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In vivo cytotoxic and *In vitro* antibacterial activities of *Kaempferia galanga*

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ABSTRACT
Kaempferia galanga (Family: Zingiberaceae) has been used for the treatment of various skin disorders and widely used in the treatment of nematocide, larvicide, colera and various inflammatory disorders. The study was aimed to investigate the cytotoxic and antibacterial activity of different extracts of the rhizome and leaf of *Kaempferia galanga*. Cytotoxicity was determined against (*Artemia salina*) brine shrimp nauplii. The antibacterial activity was performed by disc diffusion method and determination of zone of inhibition of living microorganisms. In the brine shrimp lethality bioassay all the extracts showed moderate cytotoxic activity when compared with the standard drug vincristine sulphate. For example, LC_{50} value of the acetonic leaf extract was 4.78 µg/ml while the LC_{50} of vincristine sulphate was 0.52 µg/ml. All the natural products (400 µg/disc) showed moderate antibacterial activity against both gram positive and gram negative bacteria as compared with the standard drug ciprofloxacin (5 µg/disc).

Keywords: *Kaempferia galanga*, zingiberaceae, brine shrimp nauplii, disc diffusion method, microorganisms.

1. Introduction
Kaempferia galanga (Chandramulika in Bengali) belonging to the family Zingiberaceae is an aromatic perennial herb with tuberous rootstocks. The rhizome of *Kaempferia galanga* finds an important place in indigenous medicine as carminative, expectorant, diuretic and stimulant [1]. It had been used for the treatment of various skin disorders and widely used in the treatment of diabetes mellitus, various inflammatory and lipid disorders [2]. It also possesses larvicidal activity, antioxidant, nematicidal activity, anti-inflammatory, toxicity and sedative properties [3]. A methanolic extract of the rhizome contains ethyl *p*-methoxy-*trans*-cinnamate, which is highly cytotoxic to HeLa cells [4]. Ethyl-*p*-methoxycinnamate isolated from the extracts of *Kaempferia galanga* has considerable activity against *Mycobacterium tuberculosis* and *Candida albicans* [5, 6]. As a part of our continue study [7, 8] on natural products for their pharmacological properties we investigated different extracts of *Kaempferia galanga* for its cytotoxicity and antibacterial activity.

2. Materials and Methods
2.1. Collection of the plant
The plant of *Kaempferia galanga* was collected from the local area of Mauoa, Dhaka during December 2011. Dust, dirt and the undesirable materials were then separated manually. The collected plant was then identified by Bushra Khan, Principal Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka and a voucher specimen has been deposited (DACB: 36,064) for further reference.

2.2. Extraction and fractionation of the plant material
The plant parts were extracted by a cold extraction method. The rhizome (900 g) and leaf (200 g) powder were taken and soaked with 2700 ml and 600 ml of acetone for 3 consecutive days at 25 °C. The extracts were filtered and evaporated on rotary evaporator under reduced pressure. Recovered solvent was again used for percolation for another 3 days. The process was repeated three times to obtain 58 g rhizome (yield 6.45%) and 4.14 g leaf (yield 2.07%) extract of *Kaempferia galanga*. The rhizome extract was further partitioned using petroether, chloroform and methanol. The acetone extract of the rhizome (ACR), as well as petroether fraction (PEF), chloroform fraction (CHF), methanol fraction (MEF), and acetone extract of leaf (ACL) were subjected to cytotoxic and antimicrobial activity test.

2.3. Drugs and chemicals
DMSO (dimethyl sulfoxide), ethanol and vincristine sulphate were purchased from Merck, Germany. Ciprofloxacin was collected from Oxoid Ltd., Basingstoke, England.

2.4. Phytochemical screening
The extracts of *Kaempferia galanga* were qualitatively tested for detection of carbohydrates, tannins, flavonoids, proteins, steroids, alkaloids and resins following standard phytochemical procedures [9].

2.5. Microorganisms
Simple zoological organism *Artemia salina* was used for cytotoxicity study. Gram positive *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus (B. cereus)* and Gram negative *Escherichia coli* (*E. coli*), *Pseudomonas aureus* (*P. aureus*), *Shigella dysenteriae* (*S. dysenteriae*) and *Klebsiella pneumoniae* (*K. pneumoniae*) were used for antibacterial study. These organisms were collected from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh.

2.6. Cytotoxicity studies
2.6.1. Brine shrimp lethality bioassay
Brine shrimp lethality bioassay was carried out according to Meyer *et al.* [10] to investigate the cytotoxicity of the extract. 5 mg of each 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 h to get shrimp larvae called nauplii. After 48 hr, active nauplii were attached to one side in a glass Petri dish by using a micropipette. The nauplii were then separated from the eggs by aliquoting them in another glass Petri dish containing artificial sea water and used for the assay. Suspension containing 10 nauplii was added into each test tube and was incubated at room temperature of 25±1 °C for 12 hr under the light. The tubes were then examined after 24 hr and the number of surviving larvae in each test tube was counted with the aid of a 3× magnifying glass. The percentage of mortality was plotted against the logarithm of concentration. The concentration that would kill 50% of the nauplii (LC_{50}) was determined from probit analysis [11] as well as linear regression equation using the software "Microsoft Excel-2003". Vincristine sulfate was used as standard in this bioassay.

2.7. Antibacterial studies
2.7.1 Media preparation and maintenance of bacteria
All the bacterial strains were grown and maintained on Muller Hinton agar (Hi media, India) media at 37 °C and pH (7.3±0.2). The bacteria were subcultured overnight in Muller Hinton broth, which was further adjusted to obtain turbidity comparable to McFarland (0.5) standard when required [12].

2.7.2. Antibacterial screening
2.7.3. Disc diffusion method
The antibacterial activity of the extract was determined by disc diffusion method [13]. The test microbes were taken from the broth culture with inoculating loop and transferred to test tubes containing 10.0 mL sterile distilled water. The inoculums were added until the turbidity was equal to 0.5 McFarland standards. Cotton swab was then used to inoculate the test tube suspension onto the surface of Muller Hinton agar and the plate was allowed to dry. Sterilized Whatman paper discs (6mm in diameter) were treated with the desired concentration of previously prepared ethanolic solution of extract using a micropipette and dried in air under aseptic condition and placed at equidistance in a circle on the seeded plate. The concentration of the extract was used 400 µg/disc. These plates were kept for 4-6 hr at low temperature and the test materials diffuse from disc to the surrounding medium by this time. The same was done for ethanol (negative control) as well as ciprofloxacin 5 µg/disc for positive control. The experiment was conducted in triplicates. The plates were incubated at 37 °C for 24 hr. At the end of the period, the inhibition zone against each microorganism by plant extract was measured.

2.7.4. Determination of Relative Percentage Inhibition
The relative percentage inhibition with respect to positive control was calculated by using the following formula [14]. Relative percentage inhibition of the test extract = [{100 x (a - b)}/(c - b)]. Where, a: total area of inhibition of the test extract; b: total area of inhibition of the solvent; c: total area of inhibition of the standard drug. The total area of the inhibition was calculated by using area = πr^2; where, r = radius of the zone of inhibition.

2.8. Statistical analysis
All assays were performed in triplicate under strict aseptic conditions to ensure consistency of all findings. Data of all experiments were statistically analyzed and expressed as the mean ± SEM of three replicate experiments.

3. Results
3.1. Phytochemical screening
Preliminary phytochemical group tests revealed that different extracts of *Kaempferia galanga* contain carbohydrates, tannins, flavonoids, proteins, steroids, alkaloids and resins (Table 1).

3.2. Cytotoxicity studies
3.2.1. Brine shrimp lethality bioassay
The degree of lethality shown by the extracts 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 2). The ACL was found to be maximum toxic to brine shrimp nauplii, having LC_{50} values of 4.78 µg/ml while the LC_{50} of the reference anticancer drug vincristine sulphate was 0.52 µg/ml (Table 3). The rate of mortality of the nauplii found to be increased with increasing concentration of the sample (Figure l and Figure 2).

Table 1: Results of phytochemical screening

Test	ACR	PEF	CHF	MEF	ACL
Carbohydrates	+	-	+	+	-
Tannins	+	+	+	+	+
Flavonoids	-	-	+	+	-
Saponins	-	-	-	-	-
Proteins	+	-	-	+	-
Steroids	+	+	+	+	+
Alkaloids	+	+	-	+	+
Glycosides	-	-	-	-	-
Glucosides	-	-	-	-	-
Resins	+	+	+	+	-

(+) =Presence; (–) =Absence; **ACR**= Acetone extract of rhizome, **PEF**= Petroether fraction of rhizome, **CHF**= Chloroform fraction of rhizome, **MEF**= Methanol fraction of rhizome, **ACL**=Acetone extract of leaf

Table 2: Effect of different extracts of *Kaempferia galanga* on brine shrimp lethality test in *Artemia salina.*

Sample conc. (µg/ml)	Log conc.	No. of nauplii taken	Average no. of nauplii dead					Percent of mortality					Vincristine Sulfate				
			ACR	PEF	CHF	MEF	ACL	ACR	PEF	CHF	MEF	ACL	Std.Conc. (µg/ml)	**Log conc.**	No. of nauplii taken	No. of nauplii dead	% of mortality
1.25	0.090	10	2.5	3.5	3.5	2.5	3.5	25	35	35	25	35	**0.156**	-0.806	10	3	30
2.5	0.39	10	4.5	4	4	3.5	4	45	40	40	35	40	**0.312**	-0.505	10	4	40
5	0.69	10	4.5	4.5	5	4.5	5.5	45	45	50	45	55	**0.625**	-0.204	10	5	50
10	1	10	5	5.5	5.5	5	6	50	55	55	50	60	**1.25**	0.096	10	6	60
20	1.30	10	6	6.5	6	6.5	6.5	60	65	60	65	65	**2.5**	0.397	10	8	80
40	1.60	10	6	6.5	6	6.5	7	60	65	60	65	70	**5**	0.698	10	9	90
80	1.90	10	6.5	7	6	7	7.5	65	70	60	70	75	**10**	1	10	10	100
160	2.20	10	7.5	7.5	8	8	7.5	75	75	80	80	75	**20**	1.310	10	10	100
320	2.50	10	8	8.5	8	8	9	80	85	80	80	90	**40**	1.602	10	10	100

ACR =Acetone extract of rhizome, **PEF**= Petroether fraction of rhizome, **CHF**=Chloroform fraction of rhizome **MEF**=Methanol fraction of rhizome and **ACL**=Acetone extract of leaf

Table 3: Result of *Kaempferia galanga* against on *Artemia salina.*

Sample	LC_{50} (µg/ml)	Regression equation	R^2
Vincristine Sulphate	0.52	Y = 32.61x+59.22	0.942
ACR	9.77	Y = 19.93x+30.18	0.943
PEF	6.76	Y = 20.20x+33.15	0.977
CHF	7.24	Y = 17.99x+34.36	0.923
MEF	9.77	Y = 23.25x+26.96	0.962
ACL	4.78	Y = 20.76x+35.76	0.954

ACR =Acetone extract of rhizome, **PEF**= Petroether fraction of rhizome, **CHF**=Chloroform fraction of rhizome **MEF**=Methanol fraction of rhizome and **ACL**=Acetone extract of leaf.

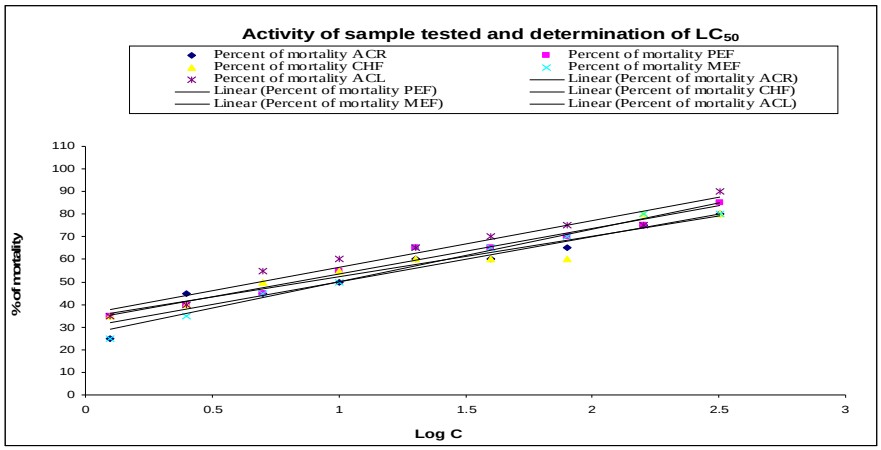

Fig 1: Effect of different extracts of *Kaempferia galanga* on brine shrimp nauplii.
ACR =Acetone extract of rhizome, **PEF**= Petroether fraction of rhizome, **CHF**=Chloroform fraction of rhizome **MEF**=Methanol fraction of rhizome and **ACL**=Acetone extract of leaf.

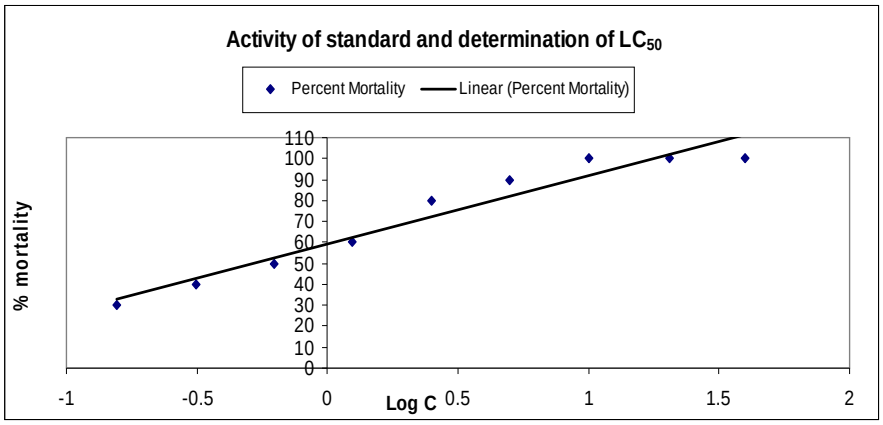

Fig 2: Effect of vincristine sulphate on brine shrimp nauplii.

Table 4: Antibacterial activity of different extracts of *Kaempferia galanga*.

Microorganism	Determination of zone of inhibition in mm					
	Cipro 5 µg/disc	ACR 400 µg/disc	PEF 400 µg/disc	CHF 400 µg/disc	MEF 400 µg/disc	ACL 400 µg/disc
Gram positive						
Staphylococcus aureus	22±0.71	10±1.47 (20.66%)	15±1.47 (46.48%)	13±1.47 (34.91%)	10±1.22 (20.66%)	14±1.47 (40.49%)
Bacillus cereus	15±0.82	8±0.71 (28.44%)	7±0.41 (21.77%)	10±0.41 (44.44%)	10±0.41 (44.44%)	6±0.41 (16.00%)
Gram negative						
Escherichia coli	21±1.08	13±1.08 (13.32%)	13±1.63 (38.32%)	15±0.71 (51.02%)	13±0.82 (38.32%)	14±0.82 (44.44%)
Pseudomonas aureus	21±1.08	8±0.41 (14.51%)	10±1.22 (22.67%)	10±0.82 (22.67%)	9±0.41 (18.36%)	10±0.71 (22.67%)
Shigella dysenteriae	21±0.41	12±0.82 (32.65%)	13±0.82 (28.32%)	10±0.41 (22.67%)	12±0.71 (32.65%)	12±0.82 (32.65%)
Klebsiella pneumonia	15±1.63	0	0	0	0	0

Values of the observed diameter zone of inhibition (mm). Incubation conditions for bacteria- 24 hours at 37 °C. The assay was performed in triplicate and the results are the mean of three values ± SEM. Within a bracket indicate the relative percentage of inhibition. ACR =Acetone extract of rhizome, PEF= Petroether fraction of rhizome, CHF=Chloroform fraction of rhizome MEF=Methanol fraction of rhizome and ACL=Acetone extract of leaf, 0= No Zone of Inhibition, Cipro = Ciprofloxacin.

3.3. Antibacterial studies

3.3.1. Disc diffusion method

In this method ACR, PEF, CHF, MEF and ACL showed moderate activity against all the tested bacteria (except *Klebsiella pneumonia)* with the zone of inhibition (Table 4 and Figure 3) range were found to be 8-13, 7-15, 10-15, 9-13 and 6-14 mm at 400 µg/disc respectively, whereas the zones of inhibition of the standard disc ciprofloxacin (5 µg/disc) was exhibited 15-22 mm. *Klebsiella pneumonia* was not inhibited by any of the extract. PEF and CHF displayed highest zone of inhibition (15 mm) against *Staphylococcus aureus* and *Escherichia coli* respectively. Maximum 32.65%, 46.48%, 51.02%, 44.44% and 44.44% relative percentage inhibitions were exhibited with ACR, PEF, CHF, MEF and ACL respectively (Table 4).

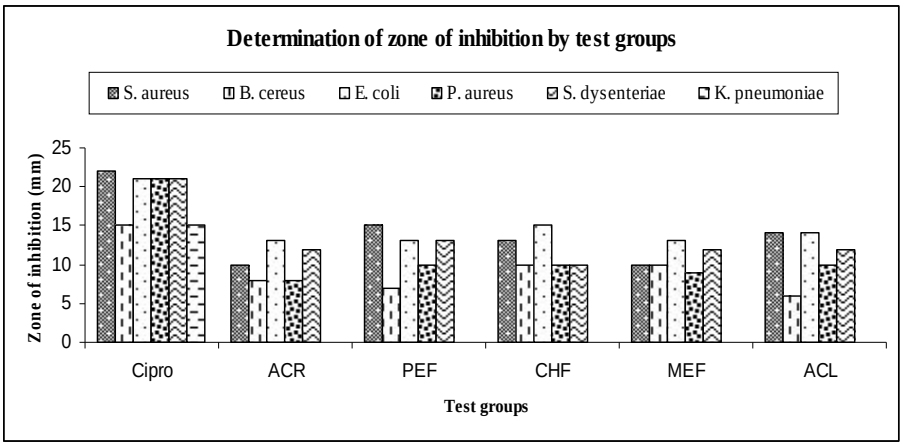

Fig 3: Antibacterial activity by *Kaempferia galanga*. *Klebsiella pneumoniae* was not inhibited by any of the extracts. ACR =Acetone extract of rhizome, PEF= Petroether fraction of rhizome, CHF=Chloroform fraction of rhizome MEF=Methanol fraction of rhizome and ACL=Acetone extract of leaf, Cipro = Ciprofloxacin.

4. Discussion

Preliminary phytochemical screening revealed that different extracts of *Kaempferia galanga* contain carbohydrates, tannins, flavonoids, proteins, steroids, alkaloids and resins (Table 1).

The cytotoxicity bioassay against *Artemia salina* is a simple and inexpensive method to test cytotoxicity, to biodirect fractionation of natural products and as a predictor of antitumor and pesticidal activity. It indicates also antiviral, antiplasmodial, antifilarial, antimalarial activities [15]. In the brine shrimp lethality bioassay all the extracts showed moderate cytotoxic activity when compared with the standard drug vincristine sulphate (Figure l and Figure 2). For example, LC_{50} value of ACL was 4.78 µg/ml while the LC_{50} of the standard anticancer drug vincristine sulphate was 0.52 µg/ml (Table 3). Control group nauplii remained unchanged (no lethality/mortality), is indicative of the cytotoxicity of all the extracts. A plot of log concentration of the test sample versus percentage of mortality on a graph paper (Figure 1) showed an approximately linear correlation between them. The inhibitory effect of the extract might be due to the toxic compounds present in the active fraction that possess ovicidal and larvicidal properties. The metabolites either affected the embryonic development or slay the eggs [16]. So the cytotoxic effects of the plant extracts enunciate that it can be selected for further cell line assay because there is a correlation between cytotoxicity and activity against the brine shrimp nauplii using extracts [16]. Antimicrobial activity was conducted against a wide range of human pathogenic microorganisms, including Gram-positive and Gram-negative bacteria. The antimicrobial activity of the compounds may be of four types: (a) they hamper cell wall synthesis; (b) they inhibit microbial protein and nucleic acid synthesis; (c) they disrupt microbial membrane structure and function; and (d) they block metabolic pathways through inhibition of key enzymes [15]. In the present study, all the extracts showed (Table 4) moderate activity against the both Gram-positive and Gram-negative bacteria (except *Klebsiella pneumoniae*). *Klebsiella pneumoniae* was not inhibited by any of the extracts. The antibacterial potency of *Kaempferia galanga* against *Escherichia coli*, *Shigella dysenteriae*, *Bacillus cereus*, and *Staphylococcus aureus*, is noteworthy, because all these bacteria have been implicated as causal agents of diarrhoea. *Shigella* species are the most important causes of acute bloody diarrhoea and account for about 15% of all deaths attributable to diarrhoea in children younger than five years [17]. It is interesting to note that the CHF showed appreciable activity against *Escherichia coli*. Diarrhoea caused by *Escherichia coli* infection is an emergent problem in both developing and developed world and is responsible for high rates of mortality in new born children and animals [18]. The significant antibacterial activities of *Kaempferia galanga* suggest that it could be useful for treating diarrhoea caused by enteropathogenic strains of *Escherichia coli*. From the results obtained, it appears that the antibacterial action of the extracts is moderate pronounced on Gram-negative than on Gram-positive bacteria.

5. Conclusion

The results of the present study, indicates that the plant extract possesses moderate cytotoxic and antibacterial activity, and therefore, suggest that the traditional use of this plant for the treatment of diarrhoea and anti-inflammatory properties can be linked to cytotoxic and antibacterial properties. However require further studies, possibly to the extent of isolating and identifying the responsible compounds.

6. Acknowledgement

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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. All listed authors read and approved the final manuscript.

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