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Investigation of analgesic and cytotoxic activities of ethanol extract of *Commelina appendiculata*

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Abstract
The present study was aimed to evaluate the possible analgesic and cytotoxic activities of the ethanol extract of *Commelina appendiculata* (EECA) (Family: Commelinaceae). Acetic acid-induced writhing, hot plate and tail immersion methods in *swiss albino* mice at the doses of 100 and 200 mg/kg body weight, p.o were used for the determination of analgesic activity. Brine shrimp lethality bioassay was carried out for assessing the cytotoxicity. Phytochemical screening of ethanol extract of *C. appendiculata* demonstrated the presence of carbohydrates, flavonoids, tannins, glycosides and alkaloids. In analgesic activity study, both doses of the extract produced a significant ($p < 0.05$ and $p < 0.001$) analgesic action in a dose dependent manner. In acetic acid induced writhing method, EECA (200 mg/kg) showed the most significant analgesic activity with writhing inhibition of 76.27% where the standard drug Diclofenac-Na (25mg/kg) and Aspirin (100 mg/kg) showed 80.72% and 61.94% inhibition. In hot plate and tail immersion tests, EECA (200 mg/kg) produced maximum 52.56% and 76.58% nociception inhibition of thermal stimulus respectively. In this study, Morphine (5mg/kg, i.p.) was used as standard. With the EECA, significant lethality was found with LC_{50} value of 26.3 µg/ml while the LC_{50} of vincristine sulphate was 0.52 µg/ml. The results from the present studies reveal the analgesic and cytotoxic properties of the ethanol extract of *C. appendiculata*. So, the plant may be further investigated to find out for its pharmacological active natural products.

Keywords: *C. appendiculata*, Commelinaceae, writhing test, hot plate test, tail immersion test, brine shrimp lethality bioassay.

1. Introduction
Medicinal plants have been used for a wide varity of purposes such as food preservation, alternative medicine, pharmaceutical, alternative medicine and natural therapies for thousands of years. Generally it is considered that compounds produced naturally rather than synthetically and will be biodegraded more easily and therefore being more environmentally acceptable. Thus, natural antioxidants, antibacterial, cytotoxic, antiviral, fungicidal agents and nutrients have gained popularity in recent years, and their use and positive image among consumers are spreading. Herbalism is a traditional or folk medicine practice based on the use of plants and plants extract [1]. Many plants synthesize substances that are useful to the maintenance of health in humans or other animals. These include aromatic substances, most of which are phenols or their oxygen-substituted derivatives such as tannins. Many of the herbs and spices used by humans to season food yield useful medicinal compounds [2, 3]. Herbal therapy is used to treat a large variety of ailment and symptoms, e.g., inflammation, fever and pain, however there are no adequate experimental evidence about their effectiveness [4, 6]. *Commelina appendiculata* (*C. appendiculata*) locally known as Kanda Loa, belonging to the family of commelinaceae is an annual herb, steam creeping, diffusely branched, 40 cm or more long, internodes slightly reddish, roots fibrous at the lower nodes. The plant is distributed in Eastern India and Sri Lanka. In Bangladesh, it occurs in Tangail, Mymensingh and Sylhet district. It is used in the Sunamganj district as a folk medicine for the treatment of cats and dogs bite [7]. Some of the species of *Commelina* are used in Chinese medicine for the treatment of wind-heat type common cold, sore throat, fever and dropsy [8]. For example, *C. communis* L., a species of the *Commelina* genus, widespread in the world especially in the tropics and subtropics, has long been used in folk medicine in China, and modern pharmaceutical investigations had revealed its anti-inflammatory, antiviral, and antihyperglycemic effects. Literature survey revealed that many plants of this genus are used in medicine for the treatment of inflammation, pain, fever, dysentery, diarrhoea, diabetes, neurological disorders, cancer, toxicity, heart diseases, asthma, oxidative stress etc. Plants belonging to the genus *Commelina* have been shown to contain, coumarins, anthocynins, alkaloids, terpenoids, steroids, iridoids, flavanoids and some other molecules such as aliphatic alcohols, polyols, and

phenolic acids [9, 12]. The group of flavonoid is famous for its anti-inflammatory, antiallergic, antithromtic, vasoprotective and protection of gastric mucosa. These properties have been attributed to influence of flavonoids on production of prostaglandins and their antioxidant effects [13]. Literature search revealed no previous report on the analgesic and cytotoxic activity of the species of *C. appendiculata*. Therefore, as a part of our continuing studies [14, 17] on natural products for their pharmacological properties we investigated the first time of ethanol extract of *C. appendiculata* for its analgesic and cytotoxic activity.

2. Materials and Methods

2.1. Collection of the plant

The plant of *C. appendiculata* was collected from local area of Sylhet sadar during January 2014. The collected plant was then identified by the taxonomist of Jahangirnagar University Herbarium, Savar and a voucher specimen has been deposited (DACB: 39,321) for further reference.

2.2. Extraction of the plant material

After shade drying, the whole plant was reduced to coarsely powder using a grinding mill. The plant was extracted by a cold extraction method. The dried and coarse powder (106 g) was extracted with ethanol (500 ml) in an air tight, clean flat-bottomed container for 3 days at room temperature with occasional stirring. The extract was then filtered and evaporated on rotary evaporator under reduced pressure to obtained 18 gm (yield = 16.98%) crude extract which was used for analgesic and cytotoxic activity study.

2.3. Drugs and chemicals

Acetic acid and DMSO (Dimethyl sulfoxide) was product of Merck, Germany. Diclofenac sodium and aspirin were purchased from Square Pharmaceuticals Ltd., Bangladesh; morphine was purchased from Gonoshasthaya Pharmaceuticals Ltd., Bangladesh; 0.9% sodium chloride solution (Normal saline) was purchased from Orion Infusion Ltd., Bangladesh and other reagents were of analytical grade.

2.4. Experimental animals

For the experiment *Swiss albino* mice of either sex, 4-5 weeks of age, weighing between 25-30 gm, were collected from the Animal Research Branch of the International Center for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). Animals were maintained under standard environmental conditions (temperature: (24.0±1.0º), relative humidity: 55-65% and 12hrs light/12 hrs dark cycle) and had free access to fed and water *ad libitum*. The animals were acclimatized to laboratory condition for two weeks prior to experimentation. The number of protocol approval by the Ethics Committee of Jahangirnagar University, Dhaka, Bangladesh for the use of laboratory animals for the experiments.

2.5. Phytochemical screening

Ethanol extract of *C. appendiculata* was qualitatively tested for detection of carbohydrates, tannins, flavonoids, saponins, proteins, steroids, alkaloids, glycosides, glucosides and resins following standard phytochemical procedures **[18-19]**.

2.6. Acute toxicity study

Mice were divided into control and test groups (n=6). The test groups received the extract per orally at the doses of 500, 1000, 1500 and 2000 mg/kg. Then the animals were kept in separate cages and were allowed to food and *ad libitum*. The control group received the water. The animals were observed for possible behavioral changes, allergic reactions and mortality for the next 72 h [20].

2.7. Analgesic activity study

2.7.1. Acetic acid induced writhing test

The method described by Dash *et al.,* (2015) [17] that was adopted to study the effect of the *C. appendiculata* extract on acetic acid induced writing test. Test samples and control (n=6) were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid but Diclofenac-sodium (i.p) and aspirin (p.o) were administered 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed (abdominal contraction, elongation of the body and extension of the hind limb were referred as writhing) for the next 10 min. Percentage inhibition of writhing was calculated using the following formula:

$$\text{Writhing inhibition (\%)} = \frac{\text{Mean no. of writhings (control)} - \text{Mean no. of writhing (test)} \times 100}{\text{Mean no. of writhings (control)}}$$

2.7.2. Hot plate test

The hot plate test was performed according to the method described by Eddy and Leimbach (1953) [21]. The animals in the control group received water (5 ml/kg, p.o.) while the standard group was treated with morphine sulphate (5 mg/kg, i.p.). The animals in the test groups were treated with 100 and 200 mg/kg, per oral of ethanol extract of *C. appendiculata.* Then the animals were placed on Eddy's hot plate kept at a temperature of 52±0.5 ^{0}C. A cut off period of 28s was observed to avoid damage to the paw. Reaction time was recorded when animals licked their fore or hind paws, or jumped prior to and 0, 30, 60 and 90 min after the administration of the standard and test drugs (n=6). Percentage of elongation was calculated using the following formula:

$$\textbf{Elongation (\%)} = \frac{\text{Latency (test)} - \text{Latency (control)}}{\text{Latency (test)}} \times 100$$

2.7.3. Tail immersion test

The procedure is based on the observation that morphine like drugs selectively prolongs the reaction time of the typical tail withdrawal reflex in mice [22].The animals were treated as discussed above. 1 to 2 cm of the tail of mice was immersed in warm water kept constant at 55±1°C. The reaction time was the time taken by the mice to deflect their tails. The first reading was discarded and the reaction time was recorded as a mean of the next three readings. A latency period of 28s was defined as complete analgesia and the measurement was then stopped to avoid injury to mice. The latent period of the tail-immersion response was determined at 0, 30, 60 and 90 min after the administration of standard and test samples (n=6).

Percentage of elongation was calculated using the same formula used in hot plate test.

2.8. Cytotoxicity studies

2.8.1. Brine shrimp lethality bioassay

Brine shrimp lethality bioassay [23] was carried out to investigate the cytotoxicity of the plant extracts. 5 mg of the extract was measured and dissolved in DMSO. Serial dilution was then carried out in order to obtain the concentration of 1.25 µg/ml to 320 µg/ml. 5 ml of artificial sea water was added into all the test tubes. *Artemia salina* was used as a convenient monitor for cytotoxic screening. The eggs of the brine shrimps were hatched in artificial seawater (prepared by using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 24 hr under the light. The hatched shrimps were allowed to grow by 48 hr to get shrimp larvae called nauplii. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. 10±2 nauplii were drawn through a glass capillary and placed in each test tube containing 5 ml of brine solution. In each experiment, 50 µl of the plant extract was added and up to volume 5 ml of brine solution and maintained at room temperature of 25±1 °C for 24 hr under the light and surviving larvae were counted. Vincristine sulfate (0.156 to 40 µg/ ml) was used as a positive control in this bioassay [24]. The mean percentage mortality was plotted against the logarithm of concentrations and the concentration killing fifty percent of the larvae (LC_{50}) was determined from probit analysis described by Finney [25] as well as linear regression equation using the software "Microsoft Excel – 2003"**.**

2.9. Statistical analysis

The statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnett's multiple comparisons. The results obtained were compared with the control group. $P < 0.05$ and $P < 0.001$ were considered to be statistically significant.

3. Results

3.1. Phytochemical screening

Preliminary phytochemical group tests revealed that ethanol extract of *C. appendiculata* contain carbohydrates, tannins, flavonoids, glycoside and alkaloids (Table 1).

Table 1: Results of phytochemical screening

Test	EECA
Carbohydrates	+
Tannins	+
Flavonoids	+
Saponins	-
Proteins	-
Steroids	-
Alkaloids	+
Glycosides	+
Glucosides	-
Resins	-

(+) =Presence, (–) =Absence, EECA = Ethanol extract of *Commelina appendiculata*.

3.2. Acute toxicity

Oral administration of ethanol extract of *C. appendiculata* at the doses of 500–2000 mg/kg did not produce any mortality or noticeable behavioral changes in mice within 72 hr observation period. Therefore, it can be suggested that *C. appendiculata* have low toxicity profile with LD_{50} greater than 2000 mg/kg.

3.3. Analgesic activity

3.3.1. Acetic acid induced writhing test

The oral administration of both doses of *C. appendiculata* extract significantly ($p < 0.001$) inhibited writhing response induced by acetic acid in a dose dependent manner. At 200 mg/kg dose showed 76.27% inhibition of writhing in comparison to control. The standard drug Diclofenac-Na (25 mg/kg, i.p.) and Aspirin (100 mg/kg, p.o.) were exhibited 80.72 % and 61.94% inhibition of writhing respectively (Table 2).

Table 2: Effect of ethanol extract of *C. appendiculata* on acetic acid-induced writhing test in mice.

Group	Dose (mg/kg)	No. of Writhings (Mean±SEM)	% of writhing	% of writhing inhibition
Control	5ml/kg	50.58±4.18**	100.00	--
Diclofenac-Na	25	9.75±0.77**	19.27	80.72
Aspirin	100	19.25±4.12**	38.06	61.94
EECA	100	14.92±3.17**	29.49	70.50
	200	12±2.22**	23.72	76.27

Control group received water 5ml/kg body weight (p.o.), standard groups received Diclofenac-Na 25mg/kg (i.p.) and Aspirin 100mg/kg body weight (p.o.).Standard drugs were administered 15 min before 0.7 % acetic acid administration. Writhing was counted for 15 min, starting after 5 min of acetic acid administration. Test group EECA was treated with 100 and 200 mg/kg body weight of the extract (p.o.) respectively. Values are mean ±SEM, (n=6); ** $p < 0.001$, *Dunnett t-test* as compared to control. **EECA=**Ethanol extract of *Commelina appendiculata.*

3.3.2. Hot plate test

Results of hot plate test are presented in Table 3. Oral administration of EECA significantly ($p < 0.05$ and $p < 0.001$) prolonged the latency period at both 100 and 200 mg/kg doses when compared to the control group. The extract increased the latency time in a dose dependent manner. Maximum effect of the extract was observed 60 and 90 min. In this study, Morphine (5 mg/kg, i.p.) was used as standard.

Table 3: Effect of ethanol extract of *C. appendiculata* on hot plate test in mice.

Group	Dose (mg/kg)	Mean Reaction Time (s)			
		0 min	30 min	60 min	90 min
Control	5ml/kg	9.67±0.98	8.83±0.60	9.83±1.04	9.33±1.11
Morphine	5	10.83±1.76	17±2.09* 48.06%	21.33±2.19* 53.91%	16±1.80 41.68%
EECA	100	9.67± 2.01	11.83 ± 1.72 25.36%	15.67±1.97 37.27%	18.67±1.34* 50.03%
	200	9.17±0.67	15±2.53 41.13%	19.67±4.29* 50.03%	19.67±2.81** 52.57%

Control group received water 5ml/kg body weight (p.o.), standard group received Morphine 5mg/kg body weight (i.p.), test group EECA was treated with 100 and 200 mg/kg body weight of the extract (p.o.) respectively. Values are mean ±SEM, (n=6); * $p < 0.05$, ** $p < 0.001$, *Dunnett t-test* as compared to control. **EECA**=Ethanol extract of *Commelina appendiculata*.

3.3.3. Tail immersion test

The tail-withdrawal reflex time of the mice to the hot water-induced pain significantly increased after administration of EECA (Table 4). The maximum effect of the extract was recorded at 60 and 90 min. The effect was statistically significant ($p < 0.001$) in comparison to control. In this test, the increase in latency was highly significant than that were observed in the hot plate test.

Table 4: Effect of ethanol extract of *C. appendiculata* on tail immersion test in mice.

Group	Dose (mg/kg)	Mean Reaction Time (s)			
		0 min	30 min	60 min	90 min
Control	5ml/kg	1.77±0.11	1.66±0.15	1.83±0.24	1.77±0.11
Morphine	5	2.44±0.11	4.94±0.63** 66.39%	5.11±0.40** 64.18%	5.88±0.25** 69.89%
EECA	100	2.49±0.13	5.05 ±0.55** 67.13%	5.94 ±1.03** 69.19%	6.22 ± 0.52** 71.54%
	200	2.77±0.25	5.05 ±0.47** 67.13%	6.22 ±0.61** 70.58%	7.56 ±1.23** 76.58%

Control group received water 5ml/kg body weight (p.o.), standard group received Morphine 5mg/kg body weight (i.p.), test group EECC was treated with 100 and 200 mg/kg body weight of the extract (p.o.) respectively. Values are mean ±SEM, (n=6); * $p < 0.05$, ** $p < 0.001$, *Dunnett t-test* as compared to control. **EECA**=Ethanol extract of *Commelina appendiculata*.

3.4. Cytotoxic activity

3.4.1Brine shrimp lethality bioassay

The degree of lethality shown by the extract was found to be directly proportional to the concentration of the extract ranging from the lowest concentration (1.25 µg/ml) to the highest concentration (320 µg/ml) (Table 5 and Figure 1). The plant extract showed mild cytotoxic potency to brine shrimp nauplii, having LC_{50} values of 26.3 µg/ml while the LC_{50} of the standard drug vincristine sulphate was 0.52 µg/ml (Table 6).

Table 5: Effect of ethanol extract of *C. appendiculata* on brine shrimp lethality test in *Artemia salina*.

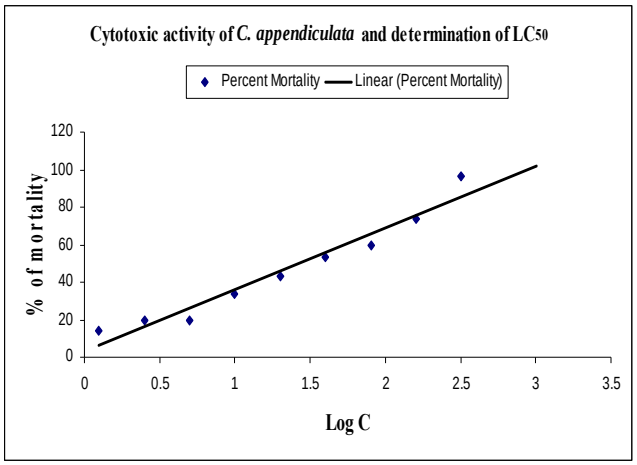
C. appendiculata									Vincristine Sulfate				
Sample conc. (µg/ml)	Log conc.	No.of nauplii taken	No. of nauplii dead				Average no. of nauplii dead	Percent of mortality	Std.Conc. (µg/ml)	Log conc.	No.of nauplii taken	No.of nauplii dead	% of mortality
			Trial-1	Trial-2	Trial-3	Trial-4							
1.25	0.09	10	2	1	1	2	1.5	15	0.156	-0.806	10	3	30
2.5	0.39	10	2	2	2	4	2.5	25	0.312	-0.505	10	4	40
5	0.69	10	2	2	2	4	2.5	25	0.625	-0.204	10	5	50
10	1	10	3	3	4	4	3.5	35	1.25	0.096	10	6	60
20	1.30	10	3	6	4	4	4.25	42.5	2.5	0.397	10	8	80
40	1.60	10	5	6	5	4	5	50	5	0.698	10	9	90
80	1.90	10	6	8	4	4	5.5	55	10	1	10	10	100
160	2.20	10	6	10	6	8	7.75	77.5	20	1.310	10	10	100
320	2.50	10	9	10	10	5	8.5	85	40	1.602	10	10	100

Mortality (%) = Number of dead brine shrimps × 100/ Total number of brine shrimps

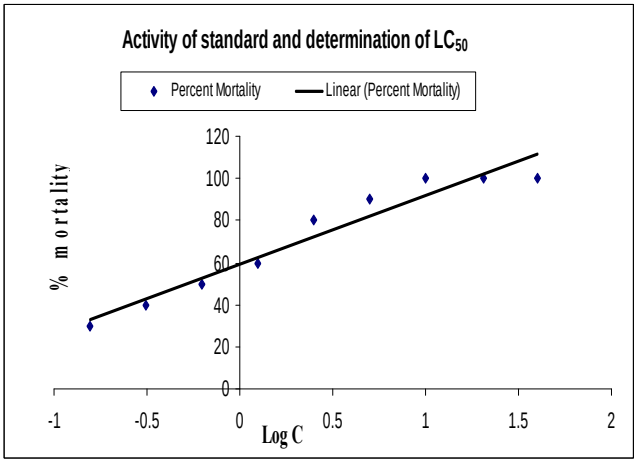
Table 6: Result of *C. appendiculata* against on *Artemia salina*.

Sample	LC_{50} (µg/ml)	Regression equation	R^2
Vincristine Sulphate	0.52	Y = 32.61x+59.22	0.942
EECA	26.3	Y = 32.721x + 3.4623	0.952

EECA =Ethanol extract of *Commelina appendiculata*.



The rate of mortality of the nauplii found to be increased with increasing concentration of the sample. From this graphical plotting the LC_{50} value found to be 26.3 µg/ml in EECA. **EECA** =Ethanol extract of *Commelina appendiculata.*



The rate of mortality of the nauplii found to be increased with increased of the concentration. From this graphical plotting the LC_{50} value found to be 0.52 µg/ml in vincristine sulphate.

Fig 1: Effect of *Commelina appendiculata* and Vincristine sulphate on *brine shrimp lethality bioassay*.

4. Discussion

The present study demonstrates that ethanol extract of *C. appendiculata* possess potent analgesic activity in chemical and heat induced models. No acute toxicity was observed after oral administration of *C. appendiculata* even at the dose of 2000 mg/kg in mice. In the present study, our results demonstrated that EECA possesses significant analgesic activity as evaluated in the acetic acid-induced writhing, hot plate and tail-flick tests. The acetic acid-induced writhing test is a chemical stimulus widely used for the evaluation of peripheral analgesic activity [26]. Intraperitoneal injection of 0.7% acetic acid can produce the peritoneal inflammation (acute peritonitis) which causes the response characterized by contraction of the abdominal muscle accompanied by an extension of the forelimbs and elongation of the body. This writhing response is considered as a visceral inflammatory pain model [27]. This method has been associated with the increased levels of prostaglandins in the peritoneal fluids [28]. In acetic acid induced writhing test, EECA at the doses of 100 and 200 mg/kg showed significant analgesic activity with writhing inhibition of 70.50% and 76.27%, where the standard drug Diclofenac-Na (25 mg/kg) and Aspirin (100 mg/kg) showed 80.72% and 61.94% inhibition (Table 2). The results in this study revealed that EECA significantly ($p < 0.001$) inhibited writhing response induced by acetic acid in a dose dependent manner. The findings of the present study indicate that the extract of *C. appendiculata* posses a significant peripheral analgesic activity. The hot plate method is one of the most common tests evaluating the analgesic efficacy of drugs in rodents [29]. The drug that reduces the nociceptive response indicated by cutaneous thermic stimuli in the hot plate test might exhibit central analgesic properties or supraspinal analgesia [30]. In hot plate method, both doses of the extract produced a dose dependent increase in latency time when compared with the control. The results were found to be statistically significant ($p < 0.05$ and $p < 0.001$). Ethanol extract of *C. appendiculata* (EECA) at the doses of 100 and 200mg/kg) displayed maximum 50.03% and 52.57 % inhibition of thermal stimulus respectively (Table 3). In this study, Morphine (41.68% inhibition) was used as standard. The tail-withdrawal reflex time of the mice to the hot water-induced pain significantly increased after administration of EECA. The maximum effect of the extract was recorded at 60 and 90 min. The effect was statistically significant ($p < 0.001$) in comparison to control. In this test, at the doses of 100 and 200 mg/kg of EECA exhibited maximum 71.54% and 76.58% inhibition of thermal stimulus respectibely, whereas the standard drug Morphine (5mg/kg, i.p) showed 69.89% inhibition (Table 4). In this study, our results indicated that the extract of *C. appendiculata* has antinociceptive effect against both the hot plate and tail-flick tests, therefore the antinociceptive is likely to mediated centrally. Phytochemical studies include the presence of alkaloids, flavonoids, carbohydrates and tannins in *C. appendiculata*. Flavonoids were reported to have a role in analgesic activity primarily by targeting prostaglandins [31]. There are also reports on the role of tannins in anti-nociceptive activity [32]. Besides alkaloids are well known for their ability to inhibit pain perception [33]. Therefore, it is assumed that these compounds may be responsible for the observed analgesic activity.

Brine shrimp lethality is a general bioassay which is indicative of cytotoxicity, antibacterial activities, pesticidal effects and various pharmacologic actions [23]. From the results of the brine shrimp lethality bioassay it can be well predicted that the extract have cytotoxic potency. The degree of lethality was directly proportional to the concentration of the extract from the lowest concentration (1.25µg/ml) to highly significant with the highest concentration (320µg/ml) (Table 5). Maximum mortalities took place at a concentration of 320µg/ml, whereas least mortalities were at 1.25µg/ml concentration. In other words, mortality increased gradually with the increase in concentration of the test samples. Control group nauplii remained unchanged (no lethality/mortality), is indicative of the cytotoxicity of the extract. A plot of log concentration of the test sample versus percentage of mortality on a graph paper showed an approximately linear correlation between them.

From this graphical plotting (Figure 1), the LC_{50} value was found to be 26.3 µg/ml in EECC whereas the LC_{50} of the standard drug vincristine sulphate was 0.52 µg/ml (Table 6). The positive response obtained in this assay suggests that the extracts may have bioactive compounds.

5. Conclusion
Based on the results of the present study, it can be concluded that the plant extract possesses analgesic and cytotoxic potential. However, further studies are needed to isolate the compound (s) responsible for such activity.

6. Acknowledgments
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7. Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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