India) as per manufacturer's instructions.

Determination of Na^+/K^+-ATPase activity

The small intestine of the sacrificed rats was also dissected out and used for evaluation of Na^+/K^+-ATPase activity. The tissue sample was rinsed and homogenized as per the method described by Gal-Garber *et al.*,[16] and the supernatant of the small intestine was used for the assay as per the method described earlier.[17]

Histopathological studies

Histopathological studies were performed on dissected colonic portion which was immediately blotted, dried, and fixed in 10% formalin. For sectioning, the samples were first dehydrated in acetone and samples embedded in paraffin wax, and sections (4-µm thickness) of the tissue samples sections were taken using microtome and stained with hematoxylin and eosin and were subjected to microscopic examination.

Statistical analysis

All the results in the experiments are expressed as mean ± standard error of mean (SEM), with six animals in each group following one-way analysis of variance (ANOVA). Newman–Keuls multiple comparison test was used for determining the statistical significance between different groups. However, two-way ANOVA followed by Bonferroni posttest was performed for determining the water content in stools and density of EPEC in stools. GraphPad Prism version 5 software (GraphPad,

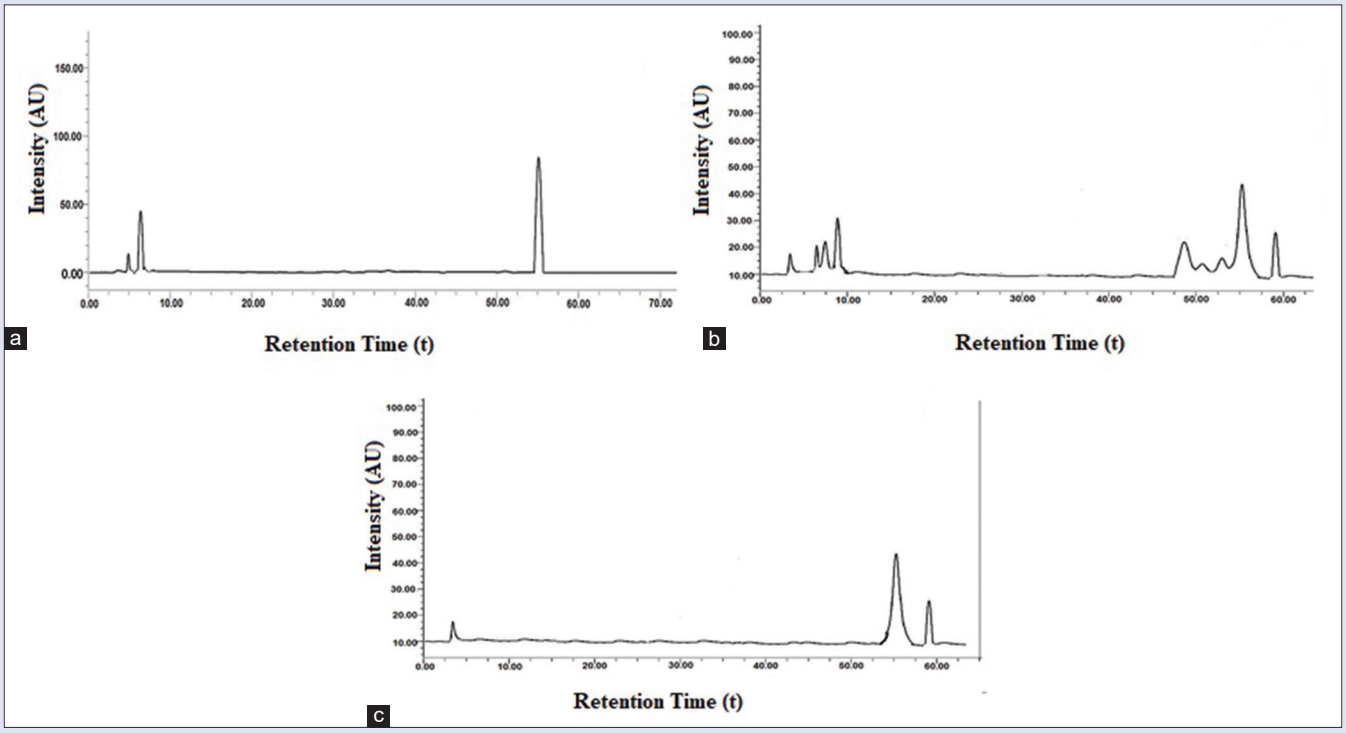

Figure 1: High-performance liquid chromatography chromatogram of eriosematin E. (a) High-performance liquid chromatography chromatogram of standard peak of eriosematin E, (b) high-performance liquid chromatography chromatogram of eriosematin E in ethanolic extract of *Eriosema chinense* and (c) high-performance liquid chromatography chromatogram of eriosematin E in chloroform fraction from ethanolic extract of *Eriosema chinense*

San Diego, CA, USA). was used for all statistical analyses. P <0.05 was considered to be statistically significant.

RESULTS

The HPLC analysis revealed the presence of eriosematin E in ethanolic extract and chloroform fraction showing similar R_t value (55 min) and was reported to be 7.48% and 5.82% (w/w), respectively [Figure 1].

From the results, it was observed that, 40 min after the induction of EPEC to the rats, diarrhea was initialized, which was found to be more pronounced after the 3rd h of induction showing greater aggressiveness among rats. However, on treatment with EEC and CEC, a significant recovery from diarrhea was observed from the 5th h of induction in case of EEC and the 4th h in case of CEC. This was confirmed through significant (P < 0.05) decline in the total number of stools, total number of diarrheal stools, weight of stools, and mean defecation rate (taken after the 6th and 24th h). It was also observed that EEC at 200 mg/kg p.o. and CEC at 100 mg/kg p.o. were found to be more effective in controlling diarrhea, where maximum recovery was observed in standard norfloxacin and CEC at 100 mg/kg p.o. treated group and was quite comparable to one another [Table 1]. The results also revealed a significant reduction in the water content of the stools calculated after the 6th and 24th h of induction of diarrhea [Figure 2]. Keeping the above results into consideration, further, evaluations were performed on most effective dose level of EEC (200 mg/kg, p.o.) and CEC (100 mg/kg, p.o.). The density of EPEC evaluated in the stools also revealed a significant decline in the EPEC level after the 4th h of treatment with CEC (100 mg/kg, p.o.) and norfloxacin, whereas EEC (200 mg/kg, p.o.) was found to be significantly effective after the 6th h of treatment [Figure 3].

Among the blood parameters evaluated, there was a significant decline in the level of WBC and Hb in EPEC control rats; however, on treatment with EEC and CEC, a significant recovery from the WBC and Hb loss was observed. Further, there was no significant difference observed in the levels of other blood parameters under observation [Table 2], except that a slight rise in the level of RBC was observed in treatment groups.

From the biochemical parameters evaluated, a significant increase in the level of NO was observed in the EPEC control rats, which was found to significantly decline on treatment with EEC and CEC. Further, the results also showed a significant increase in the levels of cellular proliferative factors such as protein, DNA, and carbohydrates along with a significant increase in the levels of *in vivo* antioxidant enzymes SOD and CAT, while a significant decrease in the level of LPO was observed [Table 3].

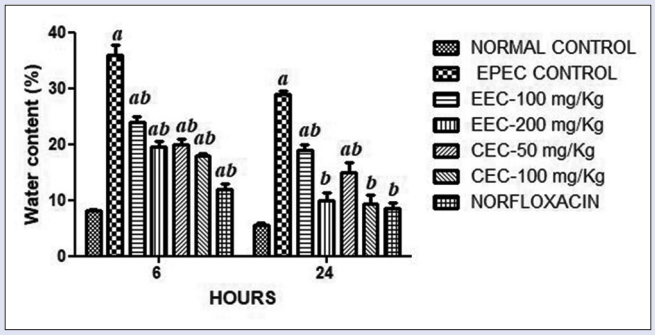

Figure 2: Effect of EEC and CEC on stool water content in EPEC-induced diarrhea rat model. Values are mean ± standard error of the mean (n = 6). Where (a) P < 0.05 versus normal control and (b) P < 0.05 versus EPEC-induced diarrhea control. EEC: Ethanolic extract of *Eriosema chinense*, CEC: Chloroform fraction from ethanolic extract of *Eriosema chinense,* and EPEC: Enteropathogenic *Escherichia coli*

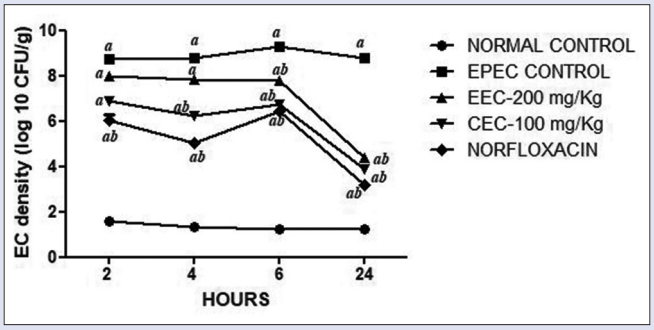

Figure 3: Effect of EEC and CEC on density of EPEC (log10 transformed) in stool of EPEC induced diarrhea rat model. Values are mean ± standard error of the mean (n = 6). Where a: P < 0.05 versus normal control and (b) P < 0.05 versus EPEC-induced diarrhea control. EEC: Ethanolic extract of *Eriosema chinense*, CEC: Chloroform fraction from ethanolic extract of *Eriosema chinense,* and EPEC: Enteropathogenic *Escherichia coli*

Table 1: Effect of ethanolic extract of *Eupatorium chinense* and chloroform fraction from ethanolic extract of *Eupatorium chinense* on various behavioral parameters in enteropathogenic *Escherichia coli*-induced diarrhea rat model

Behavioral parameters	Time (h)	Normal control	EPEC control	EEC 100 mg/kg	EEC 200 mg/kg	CEC 50 mg/kg	CEC 100 mg/kg	Norfloxacin 5.7 mg/kg
Total number	6	3.50±0.71	16.50±1.54^{a}	15.50±0.88^{a}	9.50±0.95^{a,b}	13.16±0.74^{a,b}	9.16±0.47^{a,b}	9.16±0.87^{a,b}
of feces	24	5.56±0.78	12.83±0.94^{a}	8.67±0.98^{a}	7.14±0.78^{a,b}	9.80±1.32^{a,b}	7.43±0.89^{a,b}	6.78±0.59^{a,b}
Total number	6	-	8.89±0.65	6.98±1.20^{b}	4.9±0.83^{b}	6.23±0.86^{b}	4.76±0.65^{b}	3.21±0.29^{b}
of wet feces	24	-	7.12±0.59	5.84±0.78^{b}	2.81±0.64^{b}	5.24±1.56^{b}	2.57±0.44^{b}	2.31±0.23^{b}
Loss in body	6	0.15±0.04	1.35±0.18^{a}	0.82±0.16^{a,b}	0.76±0.09^{a,b}	0.80±0.12^{a,b}	0.78±0.15^{a,b}	0.72±0.13^{a,b}
weight (g)	24	0.09±0.03	0.89±0.02^{a}	0.34±0.06^{a,b}	0.20±0.02^{a,b}	0.49±0.04^{a,b}	0.18±0.07^{a,b}	0.20±0.03^{a,b}
Total weight	6	0.40±0.04	4.12±0.32^{a}	2.23±0.20^{a,b}	1.61±0.19^{a,b}	2.28±0.24^{a,b}	1.57±0.13^{a,b}	1.26±0.08^{a,b}
of feces (g)	24	0.24±0.02	3.09±0.21^{a}	1.32±0.09^{a,b}	1.21±0.16^{a,b}	1.83±0.21^{a,b}	1.18±0.07^{a,b}	0.90±0.07^{a,b}
Mean	6	0.75±0.03	2.75±0.21^{a}	2.58±0.33^{a,b}	1.58±0.42^{a,b}	2.19±0.10^{a,b}	1.52±0.12^{a,b}	1.56±0.15^{a,b}
defecation	24	0.19±0.08	0.53±0.07^{a}	0.36±0.03^{a,b}	0.29±0.01^{a,b}	0.40±0.04^{a,b}	0.30±0.07^{a,b}	0.28±0.02^{a,b}
Diarrhea	6	-	24.31±1.91	12.23±1.03^{b}	8.30±1.09^{b}	9.21±1.44^{b}	6.01±1.10^{b}	6.23±1.32^{b}
score	24	-	18.29±1.42	7.39±1.39^{b}	4.21±0.9^{b}	6.42±1.31^{b}	3.01±1.20^{b}	3.31±1.21^{b}
Percentage	6	100	-	49.04±1.89	65.41±2.21	61.62±2.08	74.95±2.41	74.041±2.23
protection	24	100	-	59.59±1.91	76.98±2.39	64.89±2.11	88.90±3.35	88.46±3.12

Values are mean±SEM (n=6). Where ^{a}P<0.05 versus normal control and, ^{b}P<0.05 versus EPEC-induced diarrhea control. *E. chinense: Eupatorium chinense*; *E. coli: Escherichia coli*; EEC: Ethanolic extract of *E. chinense*; CEC: Chloroform fraction from ethanolic extract of *E. chinense*; EPEC: Enteropathogenic *E. coli;* SEM: Standard error of mean

Administration of EPEC caused a significant decline in the levels of ions, i.e. Cl^-, Na^+, and K^+; however, they were found to significantly recover on treatment with EEC and CEC. The results did not show any significant change in the level of Ca^{2+} ion among all the tested groups [Table 4]. The results obtained from the Na^+/K^+-ATPase activity revealed a significant decline in the enzyme activity of EPEC control rats compared to normal rats. However, treatment with EEC and CEC showed a significant increase in the enzyme activity, which was found to be higher than the normal rats. From the overall observation, the CEC-treated rats showed the most prominent effect even compared with standard norfloxacin-treated group [Figure 4].

From the histopathological examination, normal distinct and intact epithelia with normal glands was observed in the normal rat colons which were found to be distracted due to necrosis in case of EPEC-induced rat colons. However, on treatment with EEC and CEC, a very less destruction of epithelia was observed confirming their protective nature [Figure 5].

DISCUSSION

Infectious diarrhea is considered to be the major cause of the observed death among younger children belonging to developing countries like India, where the major contributor includes pathogens such as *E. coli*, *Shigella dysenteriae,* and *V. cholera.*[11] Therefore, the present investigation was an attempt to evaluate the efficacy of EEC and CEC against one of such major contributors, i.e., enteropathogenic *E. coli*-induced diarrhea model. EPEC along with other pathogenic *E. coli* has been reported as a major culprit for causing diseases or symptoms such as diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome, and thrombocytopenic purpura.[7] In case of diarrhea, EPEC binds intimately to the epithelial surface of the intestine, mainly to the colon through adhesive bundle-forming pilus, causing lesion (through attaching and effacing phenomenon), finally leading to destruction of microvilli resulting in malabsorption and diarrhea.[18,19] Similarly, in our study also, administrations of EPEC produced diarrhea in rats, approximately after 40–50 min of its administration and was found to be very severe after 3rd h. This may be attributed to a massive destruction of microvilli, as evidenced through our histopathological view of negative control group resulting in watery diarrhea. However, on treatment with EEC and CEC, a significant recovery from diarrhea was observed, which was justified through significant reduction in diarrhea score and higher percentage of protection. Further, the study also revealed a significant reduction in the water content of stools along with density of EPEC in rat stools treated

Table 2: Effect of ethanolic extract of *Eupatorium chinense* and chloroform fraction from ethanolic extract of *Eupatorium chinense* on various blood parameters in enteropathogenic *Escherichia coli*-induced diarrhea rat model

Blood parameters	Normal control	EPEC control	EEC 200 mg/kg	CEC 100 mg/kg	Norfloxacin 5.7 mg/kg
WBC (10^3/mm^3)	7.91±0.43	6.02±0.91[a]	8.03±0.38[b]	8.97±0.62[b]	8.46±0.20[b]
Hb (g/dL)	12.46±0.56	10.63±0.78[a]	13.93±0.39[a,b]	14.01±0.36[a,b]	12.63±0.34[b]
Ht (%)	46.33±2.01	45.83±2.78	45.66±2.45	45.98±3.62	46.33±2.25
RBC ($\times10^6$/mm^3)	7.12±0.32	6.79±0.45	7.14±0.46	7.81±0.21	7.97±0.64
PC ($\times10^5$/mm^3)	9.41±0.21	5.94±0.46[a]	6.81±0.65[a,b]	7.50±0.14[ab]	6.78±0.59[a,b]
MCV (μm^3)	52.83±0.94	53.00±1.33	53.43±1.07	53.33±1.02	52.06±1.32
MCH (ρg)	24.66±1.01	23.46±0.30	23.53±0.70	23.31±0.83	23.83±1.43
MCHC (%)	33.83±1.49	33.96±0.45	34.06±0.98	33.92±1.91	34.20±1.57

Values are mean±SEM (n=6). Where [a]P<0.05 versus normal control and, [b]P<0.05 versus EPEC-induced diarrhea control. WBC: White blood cell; Hb: Hemoglobin; RBC: Red blood cell; PC: Platelet cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular Hb; MCHC: Mean corpuscular Hb concentration; *E. chinense: Eupatorium chinense*; *E. coli: Escherichia coli*; EEC: Ethanolic extract of *E. chinense*; CEC: Chloroform fraction from ethanolic extract of *E. chinense* and EPEC: Enteropathogenic *E. coli*; SEM: Standard error of mean

Table 3: Effect of ethanolic extract of *Eupatorium chinense* and chloroform fraction from ethanolic extract of *Eupatorium chinense* on various biochemical parameters in enteropathogenic *Escherichia coli*-induced diarrhea rat model

Biochemical parameters	Normal control	EPEC control	EEC 200 mg/kg	CEC 100 mg/kg	Norfloxacin 5.7 mg/kg
NO (units in mole/mg of protein)	0.72±0.07	3.80±0.27[a]	2.517±0.23[a,b]	1.39±0.21[a,b]	2.14±0.19[a,b]
Total protein (units in mg/100 mg of tissue)	1.53±0.17	0.52±0.14[a]	1.12±0.13[a,b]	1.05±0.12[a,b]	1.14±0.18[a,b]
Total DNA (units in mg/100 mg of tissue)	1.41±0.04	0.87±0.02[a]	1.02±0.10[a,b]	1.14±0.10[a,b]	1.13±0.09[a,b]
Total carbohydrates (mg/g of tissue)	1.30±0.12	0.53±0.05[a]	1.69±0.17[a,b]	1.80±0.19[a,b]	1.51±0.15[a,b]
TBARS (units in mole/mg of protein)	2.97±0.60	14.25±1.38[a]	5.11±1.06[a,b]	3.94±1.30[a,b]	3.67±0.82[a,b]
CAT (μmol H_2O_2 consumed/min/mg of protein)	125.67±6.08	89.82±6.62[a]	110.12±7.01[a,b]	115.0±6.89[a,b]	120.21±7.82[a,b]
SOD (units/mg of protein)	1.39±0.22	1.13±0.13[a]	1.56±0.17[a,b]	1.54±0.09[a,b]	1.62±0.10[a,b]

Values are mean±SEM (n=6). Where [a]P<0.05 versus normal control and, [b]P<0.05 versus EPEC-induced diarrhea control. NO: Nitric oxide; TBARS: Thiobarbituric acid reactive substance; CAT: Catalase; SOD: Superoxide dismutase; *E. chinense: Eupatorium chinense*; *E. coli: Escherichia coli*; EEC: Ethanolic extract of *E. chinense*; CEC: Chloroform fraction from ethanolic extract of *E. chinense* and EPEC: Enteropathogenic *E. coli*; SEM: Standard error of mean

Table 4: Effect of ethanolic extract of *Eupatorium chinense* and chloroform fraction from ethanolic extract of *Eupatorium chinense* on concentrations of ions in enteropathogenic *Escherichia coli*-induced diarrhea rat model

Ion concentration (mmol/L)	Normal control	EPEC control	EEC 200 mg/kg	CEC 100 mg/kg	Norfloxacin 5.7 mg/kg
Cl^-	109.98±6.38	84.59±4.99[a]	108.12±5.39[b]	108.91±6.28[b]	109.09±5.94[b]
K^+	5.35±0.47	2.46±0.24[a]	4.42±0.30[b]	4.80±0.56[b]	4.91±0.39[b]
Na^+	142.50±4.73	128.21±5.32[a]	143.20±4.80[b]	143.71±5.10[b]	143.21±5.22[b]
$Ca2^+$	1.03±0.04	0.99±0.02	1.02±0.04	1.04±0.03	1.02±0.02

Values are mean±SEM (n=6). Where [a]P<0.05 versus normal control and, [b]P<0.05 versus EPEC-induced diarrhea control. *E. chinense: Eupatorium chinense; E. coli: Escherichia coli;* EEC: Ethanolic extract of *E. chinense*; CEC: Chloroform fraction from ethanolic extract of *E. chinense* and EPEC: Enteropathogenic *E. coli*; SEM: Standard error of mean

with EEC and CEC confirming the prominent antidiarrheal potential of *E. chinense* against EPEC-induced diarrhea.

It has been reported that enterohemorrhagic *E. coli* produces watery diarrhea same as that of EPEC. However, it has also been found to cause a severe blood loss as a result of hemorrhagic colitis and hemolytic-uremic syndrome.[20] To find whether EPEC have an influence on blood loss, we have evaluated the blood parameters in our investigation. The observation revealed no appearance of bloody stools in all groups including EPEC control group indicating no hemorrhagic or hemolytic activity. The results also showed no significant difference in the RBC, except that a slight rise in the treated groups was observed. However, there was a significant decline in the level of WBC and platelets in the EPEC control group, and levels significantly increased upon the treatment with EEC and CEC, which indirectly attributed to its host defense mechanism against EPEC.[19,21] The study also revealed a significant recovery from Hb loss on treatment with EEC and CEC, also suggesting the nutritional potential of *E. chinense*.

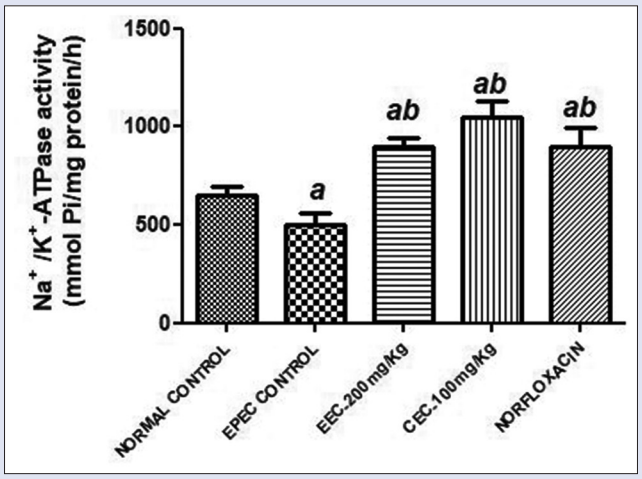

Figure 4: Effect of EEC and CEC on Na^+/K^+-ATPase activity in small intestine of EPEC induced diarrhea rat model. Values are mean ± standard error of the mean ($n = 6$). Where (a) $P < 0.05$ versus normal control and (b) $P < 0.05$ versus EPEC-induced diarrhea control. EEC: Ethanolic extract of *Eriosema chinense*, CEC: Chloroform fraction from ethanolic extract of *Eriosema chinense,* and EPEC: Enteropathogenic *Escherichia coli*

Studies have reported that diarrhea induced by EPEC results in tissue damage due to release of inflammatory mediators and accumulation of other inflammatory cells to the site of infection, which leads to stressful condition due to alteration in enzyme levels.[18,22] Such condition attributes to the expression of inducible nitric oxide synthase resulting in higher production of NO in the colonic tissue, which plays a critical role in altering physiological conditions such as blood pressure, platelet function, and host defense.[8,22] Thus, the decline in the platelets and WBC in the EPEC control group may be as a result of the higher production of NO as observed in our study along with inflammation, which was recovered on treatment with EEC and CEC. It has also been suggested that the antioxidant defense process gets impaired during inflammation due to the LPO as a result of free radical chain reaction and auto-oxidation. This attributes to release of reactive oxygen species such as peroxide anion, hydrogen peroxide, and hypochlorous acid which contribute in alleviation of inflammatory processes,[23] which was confirmed through increased level of LPO, a peroxidative enzyme and a decline in level of antiperoxidative enzymes SOD and CAT, which plays a critical role in protecting oxidative damage.[7] However, treatment with EEC and CEC showed a significant recovery from enzyme alteration, which may be attributed to a very high antioxidant potential of the roots,[5] which resulted in a suppressive action against alleviated NO, LPO and promoted the release of antioxidants SOD and CAT. During pathogenic diarrheal condition, the process of protein and DNA synthesis is impaired, causing mucosal atrophy, which lowers cell turnover.[24] The results too demonstrated a significant decline in the

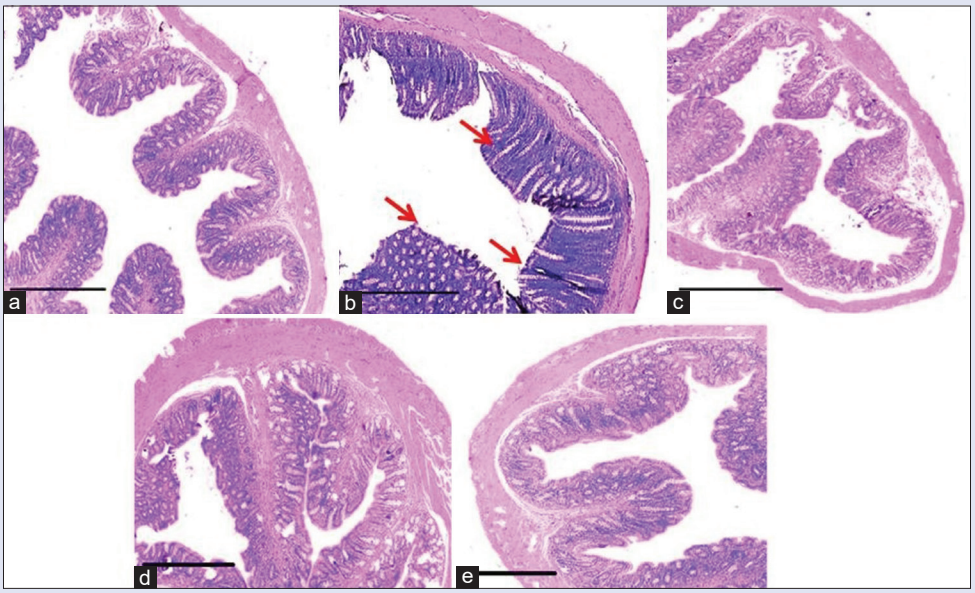

Figure 5: Histopathological view of colonic section of EPEC-induced diarrhea rat colon on treatment with ethanolic extract of *Eriosema chinense* and bioactive chloroform fraction from ethanolic extract of *Eriosema chinense* (×10, Scale Bar 100 µm). (a) Normal control rat colon, (b) enteropathogenic *Escherichia coli*-induced diarrheal control rat colon, (c) enteropathogenic *Escherichia coli*-induced diarrheal rat colon treated with ethanolic extract of *Eriosema chinense* (200 mg/kg, p.o.), (d) enteropathogenic *Escherichia coli*-induced diarrheal rat colon treated with chloroform fraction from ethanolic extract of *Eriosema chinense* (100 mg/kg, p.o.), (e) enteropathogenic *Escherichia coli*-induced diarrheal rat colon treated with norfloxacin (5.7 mg/kg, p.o.) and EPEC: Enteropathogenic *Escherichia coli* (Arrow in the figure indicates localized destruction of colonic cell including microvilli)

level of these cellular proliferative factors, i.e., protein and DNA content in the EPEC control group, which on treatment with EEC and CEC were found to significantly recover. Severe diarrhea may also lead to instant loss of energy due to dehydration,[5] which was depicted through the negative control group. However, EEC and CEC treatments significantly recovered the carbohydrate loss, suggesting the potential role of *E. chinense* in storing and transporting energy.

Studies have implicated the alteration in electrolyte transport in EPEC-infected diarrheic condition, where EPEC have found to alter the relative distribution of ions across membranes. These alteration leads to inhibition in NaCl absorption and accelerated Cl^- secretion mediated through Type III secretion system, resulting in concomitant decrease in water absorption. Increase in fluid secretion has also been reported in significant loss of K^+ due to enhanced solvent drag phenomenon.[18,21,25] The above imbalance in electrolyte may also be attributed to decrease in Na^+/K^+-ATPase activity, a basolateral protein essential for efficient nutrient and ion absorption. Literature survey suggests that EPEC mediated inflammation-induced Na^+/K^+-ATPase endocytosis in an EspF-dependent manner resulting in its inhibition.[26] Our investigation revealed a significant decline in the Na^+/K^+-ATPase activity of the EPEC control group that resulted in diminished reabsorption of ions and water. However, on treatment with EEC and CEC, a significant increase in the Na^+/K^+-ATPase activity was observed resulting in restoration of the altered levels of Na, Cl^-, and K^+. The overall findings of our investigation were well justified through histopathological examination showing normal intact colonic cells in the normal control group, whereas a localized destruction of colonic cells including microvilli was observed in the EPEC control group. On treatment with EEC and CEC, there was a marked recovery from the cellular damage confirming the protective role of *E. chinense* in EPEC induced diarrhea. The roots of the plant have been reported to have very high quantities of flavonoids, alkaloids, tannins, and carbohydrates which have proven to have a significant role directly or indirectly in treatment of diarrhea.[5] Further, the roots have also shown the presence of eriosematin E in a quite high quantity which has been proven to have a significant role in treatment of infectious diarrhea[7] and therefore was selected for standardization of EEC and CEC.

CONCLUSION

The observed antidiarrheal potential of EEC and CEC against EPEC-induced diarrhea may be due to inhibition in intestinal secretion, NO production, and reactivation of Na^+/K^+-ATPase activity. The above outcome may be attributed to the presence of eriosematin E along with other phytoconstituents in combination. Thus, we have successfully justified the potential role of *E. chinense* roots in treatment of infectious diarrhea induced by enteropathogenic *E. coli*.

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Conflicts of interest

There are no conflicts of interest.

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