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Antimicrobial Screening and Brine Shrimp Lethality Bioassay of *Calotropis gigantea* (Fam: Asclepiadaceae)

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ABSTRACT

The crude n-hexane, carbon tetrachloride, chloroform,ethanol extracts and water extract of leaves of Calotropis gugantea were evaluated for antimicrobial and cytotoxic activity. The antimicrobial screening was done against 16 microorganisms including gram-positive, gram-negative and fungi by the disc diffusion method. The carbon tetrachloride fraction and ethanolic fraction showed little antimicrobial activity with average zone of inhibition 9.5 mm and 8.4 mm respectively at a concentration of 400µg/disc. The antimicrobial activities were compared with standard antimicrobial doxycycline (30µg/ disc) which showed an average zone of inhibition of 40 mm. In the cytotoxic assay the extracts were tested for Brine Shrimp Lethality Bioassay using Brine shrimp nauplii. The administration of the crude extract induced significant cytotoxic activity where the LC_{50} and LC_{90} of crude extract were 2.08 µg/ml and 122.35µg/ml respectively after 24 hours.

Keywords: *Calotropis gigante*, Disc Diffusion Method, Brine Shrimp Lethality Bioassay.

INTRODUCTION

Plants have a great potential for producing new potential medicine [1]. Plants used as traditional medicine contains a vast array of substances that can be used to treat chronic and even infectious diseases. Statistics shows that, more than 80% population of the world depends on traditional medicine for their primary health care needs [2].

In our study, we chose *Calotropis gigantea* (common name: Milkweed, Rui(madar)) which is a common shrub of wasteland and road side and widely found in India, Sri lanka, Singapore, Malay Islands, South China and Bangladesh [3, 4]. The leaves are thick, opposite, decussate in arrangement and coated with white powder. Flowers are in umbel in colour [5]. *Calotropis gigantea* grows up to 4 m in height and possesses sessile leaves. The leaves are 10 cm in length and are 8 cm in width. Its flowers are 14-15 mm long 3-4.5 cm in diameter. Its dried root freed its outer layer is called Mudar. Ayurveda recommends the *Calotropis gigantea* in cases of cutaneous diseases, intestinal worms, cough, ascites, asthma, bronchitis, dyspepsia, paralysis, swellings, intermittent fevers, anorexia, inflammations and tumors. In large doses, *Calotropis gigantea* is known to act as a purgative and an emetic [6, 7]. The milky exudation from the plant is a corrosive poison. The leaves of this plant is useful in condition such as paralysis, swelling and intermittent fever. Fresh leaves of *Calotropis gigantean* are fried in oil and applied to improve painful joints and swellings. The oil can be applied on the paralytic parts. The leaf powder mixed with turmeric powder is applied externally for treatment eczema, skin eruptions, old sores and ulcers, paralyzed parts, lockjaw, convulsions in children, paralytic complaints, cold sweats, asthma and loss of appetite [8]. The dried whole plant is used as tonic, expectorant, depurative and anthehelmintic properties. The roots are used for febrifuge, anthehelmintic, depurative, expectorant and laxative. Roots are also used as cutaneous infections, intestinal worms, cough and ascites. The powdered roots are traditionally used as asthma, bronchitis and dyspepsia [9-12].

In the present, experiment an attempt has been made to evaluate the antibacterial activity of different extracts (n-hexane, carbon tetrachloride and ethanol crude extract) against thirteen *Calotropis gigantea* (Family: Asclepiadaceae, Vernacular name: Milkweed, Rui, Madar (Bengali)) is an important medicinal plant claimed to have potential curative properties and have been traditionally used in oriental countries. The plant is a common shrub of wasteland and roadside and widely found in India, Sri lanka, Singapore, Malay Islands, South China and Bangladesh[1,2].The plant grows upto 4m in height and possess sessile leaves. The leaves are thick, opposite, decussate in arrangement and coated with white powder. The traditional Indian medicine system Ayurveda recommends the use of *Calotropis gigantea* leaves in cases of cutaneous diseases, intestinal worms, cough, ascites, asthma, bronchitis, dyspepsia, paralysis, swelling, intermittent fevers, anorexia, inflammation and tumors. In large doses, *Calotropis gigantea* is known to act as a purgative and an emetic [4,5]. The leaves of this plant are useful in condition such as paralysis, swelling and intermittent fever. Fresh leaves of *Calotropis gigantea* are used to treat cough and ascites. The powdered roots are traditionally used in asthma, bronchitis and dyspepsia[7-10]. Fresh leaves of *Calotropis gigantean* are fried in oil and applied to improve painful joints and swellings. The leaf powder mixed with turmeric powder is applied externally for treatment of eczema, skin eruptions, old sores and ulcers, paralyzed parts [6]. The dried whole plant is used as tonic, expectorant, depurative and anthelmintic. Roots are also used to treat cutaneous infections, intestinal worms, cough and ascites.

The indiscriminate use of antibiotics in various countries has resulted in resistance to a large strain of bacterial population, thus causing a serious health problem. A lot of potential antibiotics are now not effective against some frequently attacking bacteria for example *Staphylococcus aerious*. In face of this scenario, the search for substance from natural source, including plants,

has been gaining importance. The herbal antibacterial will be relatively cheap and locally available and most of the cases devoid of serious side-effects.

Currently available anti-tumor drugs: nitrogen mustards, mercaptopurine, carboplatin, azathioprine have been associated with serious side effects. If any significant cytotoxic effect exerting herbal medicine can be obtained which is locally available and relatively cheap then it will be very helpful in the treatment of cancer.

Although *Calotropis gigantea* have various ethnopharmacological uses, the plant have not been undergone any chemical and pharmacological investigation. Therefore the present study was designed to investigate antimicrobial and cytotoxic activity leave extract of *Calotropis gigantean*. prominent gram-positive and gram-negative human pathogenic bacteria and three fungal strains. Besides, cytotoxic activity screening of the extracts was also carried out with view to assess the presence of antitumor activity of different extracts.

MATERIALS AND METHODS

Plant Materials:
The collected plant parts (leaves) were separated from undesirable materials or plants or plant parts. They were dried in open air under shade for two weeks. The shade dried plants part ground into a coarse powder with the help of a suitable grinder from. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced. About 850 gm of powder material of leaf were taken in a clean glass container and soaked in 4 Litre of Ethanol. The container with it contain was sealed and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white, cotton material. Then it was filtrate through Whatman filter paper. The filtrate (ethanol extract) thus obtained was evaporated under ceiling fan and in a water bath until dried. It rendered a greenish black color. The greenish black color extract was designated as crude extract of ethanol. The percent of yield is 1.88%. The crude extract was then fractionated into n-hexane, chloroform, water and carbon tetrachloride by using kupchan partitioning method [11].

Methods for antimicrobial assay: Collected all fractions, i.e., n-hexane, carbon tetrachloride, chloroform,ethanol extracts and water were tested for antimicrobial study by using standard disc diffusion method [14, 15]. In this study, 16 microorganisms were obtained from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka, Bangladesh. Standard Kanamycin (30 µg/disc) and blank sterile filter paper disc (diameter, 6 mm) were used as positive and negative control. These plates are kept at low temperature (4^0C) for 24 hours to allow maximum diffusion of the test. Nutrient agar medium (DIFCO) was used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures. The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates, pre-inoculated with test bacteria. The discs were then incubated on the plate aerobically at 37^0C for 24 hours for optimum growth of the organisms. The antimicrobial activity expressed in millimeter [16].

Methods for Cytotoxic study: The study was performed according to the Brine shrimp lethality bioassay method. 4 mg of dried crude extract was taken in 10 ml volumetric flask and volume

was adjusted by 5ml water. The concentration of this solution was 400 µg/µl and added by 100 µl DMSO solution. Similarly we made concentration of 400 µg/µl like as n-hexane, CCl_4. 20 g of NaCl and 18 g of table salt were weighed accurately, dissolved in distilled water to make one liter and then filtered off to get a clear solution. Sea water was taken in the small tank and shrimp eggs were added to the one side to the divided tank and the side was covered. The shrimps were allowed for 24 hours to hatch and mature as nauplii (larvae). The hatched shrimps were attracted to the lamp through the perforations in the dam and they were taken for bioassay. In hatching time was 22 hours. Seven (07) clean test tubes were taken, six (06) of test tube contain different samples concentration and one (1) for negative control test. Then we taken ½(half) portion of stock solution in fist test tube and added 100 µl DMSO solution and added 2.5ml of sea water was given of this test tubes. Similarly, this process contain next test tube and negative control test tube contain only 5ml of sea water. These six test tubes contain concentrations of 200 µg/µl, 100 µg/µl, 50 µg/µl, 25 µg/µl, 12.5 µg/µl, 6.75 µg/µl, 3.125 µg/µl respectively. Finally with the help of a Pasteur pipette 15 living shrimps were kept to each of the test tubes [12, 13]. For n-hexane, CCl_4, solution prepared by the following above this procedure. After 18 hours the test tubes were observed and the number of survived nauplii in each test tube was counted and the results were noted. Form this, the percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample. Like above procedure after 24 hour's the percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample

RESULTS AND DISCUSSION

Table 1: Antibacterial and antifungal activity of different extracts of *C. Gigantea*

Microbial antibiotic sensitivity test	Standard	N-Hexane fraction 400µg/ disc	CCl_4 fraction 400µg/disc	Chloroform fraction 400µg/ disc	Ethanolic fraction 400µg/ disc	Water fraction 400µg/ disc
Gram positive bacteria						
Bacillus Sereus	40	-	9	-	9	7
Staphylococcus aureus	41	-	9	-	9	7
Bcillus megaterium	41	7	9	8	8	7
Bacillus subtilis	40	7	11	8	9	7
Sarcina lutea	40	-	10	-	8	6
Gram negative bacteria						
Escherichia coli	42	-	9	-	8	6
Pseudomonas aureus	40	-	10	-	8	6
Salmonella typhi	40	7	10	8	8	7
Vibrio mimicus	40	-	9	-	8	6
Shigella boydii	40	-	10	-	8	7
Shigella dysenteriae	40	-	10	-	8	6
Salmonella paratyphi	40	7	10	9	8	6
Vibrio parahemolyticus	40	7	10	9	8	6
Fungi						
Saccharromyces cerevaceae	41	-	10	-	10	6
Candida albicans	41	-	9	-	10	6
Aspergillus niger	41	-	14	-	10	6

Antimicrobial study:
The n-hexane, carbon tetrachloride, chloroform,ethanol extracts and water extract exhibited antimicrobial activity against most of the test organisms. The zones of inhibition produced by n-hexane, carbon tetrachloride, chloroform,ethanol extracts and water extract showed average zones of inhibition of 7mm, 9.5mm, 8.4mm, 8.25mm, 6.38mm respectively at a concentration of 400 µg/disc where standard doxycycline (30µg/disc) showed zone of inhibition of 40-42 mm (Table 1). The experiment was done for two times for the confirmation of no inhibitory effect.

Cytotoxic study: All of the extracts (n-hexane, carbon tetrachloride and crude extract) showed positive results indicating that the test samples are biologically active. Plotting of log of concentration (log C) versus percent mortality (% Mortality) for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) were determined and LC_{90} values were also determined to establish the therapeutic index.

After 18 hours later result of Brine shrimp lethality bioassay of distilled crude extract, n-hexane and CCl_4 showed significant cytotoxic activity against brine shrimp nauplii and LC_{50} value was found to be 43.92 µg/ml, 106.09 µg/ml and 80.76 µg/ml (Table 2, Figure 1, 2 & 3) . To get the therapeutic index, LC_{90} (90% mortality) values were calculated for all fractions and the values were 136.02 µg/ml, 248.54 µg/ml and 216.35 µg/ml (Table 2, Figure 1, 2 & 3) for distilled crude extract, n-hexane and carbon tetrachloride fraction respectively. After 21 hours later result of Brine shrimp lethality bioassay of distilled crude extract, n-hexane and CCl_4 showed significant cytotoxic activity against brine shrimp nauplii and LC_{50} value was found to be 20.59 µg/ml, 77.37 µg/ml and 41.44 µg/ml (Table 3, Figure 4, 5 & 6) . To get the therapeutic index, LC_{90} (90% mortality) values were calculated for all fractions and the values were 128 µg/ml, 225.05 µg/ml and 173.23 µg/ml (Table 3, Figure 4, 5 & 6) for distilled crude extract, n-hexane and carbon tetrachloride fraction respectively. After 24 hours later result of Brine shrimp lethality bioassay of distilled crude extract, n-hexane and CCl_4 showed significant cytotoxic activity against brine shrimp nauplii and LC_{50} value was found to be 2.08 µg/ml, 31.75 µg/ml and 6.841 µg/ml (Table 4, Figure 7, 8 & 9) . To get the therapeutic index, LC_{90} (90% mortality) values were calculated for all fractions and the values were 122.35 µg/ml, 175.71 µg/ml and 135.37 µg/ml (Table 4, Figure 7, 8 & 9) for distilled crude extract, n-hexane and carbon tetrachloride fraction respectively. For the conformity of the result, the test was done for two times.

TABLE 2: Brine shrimp lethality bioassay of *C. Gigantea* (after 18 hour)

Conc. (µg/ml)	LogC	% Mortality			LC_{50} (µg/ml)			LC_{90} (µg/ml)		
		DCE	Hex	CTC	DCE	Hex	CTC	DCE	Hex	CTC
o (blank)	∞	6.66	6.66	6.66	43.92	106.09	80.76	136.02	248.54	216.35
200	2.3	100	66.66	73.33						
100	2	100	60	66.66						
50	1.698	66.66	46.66	60						
25	1.397	53.33	33.33	46.66						
12.5	1.096	40	26.6	33.33						
6.75	0.829	33.33	20	26.66						
3.125	0.495	20	13.34	13.34						

An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper and the values of LC_{50} and LC_{90} were calculated using Microsoft Excel 2007.

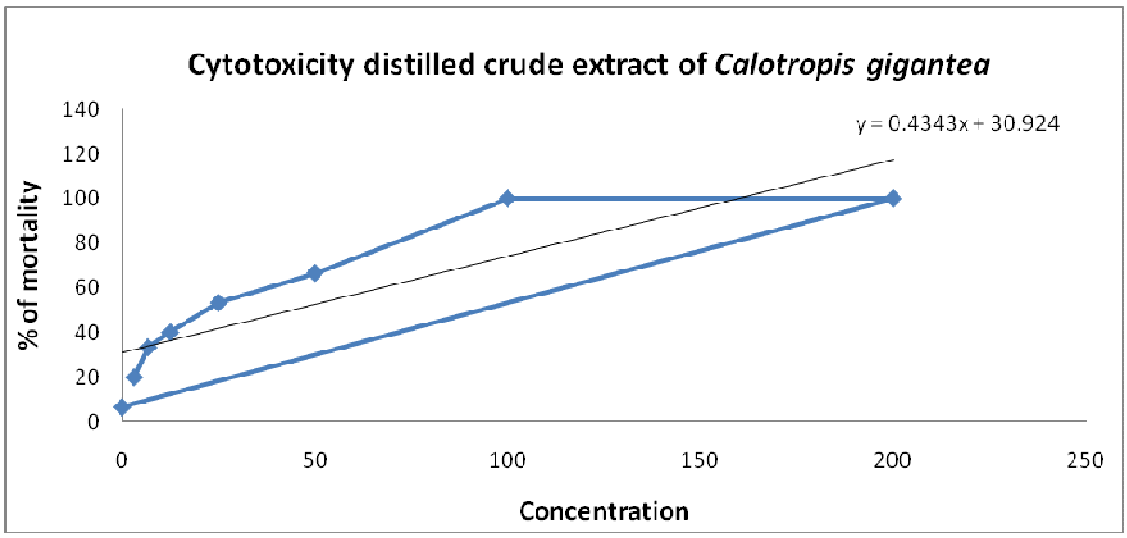

FIG. 1: Determination of LC_{50} and LC_{90} of distilled crude extract of *C. gigantea* (after 18 hour)

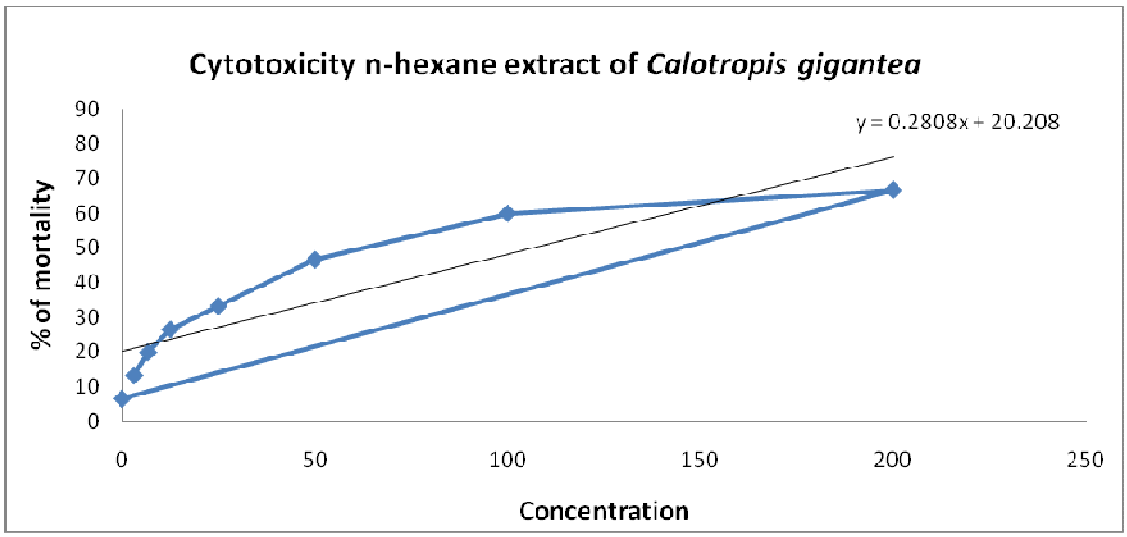

FIG. 2: Determination of LC_{50} and LC_{90} of n- hexane extract of *C. gigantea* (after 18 hour)

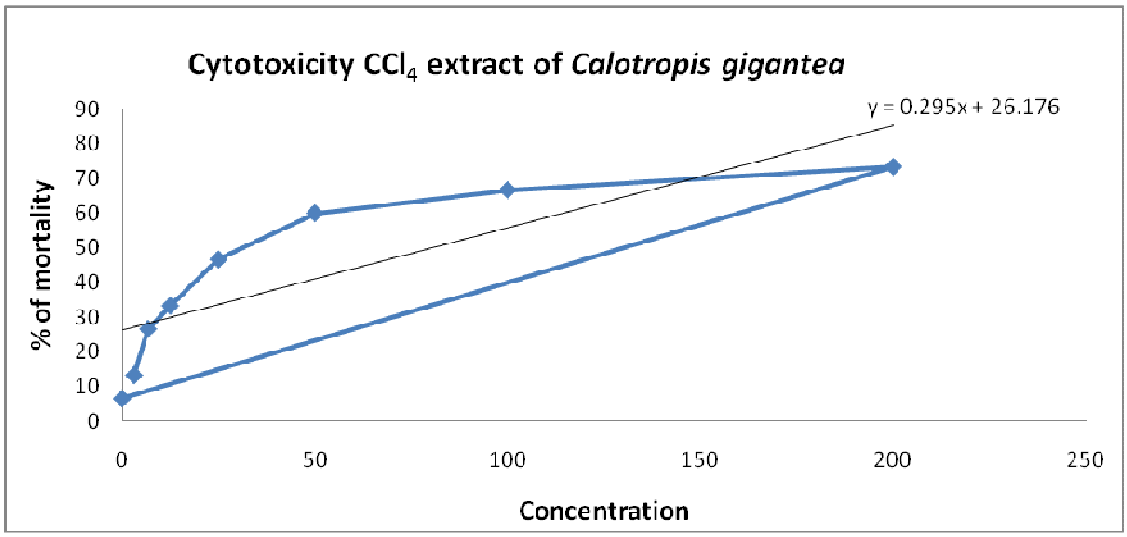

FIG. 3: Determination of LC_{50} and LC_{90} of CCl_4 extract of *C. gigantea* (after 18 hour)

TABLE 3: Brine shrimp lethality bioassay of *C. Gigantea* (after 21 hour)

Conc. (µg/ml)	LogC	% Mortality			LC_{50} (µg/ml)			LC_{90} (µg/ml)		
		DCE	Hex	CTC	DCE	Hex	CTC	DCE	Hex	CTC
o (blank)	∞	20	20	20	20.59	77.37	41.44	128	225.05	173.23
200	2.3	100	73.33	86.66						
100	2	100	66.66	80						
50	1.698	80	60	66.66						
25	1.397	66.66	40	60						
12.5	1.096	53.33	33.33	46.66						
6.75	0.829	40	26.67	33.33						
3.125	0.495	26.66	20	26.66						

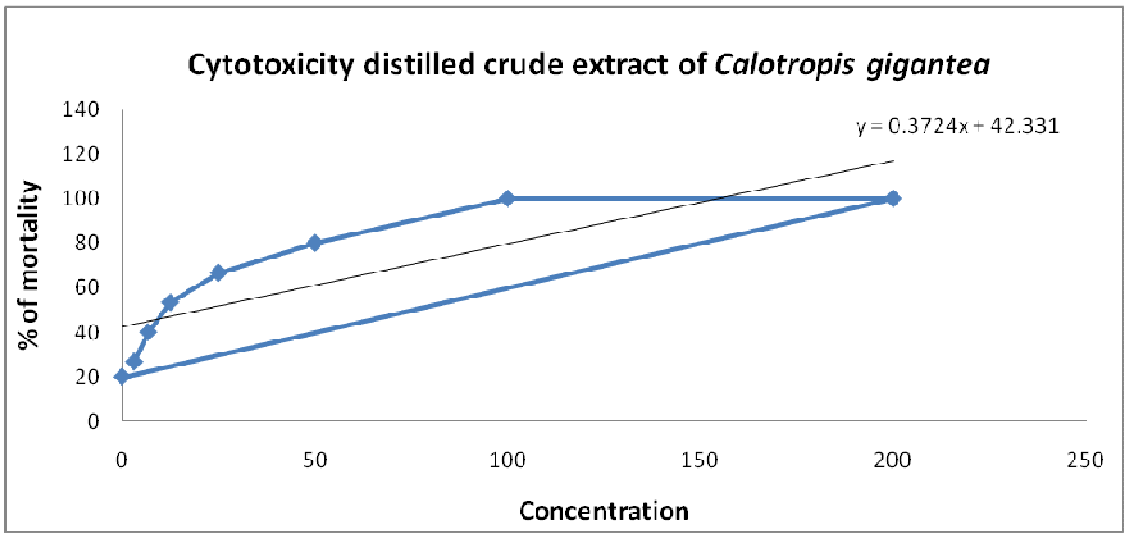

FIG. 4: Determination of LC50 and LC_{90} of distilled crude extract of *C. gigantea* (after 21 hour)

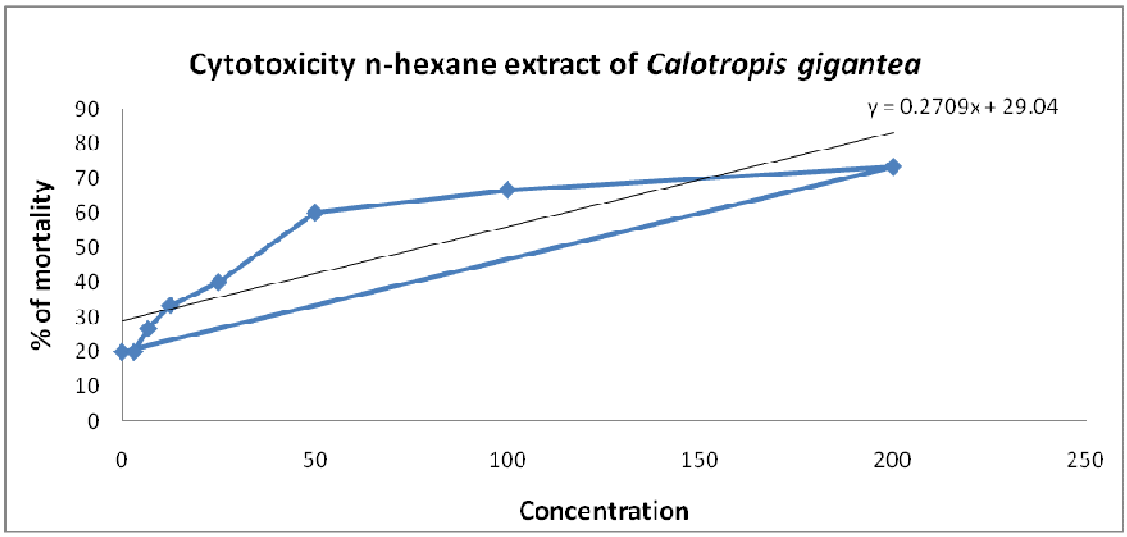

FIG. 5: Determination of LC_{50} and LC_{90} of n- hexane extract of *C. gigantea* (after 21 hour)

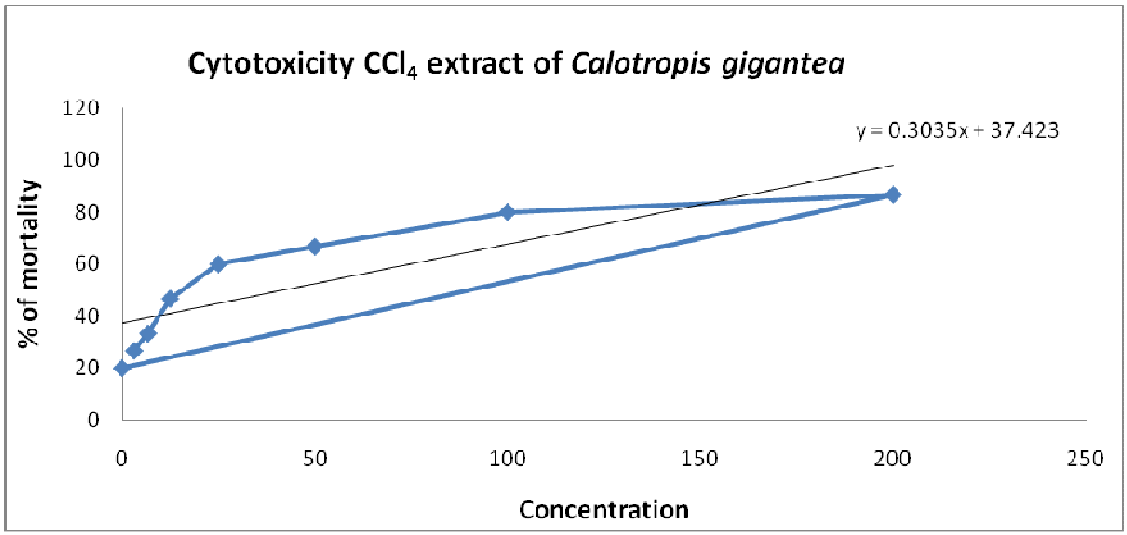

FIG. 6: Determination of LC_{50} and LC_{90} of CCl_4 extract of *C. gigantea* (after 21 hour)

TABLE 4: Brine shrimp lethality bioassay of *C. gigantea* (after 24 hour)

Conc. (µg/ml)	LogC	% Mortality			LC_{50} (µg/ml)			LC_{90} (µg/ml)		
		DEC	Hex	CTC	DEC	Hex	CTC	DEC	Hex	CTC
0(blank)	∞	26.66	40	33.33	2.08	31.75	6.841	122.35	175.17	135.37
200	2.3	100	86.66	100						
100	2	100	80	86.66						
50	1.698	86.66	73.33	80						
25	1.397	73.33	53.33	73.33						
12.5	1.096	60	46.66	60						
6.75	0.829	53.33	33.33	40						
3.125	0.495	33.33	26.66	33.33						

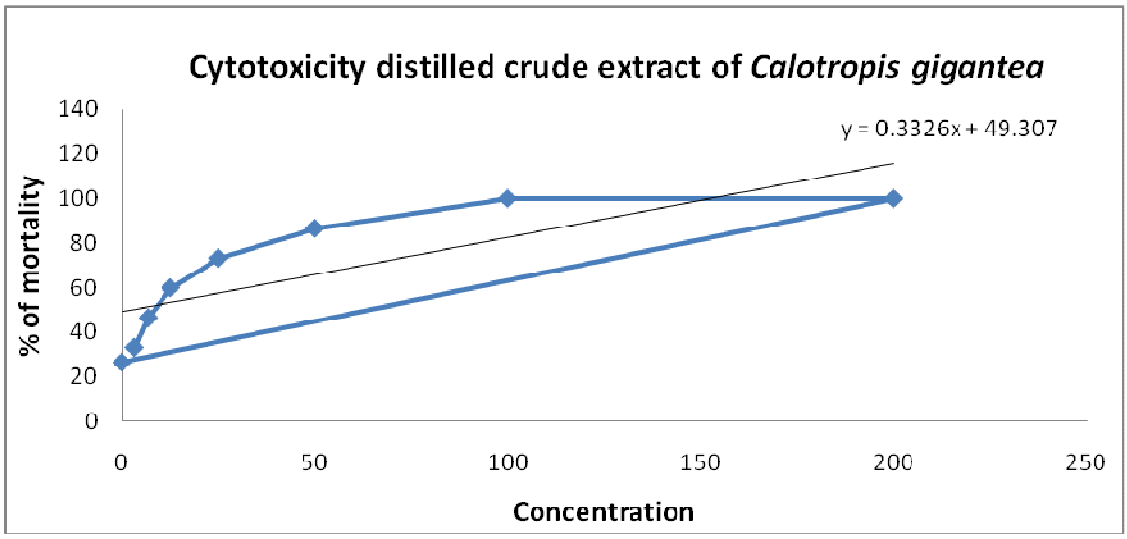

FIG. 7: Determination of LC_{50} and LC_{90} of distilled crude extract of *C. gigantea* (after 24 hour)

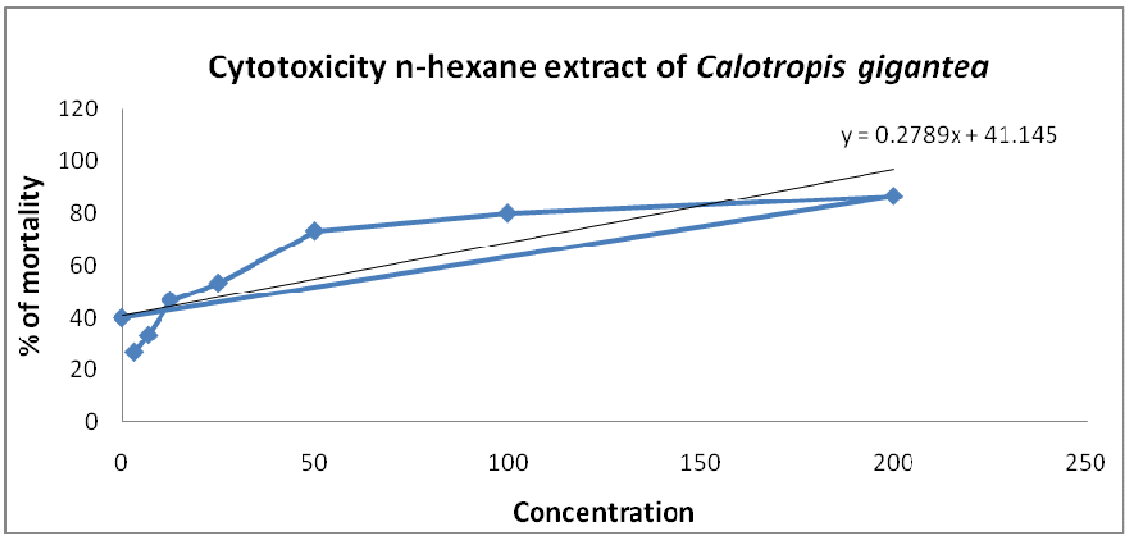

FIG. 8: Determination of LC_{50} and LC_{90} of n- hexane extract of *C. gigantea* (after 24 hour)

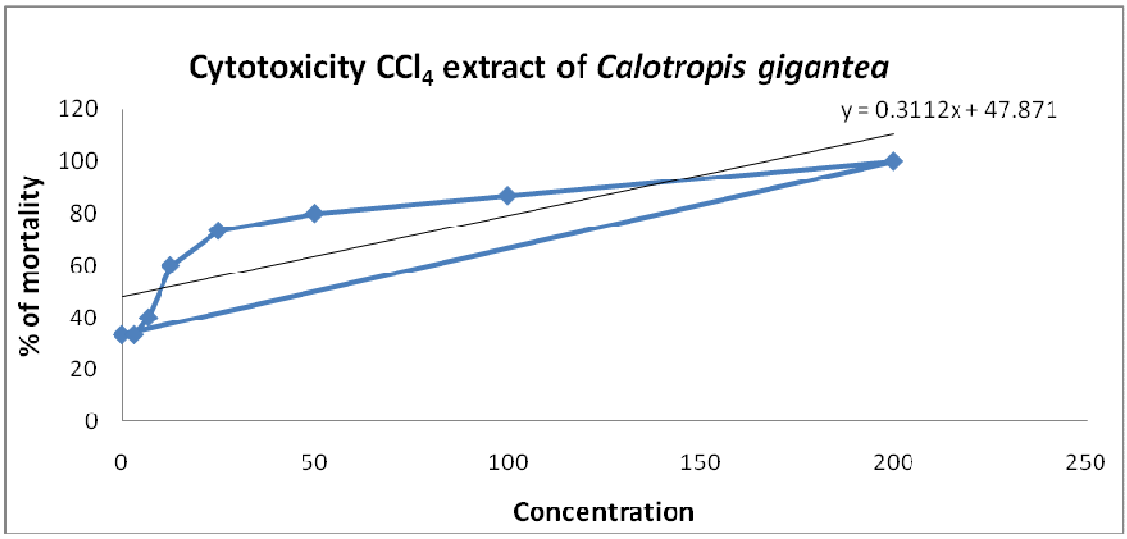

FIG. 9: Determination of LC_{50} and LC_{90} of CCl_4 extract of *C. gigantea* (after 24 hour)

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