

## The study of Analgesic, Antidiarrhoeal and Anti-oxidant Effect of Ethanolic Extracts of *Ecbolium linnaenum* in Albino Mice

Md Shamsuddin Sultan Khan<sup>\*1</sup>, Rabiul Islam<sup>2</sup>, Md. Kamrul Hasan Chowdhury<sup>3</sup>

<sup>1</sup>Research Associate, Department of Pharmacy, The University of Asia Pacific, Bangladesh

<sup>2</sup>Department of Pharmacy, Khulna University, Bangladesh

<sup>3</sup>Department of Pharmacy, University of Development Alternative, Bangladesh

### \*Correspondence Info:

Md Shamsuddin Sultan Khan,

Research Associate, Department of Pharmacy,

The University of Asia Pacific, Bangladesh

E-mail: [jupitex@gmail.com](mailto:jupitex@gmail.com)

### Abstract

The *Ecbolium linnaenum*(leaves) is used as a folk medicine in Bangladesh for pain, diarrhea and infectious diseases. Phytochemical evaluation of the ethanolic extracts of *Ecbolium linnaenum* leaves demonstrates these pharmacologic effects for the presence of alkaloids, tannins, gums, flavonoids and absence of carbohydrates, steroids, saponins. In this present study an attempt was made to determine the analgesic, antidiarrhoeal, anti oxidant and antimicrobial effect in *Swiss Albino* mice. Ethanolic extracts of 250 and 500 mg/kg showed significant inhibition of writhing reflex 36.20% (P< 0.01) and 54.48% (P< 0.001), respectively while the standard drug diclofenac-Na was 75.52% (P< 0.001) at a dose of 25 mg/kg body weight. In the castor oil-induced diarrhoeal mice, the ethanolic extracts of 250 mg/kg & 500 mg/kg raised the latent period and reduced the number of stools comparing with standard drug Loperamide. 0.02% DPPH solution of ethanol on TLC plate showed the presence of anti-oxidant components in the *Ecbolium linnaenum*. From the percent inhibition of ascorbic acid and *Ecbolium linnaenum* we observed that it has anti-oxidation effect. The IC<sub>50</sub> (inhibitory conc. 50%) of ascorbic acid is approximately 1 µg/ml and it is more than 500 µg/ml for the sample. The ethanolic extract of *Ecbolium linnaenum* was tested for antimicrobial activity against a number of both gram positive and gram-negative bacteria but it does not show any anti-microbial effect.

**Key Words:** Analgesic, Antidiarrhoeal, Anti-oxidant, Ethanolic, Extracts, *Ecbolium linnaenum*, Mice

### 1. Introduction

*Ecbolium linnaenum* is used as a local medicinal plant of Acanthaceae family. The Acanthaceae are mostly herbs or shrubs comprising about 250 genera and 2,500 species, including twining forms. It is known as Bakos, Neel Kantha to the local people. *Ecbolium linnaenum* is a shrubby plant, with 4-sided flower-spikes at the end of branches. Leaves are often 3 by 1/3 in., tip triangular, obtuse, base narrowed, glabrous or obscurely puberulous; petiole 0– 1/6 inches long, leaves varying from narrowly oblong to broadly ovate, also much in size. The leaves are simple, opposite and decussate, stipules are lacking. Flowers are large, greenish blue. Upper lip of the flower is linear. Plant is used in gout and dysuria, decoction of leaves for stricture. Roots are given in jaundice, menorrhagia and rheumatism. *E. linnaenum* is highly useful for the treatment of jaundice, menorrhoea, rheumatism<sup>1</sup> and anti-inflammatory activity<sup>2</sup>. Anti-helminthic and premenstrual colic effect is found from Root juice<sup>3</sup>. No report has been found on the study of analgesic, antidiarrhoeal, anti-oxidant effect of *E. linnaenum*. Therefore the present study was considered to find out these effects.

### 2. Materials & Methods

## 2.1 Materials

**2.1.1 Chemicals and Reagents:** Ascorbic acid, potassium ferricyanide, trichloroacetic acid, acetic acid and magnesium sulfate were purchased from Merck, Germany. Folin-Ciocalteu's reagent, quercetin and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. Ltd, (St. Louis, MO, USA). Tween-80 and castor oil were purchased from Loba Chemie Pvt Ltd, India. Solvents and all other reagents were of analytical grade.

**2.1.2 Standard Drugs:** Diclofenac sodium was bought from Yung Zip Chemical Co. Limited (Taiwan) and Loperamide active ingredients were obtained from Aceto Pharma GmbH (Germany). Morphine sulfate was collected from Renata Pharmaceuticals Ltd, Bangladesh.

**2.1.3 Phytochemical Screening:** The ethanol leaves extract of *E. linneanum* was treated to different preliminary phytochemical tests to identify major functional groups<sup>4,5</sup>.

**2.1.3 Plant Materials:** For this present investigation the plant *Ecbolium linneanum* (Acanthaceae) was collected from Jessore, Bangladesh, and identified by Bangladesh National Herbarium, Mirpur, Dhaka "Accession No: 35053" and a voucher specimen also deposited there for future experiment.

## 2.2 Methods

**2.2.1 Preparation of Plant Extract:** The collected plant parts were separated from undesirable materials or plant parts. They were air dried for three weeks. The plant parts were ground into a fine powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powder was stored in an airtight container and kept in a cool, dark and dry place.

A glass jar with plastic cover was taken and washed thoroughly. The jar was rinsed with ethanol and dried. Then 150gm of the dried powder was taken in the jar. After that 95% ethanol (750ml) was poured into the jar up to 1 inch height above the sample surface as it can sufficiently cover the sample surface. The plastic cover with aluminium foil was closed properly to resist the entrance of air into the jar. This process was performed for 8 days. The jar was shaken and stirring several times during the process to get better extraction. After the extraction process the extract was filtered by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper (Whatman Ltd, Germany). The filtrate was collected in a beaker. The filtrate (ethanolic extracts) obtained was evaporated by Rotary Evaporator and after that it was kept under ceiling fan to evaporate the ethanol completely. It rendered a gummy concentrate (5.2 gm) of greenish black color. The gummy concentrate was designated as crude extract of ethanol.

**2.2.2 Experimental Animals:** *Swiss albino* mice (weighing 15-25 g) were obtained from the animal house, *International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B)*. Mice were housed in laboratory of Khulna University with proper cages with adequate lighting and ventilation with an ambient temperature of 23-26°C. All experimental studies were conducted following the ethical guidelines of the National Institute of Health, USA and appropriate permissions obtained from the Institutional Animal Ethics Committee (IAEC, approval no. 343/09/ab/KURS) of ethical review committee of the University of Khulna before commencing the experiments.

### 2.2.3 *In-vitro* Anti-oxidant Activity test

**2.2.3.1 Thin Layer Chromatography Technique:** Thin-layer chromatography (figure 3) was used to observe the characteristics of the plant extract, identify polar, non-polar and medium polar groups present in the plant extract using suitable solvent system, detect those groups under UV light at short (264 nm) and long (360 nm) wavelength.

A little amount of ethanolic extract of *E. Linneanum* leaves was dissolved in ethanol and diluted suitably and was applied on the two different TLC plate by spotter. A small amount of ascorbic acid was also dissolved in ethanol and diluted suitably and was applied on the former TLC plate by spotter at the same way. The plate was then kept in a TLC jar containing a solvent system (table 1). A filter paper was kept in the TLC jar and was closed the jar properly. After about 5 minutes spotted TLC plate was kept into the jar dipping into the solvent system. When the solvent system was reached at the desired level then it was removed from TLC jar and was subjected to air drying. At first two chromatograms were developed into the solvent system of n-hexane and Acetone (2:1). This is a non-polar solvent system. After drying chromatograms were observed under UV detector at longer wavelength (306 nm) and shorter wavelength (256 nm) and colored spots was marked by pencil. Spots which was observed into longer wavelength was manifested by the symbol of ( ) and spots which was observed into shorter wavelength was evident by the symbol of <>. Later than one of the chromatograms were taken and 0.02 % DPPH solution of ethanol was sprayed on it by a spray gun. Yellow color was formed on the chromatogram. Other chromatogram was treated with 10% H<sub>2</sub>SO<sub>4</sub> at the same way. Then the plate was heated on hot plate and was developed.

Then the sample was compared with standard. Another two chromatograms were developed into the solvent

system of Chloroform, Methanol and Water (40:10:1) and two chromatogram were developed using the solvent system Chloroform and Methanol (5:1) (table 1). This was polar solvent system. Then by the similar procedure described above was applied to compare the sample with standard.

**Table 1: Solvent used in thin layer chromatography**

Groups	Solvent	Ratio
Non polar	n-hexane : acetone	2:1
Medium polar	CHCl <sub>3</sub> : CH <sub>3</sub> OH	5:1
Polar	CHCl <sub>3</sub> : CH <sub>3</sub> OH : H <sub>2</sub> O	40:10:1

**2.2.3.2 DPPH radical scavenging assay:** The anti-oxidant potential of the ethanolic extract was determined on the basis of their scavenging activity of the stable 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. The aliquots of the different concentrations of 1, 5, 10, 50, 100, 500 µg/ml of the extract were added to 3 ml of a 0.004% Ethanol solution of DPPH. Absorbance at 517 nm was determined after 30 min, and IC<sub>50</sub> (inhibitory conc.50%) was determined. IC<sub>50</sub> value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals. The formula used for % inhibition ratio is:

$$\% \text{ inhibition} = \frac{\text{Blank OD} - \text{Sample OD}}{\text{Blank OD}} \times 100$$

**2.2.4 Analgesic activity test:** Acetic acid induced writhing method was used to find out the analgesic effect of extract of *E. Linneanum* leaves. The mice were randomly screened and divided into four groups (n = 6) to carry out the study. 250 and 500 mg/kg ethanol leaves extract was administered to the test groups orally. Standard analgesic drug Diclofenac sodium<sup>6</sup> at the dose of 25 mg/kg, p.o. and 1% tween-80 in distilled water at the dose of 10 mL/kg were administered to the positive control group and control group orally, respectively. Each animal was given an intraperitoneal (i.p.) injection of 0.6% v/v acetic acid at the dose of 10 mL/kg to induce the characteristic writhing after 30 min. The number of writhing was recorded for the period of 10 min for each mouse. The percent writhing inhibition was calculated and compared with control to assess analgesic activity.

### 2.2.5 Antidiarrhoeal activity test

**2.2.5.1 Castor Oil-Induced Diarrhea:** Mice were randomly chosen and divided into four groups having six mice in each. The control received only distilled water containing distilled water. The positive control received standard antitomotility drug Loperamide<sup>7</sup> at a dose of 3 mg/kg-body weight as oral suspension. The test groups were treated with suspension of ethanolic extracts of *E. Linneanum* leaves at the oral dose of 250 mg/kg-body weight and 500 mg/kg-body weight. Control group was supplied with 1% tween-80 in distilled water (10 mL/kg, p.o.). After the interval of 60 min, each animal was given 0.5 mL of castor oil in oral route to induce diarrhea. Each animal was placed in individual plastic transparent cage and floor was lined with white blotting paper which was changed in every hour throughout the observation period of 4 h. Onset of diarrhea and total number of faces for each animal was recorded. Latent period and percent inhibition of defecation were compared with control group to assess antidiarrheal activity.

## 3. Results

**3.1 Phytochemical Screening:** The experimental findings showed that the ethanolic extract of the leaves of *Ecbolium linnaenum* constituted with Gum, Alkaloid, Flavonoid showed in table 2.

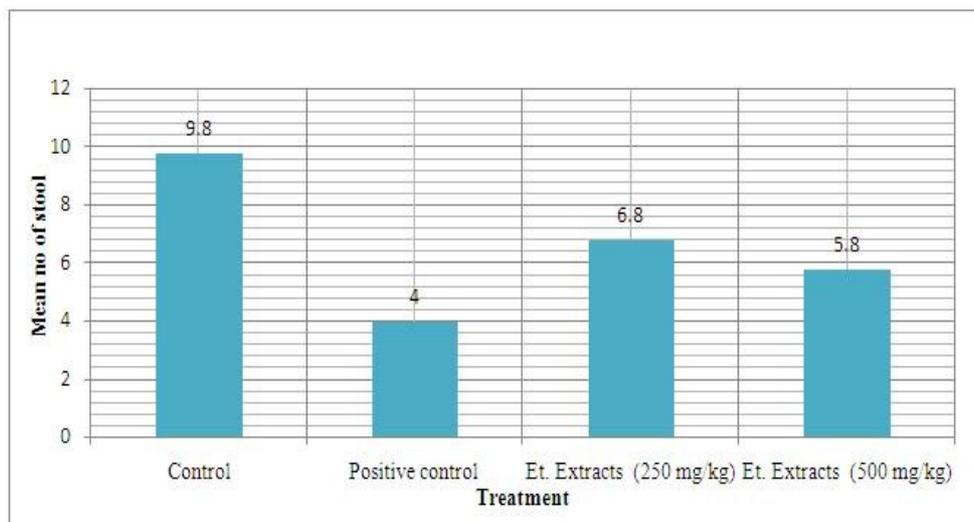
**Table 2: Phytochemical screening of extract of *Ecbolium linnaenum* leaves**

Reducing Sugar	Steroid	Gum	Tannin	Alkaloid	Saponin	Flavonoid	Glycoside
-	-	+	-	+	-	+	-

(+ = Presence, - = Absence)

**3.2 In Vivo Antidiarrheal Activity: Effect On Castor Oil-Induced Diarrhea:** The ethanol leaves extract of *Ecbolium linnaenum* significantly and dose dependently increased onset of diarrhea as compared with control ( $P < 0.001$ ) in castor oil induced diarrhea. The extract showed Mean no stool value 6.8 and 5.8 inhibition of defecation at the doses of 250 and 500 mg/kg, respectively (Figure 1). Standard drug Loperamide (3 mg/kg) also increased onset of diarrhea and exhibited mean no of stool value of 4 on the inhibition of defecation.

Figure 1: Effect of ethenol leaves extract of *Ecbolium linnaenum* On Castor Oil-Induced Diarrhea

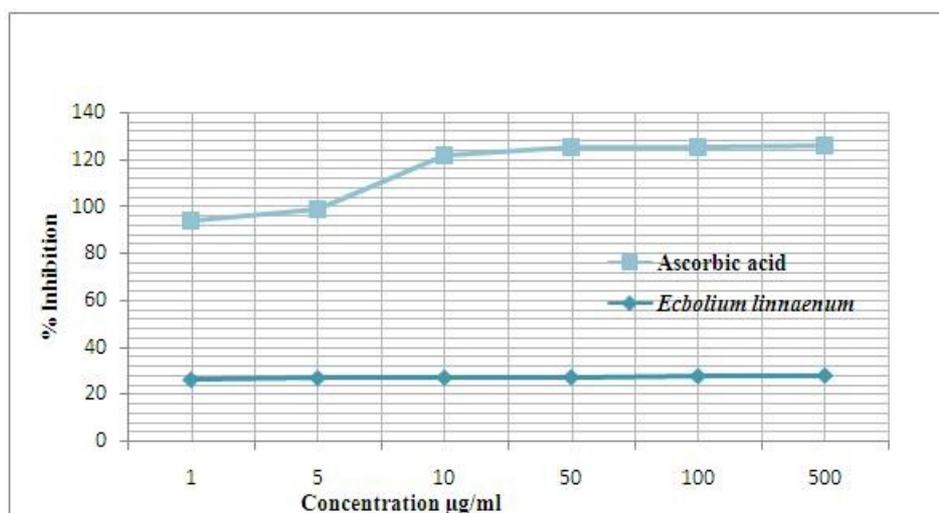


3.3 DPPH radical scavenging assay: The percent inhibition was increased according to dose but at very low rate. Ethanolic extract of *Ecbolium linnaenum* showed 28.25% inhibition at the dose of 500 µg/ml.

Table 3: scavenging activity of ascorbic acid

Conc.(µg/ml)	ascorbic acid (% inhibition)	ethanolic extract of <i>Ecbolium linnaenum</i> (% inhibition)
1	67.51	26.33
5	71.32	27.14
10	94.19	27.25
50	97.36	27.44
100	97.36	27.95
500	97.54	28.25

Figure 2: Comparison of % inhibition of Free radicle by standard ascorbic acid and ethanolic extract of *Ecbolium linnaenum*



3.4 Analgesic activity test: 54.48% and 36.20% inhibition of writhing was found for 500 mg/kg and 250 mg/kg of extract leaves. This rate of inhibition was very close to 75.52% inhibition of Diclofenac-Na. The inhibition is significant at the leaves extract dose of 500 mg/kg (P<0.001).

**Table 4: Effects of ethanolic extracts of *Ecbolium linnaenum* on acetic acid induced writhing in mice**

Treatment (no. = 6)	Dose (mg/kg)	Mean writhing	% Inhibition of writhing	SD	SE	t- test (p values)
Control	--	29	--	7.45	3.72	--
Positive control (Diclofenac Na)	25	7.1	75.52	1.98	0.99	5.68(P<0.001)
Extract of leaves	250	18.5	36.2	10.81	5.43	2.65(P<0.10)
Extract of leaves	500	13.2	54.48	1.45	0.72	3.78(P<0.001)

(SD- Standard Deviation; SE- Stand Error)

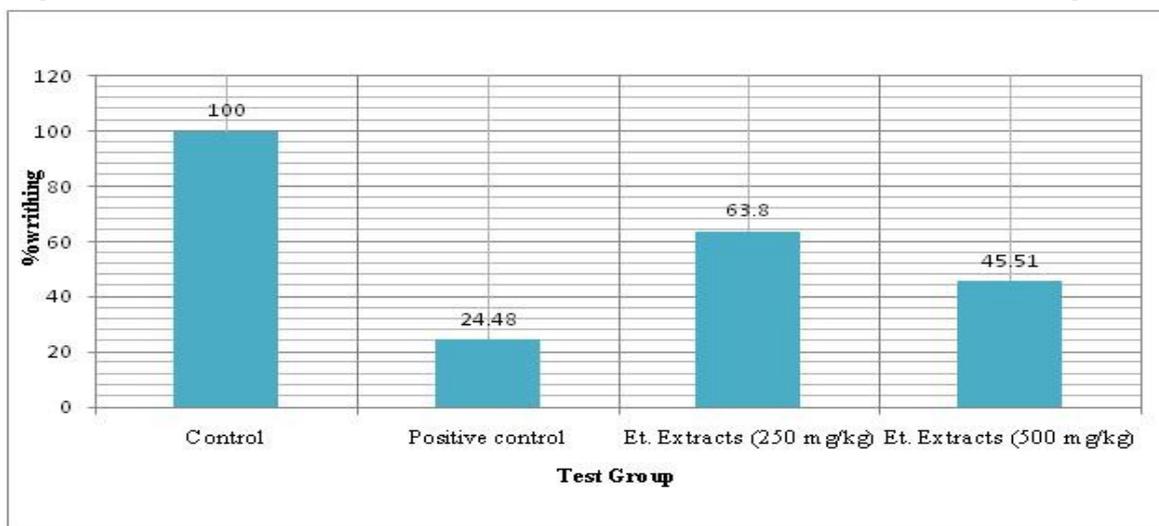
**Figure 3: Observed colour after treating with DPPH on the TLC plate**



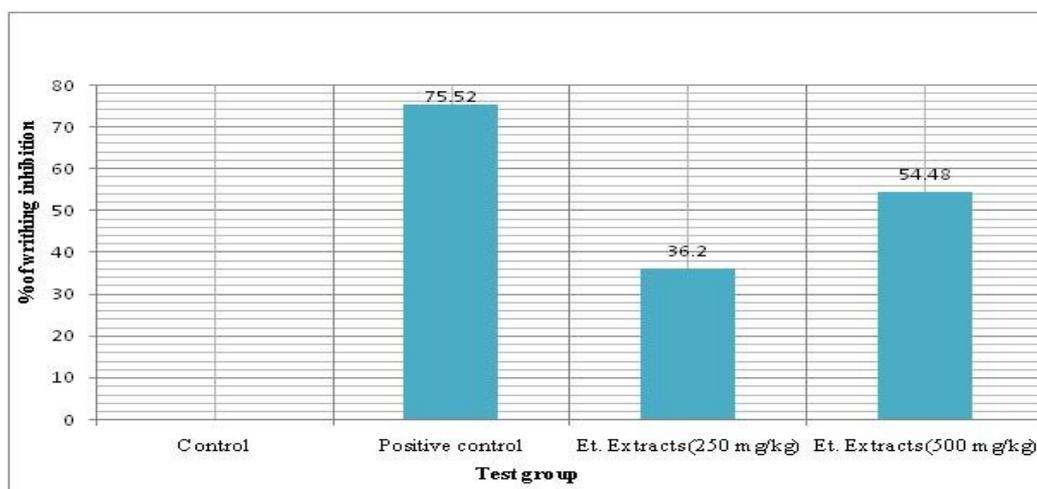
CHCl<sub>3</sub>: CH<sub>3</sub>OH =5:1

CH<sub>3</sub>OH:CHCl<sub>3</sub>: H<sub>2</sub>O =40:10:1

**Figure 4: Effects of ethanolic extracts of *Ecbolium linnaenum* on acetic acid induced writhing in mice**



**Figure 5: Percentage of writhing inhibition of acetic acid induced writhing in mice by the standard drug Diclofenec Na and *Ecbolium linnaenum***



Ethanol extracts of *Ecbolium linnaenum* (250 and 500 mg/kg) exhibited significant inhibition of writhing reflex of 36.20% ( $P < 0.01$ ) and 54.48% ( $P < 0.001$ ) respectively while the standard drug diclofenac inhibition was found to be 75.52% ( $P < 0.001$ ) at a dose of 25 mg/kg body weight.

**3.5 Antimicrobial activity:** Antimicrobial activity of extract was not found at the dose of 250 and 500  $\mu\text{g}/\text{disc}$ .

**Table 5: *In-vitro* antibacterial activity of ethanolic extracts of *Ecbolium linnaenum***

Bacterial Strains	Type of Bacterial Strains	Diameter of Zone of Inhibition (mm)			
		Blank	Kanamycin (30 $\mu\text{g}/\text{disc}$ )	Extracts (250 $\mu\text{g}/\text{disc}$ )	Extracts (500 $\mu\text{g}/\text{disc}$ )
<i>Escherichia coli</i>	Gram(-)	--	32	--	--
<i>Staphylococcus epidermidis</i>	Gram(+)	--	21	--	--
<i>Shigella flexneri</i>	Gram(-)	--	25	--	--
<i>Enterococcus faecalis</i>	Gram(+)	--	28	--	--
<i>Shigella sonnei</i>	Gram(-)	--	23	--	--
<i>Shigella boydii</i>	Gram(-)	--	20	--	--
<i>Streptococcus pyrogenes</i>	Gram(+)	--	31	--	--
<i>Shigella dysenteriae</i>	Gram(-)	--	20	--	--

(Gram (-): Gram Negative Bacteria; Gram (+): Gram Positive Bacteria; (-): No inhibition)

#### 4. Discussion

*In vitro* study of the ethanol extract of *Ecbolium linnaenum* leaves demonstrated its antioxidant activity. In this study DPPH free radical scavenging assay showed potential DPPH scavenging activity 28.25% for 500  $\mu\text{g}/\text{ml}$ . DPPH is most widely used as scavenging test. The ethanol extract of leaves showed also antioxidant activity for the presence of Phenols and flavonoids. The presence of hydroxyl group in phenol is the reason for anti-oxidant activity as well as scavenging solution<sup>8</sup>. The phenolic groups are good antioxidants due to effective hydrogen donor ability<sup>9</sup>. Flavonoids work as scavengers of different “reactive oxygen species (ROS)” like superoxide anion, hydroxyl, peroxy, and hydrogen peroxide. It is also reported as quenchers of singlet oxygen<sup>10</sup>. The extract showed the presence of flavonoids for radical scavenging activity in DPPH assay. Phenols and flavonoids of plants exhibit antioxidant potential due to their redox properties with metal chelating potential<sup>11</sup>.

Acetic acid-induced writhing method showed Analgesic activity of the extract. This method assessed analgesic activity of the plant extract for the release of prostaglandins from receptors<sup>12, 13</sup>. The analgesic activity of the extract was 54.48% for 500 mg/kg and 36.20% for 250 mg/kg than control group. The probable mechanism may be the inhibition of prostaglandins (PGE2 and PGE2 $\alpha$ ) synthesis. The analgesic activity was dependent on the dose. Opioid and steroid

analgesic drugs act in the spinal cord level by binding with different receptors like  $\mu$ ,  $\delta$  and  $\kappa$  in pre and post synaptic membrane as well as inhibiting neurotransmitter release and transmission. Probable mechanism of the extract may be same as opioid analgesics like standard morphine<sup>14, 15, 16</sup>.

The Castor oil induced method was showed antidiarrheal effect of 6.8 and 5.8 Mean inhibition of defecation at the doses of 250 and 500 mg/kg of leaves extract. The model was rational due to prostaglandins in causation of diarrhea by castor oil through the release of ricinoleic acid on the irritation of the intestinal mucosa<sup>17</sup>. The mechanism may be the inhibition of intestinal  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity<sup>18</sup>, stimulation of prostaglandins formation through irritation of the intestinal mucosa<sup>19</sup>, activation of adenylatecyclase mediated active secretion<sup>20</sup> and contribution of nitric oxide<sup>21</sup>.

## 5. Conclusion

Taken together, the ethanol extract of *Ecbolium linnaenum* leaves can justify the use of *Ecbolium linnaenum* as a folk medicine in Bangladesh for the treatment of pain, diarrhea, antioxidant and suggest for the administration of leaves of *Ecbolium linnaenum* in order to use as an anti-diarrheal, analgesic and antioxidant.

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