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Cytotoxic activity of Ethanolic root extract of *Calotropis gigantea* Linn

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Abstract

The present study deals with the cytotoxic activity of the ethanolic root extract of *C. gigantea Linn*. The different concentrations of ethanolic extract of the roots of *C. gigantea Linn* were used for cytotoxic activity by Brine shrimp lethality bioassay (BSLB) and *Allium cepa* root meristem (ACRM) models. In BSLB, LC_{50} value was found to be 62.12µg/ml while LC_{50} value of cyclophosphamide was found to be 41.54µg/ml. In ACRM model, incubation of onion bulbs in different concentrations of extract produced a growth retarding effect that was associated with a decrease in the root number. ACRM growth inhibition was highest with significance of ($p<0.01$) at the 10 mg/ml concentration after 48 hrs incubation for ethanolic root extract. Extract produced dose and time dependent growth inhibition. The ethanolic root extract of *C. gigantea* exhibits potent cytotoxic property comparable to that of standard drug. Therefore, this might be utilized for the development of novel anticancer drug leads.

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Key words:
Cytotoxicity, *Calotropis gigantea*, BSLB, ACRM, *Allium cepa,* anticancer.

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Introduction

It is well established that plants have been useful sources of clinically relevant antitumor compounds. Indeed there have been worldwide efforts to discover new anticancer agents from plants [1]. *Calotropis gigantea* Linn. (Asclepiadaceae) is a perennial under shrub found chiefly in wastelands throughout India. The traditionally *C. gigantea* is used as analgesic [2], cures toothache and earache [3, 4], sprain [5], anxiety [6, 7] epilepsy [8] and in mental disorders [9]. Various scientific studies reported this plant as contraceptives for human [10], sedative, anxiolytic, anticonvulsant [11],

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analgesic [12] and wound healer [13]. Our study demonstrated the cytotoxic activity of ethanolic extract of roots of *C. gigantea* plant by reported models viz Brine shrimp lethality bioassay (BSLB) and *Allium cepa* root tip meristem (ACRM) model. *Artemia salina L. (Artemiidae)*, the brine shrimp, is an invertebrate of the fauna of saline aquatic and marine ecosystems. It can be used in laboratory bioassay in order to determine toxicity through the estimation of medium lethal concentration (LC_{50} values) which has been reported for series of toxins and plant extracts. Several naturally extracted products which had LC_{50} < 1000 µg/ml using BSLB were known to contain physiologically active principles [14, 15]. BSLB and other *in vivo* lethality test have been successively employed for bioassay guide fractionation of active cytotoxic and antitumor agents [16]. The technique has been used to screen and identify over 300 novel antitumor and pesticidal natural products and found to have a positive correlation with human nasopharyngeal carcinoma cytotoxicity [17, 18]. BSLB also has been reported to be useful in predicting other biological activities such as phototoxicity, trypanocidal, enzyme inhibition, ion regulation activities [19, 20] and hepatotoxicity [21]. ACRM has been widely used for the evaluation of cytotoxicity, anti-mitotic [22, 23], genotoxicity [24, 25], antimutagenic [26] and antioxidant activity [27, 28] by employing the growing roots of *Allium cepa*.

Materials and Methods

The *C. gigantea* roots were collected from Nagpur District, Maharashtra, India in the month of August 2008. The plant was identified and authenticated by Dr. Alka Chaturvedi, "Post graduate Teaching Department of Botany, Rashtrasant Tukadoji Maharaj, Nagpur University, Nagpur. A voucher specimen (No.9199) was deposited with the, "Post graduate Teaching Department of Botany, RTM Nagpur University, Nagpur.

All the chemicals used for experimental purpose were of laboratory grade. Brine shrimp (*Artemia Salina*) eggs were purchased from Matsyakanya Aquarium, 480/58, Cannada Corner, Nasik-5, Maharashtra and the standards drug i.e. cyclophoshpamide was provided as gift sample by VHB life sciences Rudrapur, Uttaranchal, India.

The root of *C. gigantea* was subjected to extraction with ethanol in a soxhlet apparatus. Extract was filtered, through Whatmann filter paper #42 and evaporated to dryness at 50 °C in oven. The extract was then stored in desiccators till further use.

Cytotoxic activity

Brine Shrimp Lethality Bioassay (BSLB)

To evaluate cytotoxicity of the extracts by using BSLB, the as eggs (150 mg) were kept for hatching in a conical shaped vessel (1L), filled with sterile artificial sea water (prepared using sea salt 38 g/L) under constant aeration for 72 hrs. To avoid risk of death to larvae by decrease of pH during incubation, pH was adjusted to 8.5 using 1N NaOH [29, 30, 31].

After 48 hrs, 15ml of yeast solution 0.06% was added to vessel for every litre of salt water in order to feed larvae after 72 hours hatching takes place, active nauplii free from egg shells were collected and used for the assay [32, 33]. Eight concentrations of the plant extract (10, 20, 50, 100, 500, 1000, 2000, 5000 µg/ml.) were tested in order to determine dose response relationship, and a control group was set with vehicle used for dilutions. Ten nauplii were drawn through a glass capillary and placed in test tube containing sample, filled with 5 ml total volume of artificial sea water. Experiment was conducted along with treated (extract) control (vehicle) and standard drug (Cyclophosphamide) at above mentioned concentrations of test substances in a set of three test tubes per dose. After 24 hours, live nauplii were counted and LC_{50} value was estimated.

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The percentage lethality was determined by comprising the mean surviving larvae of the test and control tubes. In toxicity evaluation of ethanolic extract by BSLB, LC_{50} value lower than 1000 µg/ml is considered bioactive. LC_{50} values were obtained from, concentration verses percentage lethality by using statistical method of Finney's probit analysis.

Allium cepa Root Tip Meristem (ACRM) Model

Locally available onion bulb (*Allium cepa* 50 ± 10 g) were obtained and grown in the dark over 100 ml tap water at ambient temperature until the roots have grown to approximately 2-3 cm length. The base of each of the bulbs were suspended on the extract inside 100 ml beakers, root length (newly appearing roots not included) and root number at 0, 48, 96 hrs for each concentration of extract and control was measured. The percentage root growth inhibition after treating with ethanolic extract at 48 and 96 hr. was determined. Working dilutions of all the drugs were made in water. Cyclophosphamide (standard) as well as extracts of root was used at 1 mg/ml and 10 mg/ml concentration. [22]

Results

Brine shrimp lethality activity of ethanolic extracts of *C. gigantea*, shows that mortality is increased as the concentration of extract is increased. The degree of lethality was found to be directly proportional to the concentration of the extract. The ethanolic extract of *C. gigantea* have shown the LC_{50} value of 62.12 µg/ml (Table 3) while LC_{50} value of cyclophosphamide was found to be 41.54 µg/ml (Table 2). Results showed that ethanolic extract of *C.gigantea* has potent cytotoxic effect which is very similar to standard drug. The LC_{50} value for extract of root in our study was found to be lower than 1000µg/ml.

Table 1: Observations of average mortality for control

Control	Concentration (µg/ml)	No. of test tube	No. of Shrimp tested	Average mortality after (24 hrs)	% Mortality after(24hrs)
Without drug	----	1-a	10	00	00
		1-b	10		
		1-c	10		

Table 2: Observations of Average mortality and LC_{50} value: Cyclophosphamide

S. No.	Concentration (µg/ml)	No. of test tube	No. of Shrimp test	Average mortaliy after 24 hrs	% Average mortality	LC_{50} (µg/ml)
1	10	1-a	10	01	10	41.54
		1-b	10			
		1-c	10			
2	20	2-a	10	02	20	
		2-b	10			
		2-c	10			
3	50	3-a	10	06	60	
		3-b	10			
		3-c	10			
4	100	4-a	10	08	80	
		4-b	10			
		4-c	10			
5	500	5-a	10	09	90	
		5-b	10			
		5-c	10			
6	1000	6-a	10	10	100	
		6-b	10			
		6-c	10			
7	2000	7-a	10	10	100	
		7-b	10			
		7-c	10			
8	5000	8-a	10	10	100	
		8-b	10			
		8-c	10			

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Table 3: Observations for average mortality and LC_{50} value of ethanol extract of root

S. No.	Concentration (µg/ml)	No. of test Tube	No. of Shrimp test	Average mortality after 24 hrs	% Average mortality	LC_{50} (µg/ml)
1	10	1-a	10	00	00	62.12
		1-b	10			
		1-c	10			
2	20	2-a	10	01	10	
		2-b	10			
		2-c	10			
3	50	3-a	10	04	40	
		3-b	10			
		3-c	10			
4	100	4-a	10	06	60	
		4-b	10			
		4-c	10			
5	500	5-a	10	08	80	
		5-b	10			
		5-c	10			
6	1000	6-a	10	09	90	
		6-b	10			
		6-c	10			
7	2000	7-a	10	10	100	
		7-b	10			
		7-c	10			
8	5000	8-a	10	10	100	
		8-b	10			
		8-c	10			

The degree of lethality was found to be directly proportional to concentration of ethanolic extract. In this study LC_{50} value for the Ethanolic Extract lesser than 1000µg/ml. LC_{50} value has been estimated (after 24 hours, live nauplii were counted and analysed) statistically by Finney's Probit Analysis Method and was found to be 62.12µg/ml.

The cytotoxic effect of ethanolic extract of *C. gigantea* root was also evaluated on the *Allium cepa* root meristems. A progressive increase in root number and root length was observed in control group. The root length in control group at 0, 48 and 96 hrs was 3.33±0.67 cm (n=16), 4.19±0.81 cm (n=22) and 4.63±1.10 cm (n=28) respectively (Table-4,5). The root extract produced dose and time dependent growth inhibition. Incubation of bulbs in different concentrations of cytotoxic agents produced a growth retarding effect that was associated with a decrease in the root number (Fig.1). *Allium cepa* root tip meristem growth inhibition was highest with significance of ($p<0.01$) at the 10 mg/ml concentration after 48 hrs for ethanolic root extract. The root length after 0, 48 and 96 hrs with significance of ($p<0.01$) at 10 mg/ml was found to be 2.48±0.92 (n =18), 2.62±0.97 (n =15), 2.73±0.97(n =14) respectively (Table 4). Extract produced dose and time dependent percentage growth inhibition, shows maximum 83.72% root growth inhibition after 48 hrs (Table 6). The ethanolic root extract of *C. gigantea* exhibits potent cytotoxic property comparable to standard anticancer drug.

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***Allium cepa* root model**

(A) Control (B) Cyclophosphamide treated (C) Extract treated

Figure 1: *Allium cepa* bulbs showing the effect of different extracts of roots of *C.gigantea* on root length following 96 hrs of incubation.

Table 4: Observations for *Allium cepa* root length and root number attained following incubation with extract of *C. gigantea* root and standard drug.

Groups	Concentration	Root length (cm)		
		0 hr	48 hrs	96 hrs
Control	----------	3.33±0.67 (n=16)	4.19±0.81 (n=22)	4.63±1.10 (n=28)
Ethanol	1 (mg/ml)	2.86±0.57 (n=16)	3.58±0.30 (n=21)	3.50±1.04 (n=24)
	10 (mg/ml)	2.48±0.92 (n=18)	2.62±0.97 (n=15)	2.73±0.97** (n=14)
Cyclophosphamide	1(mg/ml)	2.35±0.26 (n=13)	2.72±0.27 (n=19)	3.05 ± 0.34* (n=25)
	10 (mg/ml)	2.47±0.25 (n=12)	2.60±0.22 (n=12)	2.70 ± 0.26** (n=11)

* Indicates Significant One-way Analysis Variance of P value < 0.05 for root number (n) and root length.
** Indicates Significant One-way Analysis Variance of P value < 0.01 for root number (n) and root length.

Table 5: Root growth after treating with drugs

Groups	Concentration	Root growth After	
		48 hrs	96 hrs
Control	----------	0.86 cm	1.3 cm
Cyclophosphamide	1 mg/ml	0.37 cm	0.8 cm
	10 mg/ml	0.13 cm	0.23 cm
Ethanol	1 mg/ml	0.72 cm	0.64 cm
	10 mg/ml	0.14 cm	0.24 cm

Table 6: % Inhibition of root for different extract at 46 & 96 hrs

Extract	Concentration	48 hrs	96 hrs
		% inhibition	% inhibition
Control	.....	……..	……..
Ethanol	1 mg/,ml	16.27	50.76
	10 mg/ml	83.72	72.09
Cyclophosphamide	1 mg/ml	53.16	68.75
	10 mg/ml	83.54	91.01

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Discussion

In the present study BSLB has been used. It is simple bioassay useful for screening large number of extracts in the drug discovery process and has been used in number of previous studies. [34, 35, 36, 37] The brine shrimp lethality activity of ethanolic extracts of *C. gigantea*, after testing the extract with brine shrimp (Table 3) showed that mortality is increased as the concentration of extract is increased means there is linear dose-effect relationship between extract concentrations and LC_{50} value. The degree of lethality was found to be directly proportional to the concentration of the extract. The ethanolic extract of *C. gigantea* has shown the LC_{50} value of 62.12µg/ml while LC_{50} value of Cyclophosphamide was found to be 41.54µg/ml. Results showed that ethanolic extract of *C.gigantea* has potent cytotoxic effect comparable to that of standard drug (Cyclophosphamide). The concentration required for produce the maximum mortality in the ethanolic extract of root is 2000µg/ml and 1000µg/ml for cyclophosphamide.

LC_{50} lower than1000µg/ml in the BSLB is considered to biologically active. The LC_{50} value for extract of root in our study was found to be lower than 1000µg/ml. Hence, ethanolic extract of root is bioactive by BSLB method with LC_{50} = 62.12µg/ml and it can have biological activity. Several naturally extracted products which had LC_{50} < 1000µg/ml using BSLB were known to contain physiologically active principles. [14, 15] BSLB shows positive correlation with human nasopharyngeal carcinoma cytotoxicity. [17, 18]

ACRM model, a standardized test for cytoxicity monitoring and has been used by various authors. [26, 38, 39] The cytotoxic effect of ethanolic extract of *C. gigantea* root was also evaluated on the *Allium cepa* root meristems. A progressive increase in root number and root length was observed in control group. The root extract produced dose and time dependent growth inhibition. Incubation of bulbs in different concentrations of cytotoxic agents produced a growth retarding effect that was associated with a decrease in the root number (Fig.1). *Allium cepa* root tip meristem growth inhibition was highest with significance of ($p<0.01$) at the 10 mg/ml concentration after 48 hrs for ethanolic root extract. Ethanolic root extract of *C. gigantea* and cyclophosphamide arrested the root growth. Its cytotoxic effect was also evident in the form of shortening and decaying of roots. However, the root number did not increase any further at 10 mg/ml concentration. Cytotoxic effect of *C. gigantea* ethanolic extract was comparable to that of cyclophosphamide.

It confirmed cytotoxic activity of ethanolic extract of *C. gigantea* root. Two different models were used for evaluation of cytotoxic activity are having different sensitivity which confirms the activity. ACRM model, root tips which are growing have been used, probably drug might have mitotic inhibitory activity further study is required to clear this aspect. Thus, this study demonstrates that ethanolic root extract of *C. gigantea* exhibits potent cytotoxic property comparable to standard anticancer drugs.

Conclusion

The results of our study revealed that the ethanolic extract of *C. gigantea* exhibit potent cytotoxic activity. Further *in vivo* and *in vitro* with different human cell lines study is required to demonstrate the antitumor activity of this plant and isolated the lead compound responsible for this activity, might be utilized for the development of novel anticancer drug.

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