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Cytotoxic activity of certain medicinal plants extracts against sea monkey: *Artemia salina*

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ABSTRACT

Cytotoxicity assays are widely used by the pharmaceutical industry to screen compound libraries. *Artemia* populations are found in about 500 natural salt lakes and man-made salterns scattered throughout the tropical, subtropical and temperate climatic zones, along coastlines as well as inland. This general bioassay is not only rapid but it is also reliable. In the present investigation, the leaves of angiosperm plants *Andrographis paniculata, Argemone mexicana*, and *Vitex negundo* were extracted with solvents of varying polarity such as hexane, ethyl acetate, acetone, and methanol and their yield was calculated. The extracts were tested for hatch inhibition (HI) of cysts of *Artemia salina* and cytotoxic activities against *A. salina* nauplii. The overall values of HI percentage ranged from 85.6% to 100%. Among the different extracts of three plants studied, the methanol extract showed a more potent cytotoxic activity than other extracts.

KEY WORDS: *Artemia salina*, medicinal plants, crude extracts, cytotoxic activities

INTRODUCTION

Cancer is a major public health burden in both developed and developing countries. It is an abnormal growth of cells in body that can lead to death. Cancer cells usually invade and destroy normal cells. These cells are born due to an imbalance in the body and by correcting this imbalance, cancer may be treated. Billions of dollars have been spent on cancer research and yet we do not understand exactly what cancer is. Every year, millions of people are diagnosed with cancer, leading to death. According to the American Cancer Society, deaths arising from cancer constitute 2-3% of the annual deaths recorded worldwide. Thus, cancer kills about 3500 million people annually worldwide (Prakash *et al.*, 2013).

In the investigation of the biological activity of plant extracts and natural products isolated from plants, the brine shrimp assay is a valuable tool for establishing general toxicity and cytotoxicity parameters. This assay consists of exposing brine shrimp larvae to plant extract in saline solution and larval mortality is evaluated after 1 day. A very positive correlation between the lethality to brine shrimp and cytotoxicity has been established by researchers working on the development of new anticancer drugs from plants (Anderson *et al.*, 1991).

Herbal medicines are being used by about 80% of the world population primarily in the developing countries for primary health care due to their safety, efficacy, cultural acceptability, and lesser side effects. India, despite its rich traditional knowledge, heritage of herbal medicines and large biodiversity has a dismal share of the world market due to the export of crude extracts and drugs (Kamboj, 2000). It is one of the leading countries in Asia, in terms of the wealth of traditional knowledge related to the use of plant species (Kala *et al.*, 2004). The screening of the toxic nature of medicinal plants is in-dispensable to consider a treatment safe and it enables the definition of the intrinsic toxicity of the plant and the effects of acute overdose (Padmaja *et al.*, 2002).

In Kanchipuram district, Tamil Nadu, India, the leaf paste of *Andrographis paniculata* is applied topically at the bitten site of snake, beetle, and scorpion. Powdered leaf is mixed with cow's or goat's milk and taken orally to treat diabetes (Muthu *et al.*, 2006). It has traditionally been used over centuries in Asia as a folklore medicine for a variety of ailments or as herbal supplements for health promotion. Indian pharmacopoeia narrates that it is a predominant constituent of at least 26 Ayurvedic formulations and used to treat various diseases (Kumar *et al.*, 2004).

Argemone mexicana is used as a medicinal plant in several countries. In Mexico, the seeds are considered as an antidote to snake venom. In India, the smokes of the seeds are used to relieve toothache. The fresh yellow, milky seed extract contains protein-dissolving substances, effective in the treatment of warts, cold sores, cutaneous infections, skin diseases, itches and also dropsy and jaundice (Chopra *et al*., 1986).

In Ayurvedic medicine, a drug, namely Nirgundi, has been prepared from *Vitex Negundo*, and the drug is astringent, bitter, and cold. Nirgundi improves the receptive and retentive power of mind, complexion, and growth of hair. It also cures cough, asthma, eye diseases, inflammation, glandular and rheumatic swelling, intestinal worms, fever, ulcers, skin diseases, nervous disorders and leprosy (Sivarajan and Balachandran, 2002). In the present investigation, the leaves of *A. paniculata, A. mexicana*, and *V. negundo* were studied for their toxic potentials of the selected plants using *Artemia salina*.

MATERIALS AND METHODS

Collection of Plant Materials

The fresh leaves of angiosperm plants *A. paniculata*, (Burm.f.) Wall. ex Nees, *A. mexicana* L., and *V. negundo* L. were collected from local habitat.

Preparation of Extracts

The collected plant materials were immediately brought to the laboratory in polythene bags. The leaves were washed three times with water to remove all the unwanted impurities. Then, the leaves were shade dried under room temperature and kept in a hot air oven for 50°C for ½ h. After that, the material was ground using an electric blender. The powdered materials were stored in air tight container. The powdered plant materials were extracted by soaking 25 g plant powders at room temperature in Erlenmeyer flasks containing 250 ml of solvents such as hexane, ethyl acetate, acetone, and methanol for 72 h. The extraction process was carried out in triplicates. After 72 h, the extract was filtered through Whatman No. 1 filter paper and the solvent was evaporated under vacuum in a rotary evaporator and the dried extracts were stored at 4°C until further assay.

Brine Shrimp Hatchability Test

The brine shrimp hatchability test is based on the procedure done by Migliore *et al*. (1997). Dried cysts of about 100 mg were allowed to hatch in 100 ml seawater at 28°C, under conditions of continuous illumination and strong aeration. The concentration used was 25 ppm. To get the concentration, 10 mg of each extract was dissolved in 10 ml, i.e. 1 ml containing 1000 µg/ml. After 2 h, 25 µl aliquots were placed in each well where the extracts had previously been deposited. They were incubated at the same conditions of temperature and illumination under gentle shaking. The free nauplii in each well were counted under a stereoscopic microscope after 24 exposures. Five replicates were used for each treatment and control. The percentages of hatchability were calculated by comparing the number of free nauplii in each treatment with the number of free nauplii in the control. Later the percentage of hatch inhibition (HI%) was calculated as follows:

HI (%) = % Hatchability in the control − % Hatchability in each treatment

Brine Shrimp Lethality Bioassay (Cytotoxicity Test)

Hatching the brine shrimp

Brine shrimp eggs were hatched in artificial sea water prepared from commercial sea salt 40 g/L and supplemented with 6 mg/L dried yeast. The two unequal compartments plastic chamber with several holes on the divider was used for hatching. The eggs were sprinkled into the larger compartment which was darkening, while the smaller compartment was illuminated. After 48 h incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette from the lighter side, whereas their shells were left in another side.

Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is considered a useful tool for preliminary assessment of toxicity. The method is attractive, because it is very simple, inexpensive and sensitive (Krishnaraj *et al.,* 2005). 10 nauplii were drawn through a glass capillary and placed in test tubes containing 10 ml of artificial seawater solution and 0.5 ml of the diluted plant extract (5.0, 10, 25, 50, 100 ppm) was added to it and maintained at room temperature for 24 h under constant aeration and light source. The test was also carried out on control (artificial sea water). The *Artemia* mortality in both treated and control was recorded after 24 h and the percentage of mortality calculated.

$$\% \text{ Mortality} = \frac{\text{Mortality at treatment - mortality at control}}{100 - \text{mortality at control}} \times 100$$

Statistical Analysis

The lethal concentrations, LC_{50} and LC_{90} at which concentrations (ppm) 50% and 90% larvae showed

mortality, 95% confidence limit of upper and lower confidence levels were calculated by Probit analysis (SPSS, version 11.5).

RESULTS AND DISCUSSION

The 12 different extracts of three plants selected for this study were tested for their HI activity against the cysts of *A. salina*. The results of HI were presented as HI percentage in Table 1. The overall values of HI percentage ranged from 85.6% to 100%. The hexane extract of *A. paniculata* exhibited the highest HI (100%) followed by the methanol extract (98.2%). The acetone extract of *A. paniculata* showed 94.3% HI. The ethyl acetate extract recorded 85.6%. In the HI action of *A. mexicana*, the highest HI (98.4%) was noticed in the methanol extract followed by ethyl acetate (92.4%), acetone (90.1%), and hexane (89.2%). The leaf crude extracts of leaves *V. negundo* were analyzed for their HI nature against the eggs of *A. salina,* and the results showed that the methanol extract of *V. negundo* showed highest HI (100%). The HI percentage for ethyl acetate was 97.2% and followed by acetone extract (94.4%) and hexane (92.3%).

Table 1: Effect of different plant extract on HI of *A. salina* eggs

Name of plant	Name of extract	Percentage of mortality*
A. paniculata	Hexane	100
	Ethyl acetate	85.6
	Acetone	94.3
	Methanol	98.2
A. mexicana	Hexane	89.2
	Ethyl acetate	92.4
	Acetone	90.1
	Methanol	98.4
V. negundo	Hexane	92.3
	Ethyl acetate	97.2
	Acetone	94.4
	Methanol	100

*Results of five replicate; 0% HI observed in control.
A. paniculata: *Andrographis paniculata, A. mexicana*: *Argemone mexicana, V. negundo*: *Vitex negundo, A. salina*: *Artemia salina,* HI: Hatch inhibition

Among all the three plants studied, the methanol extract showed more potent cytotoxic activity than other extracts. The brine shrimp mortality recorded with the hexane, ethyl acetate, acetone, and methanol extracts of *A. paniculata* are presented in Tables 2-5. The hexane extract at 200 ppm showed 100% mortality with an LC_{50} and LC_{90} values of 28.73 and 115.27 ppm, respectively. The methanol extract at the same concentration also showed 100% mortality. However, the LC_{50} and LC_{90} values were slightly higher (36.64 and 156.93 ppm). The brine shrimp mortality by ethyl acetate and acetone extracts was maximum at 200 ppm with 96.0% and 92.5%. The LC_{50} and LC_{90} values were 52.95 and 145.29 ppm for ethyl acetate and 69.72 and 171.56 ppm for acetone extract.

The brine shrimp mortality of *A. mexicana* leaf extract is presented in Tables 6-9. The highest brine shrimp mortality of 100% was recorded with the methanol extract at 200 ppm with an LC_{50} and LC_{90} values of 33.90 and 152.19 ppm, respectively. It was followed by the acetone extract which showed 84.7% mortality and LC_{50} and LC_{90} values of 81.3 and 213.29 ppm. The hexane and ethyl acetate extracts resulted in a moderate cytotoxic activity

Table 2: LC of hexane extract of *A. paniculata* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	24.0					
25	47.6					
50	61.8	28.73	115.27	15.05-45.48	67.28-472.75	0.763
100	85.2					
200	100.0					
Control	0.0					

LC: Lethal concentration, *A. paniculata*: *Andrographis paniculata, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit

Table 3: LC of ethyl acetate extract of *A. paniculata* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	27.4					
25	36.8					
50	48.4	52.95	145.29	16.24-87.53	22.62-93.13	0.924
100	76.8					
200	96.0					
Control	0.0					

LC: Lethal concentration, *A. paniculata*: *Andrographis paniculata, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit

Table 4: LC of acetone extract of *A. paniculata* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	14.3					
25	31.8					
50	46.0	69.72	171.56	40.65-103.07	129.27-283.48	8.61*
100	68.9					
200	92.5					
Control	0.0					

*Significant at $P<0.05$. LC: Lethal concentration, *A. paniculata*: *Andrographis paniculata, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit

Table 5: LC of methanol extract of *A. paniculata* against brine shrimp (*A. salina)*

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	23.2					
25	37.4					
50	48.2	36.64	156.93	16.07-69.45	79.08-1944.81	17.03*
100	72.1					
200	100					
Control	0.0					

*Significant at $P<0.05$. LC: Lethal concentration, *A. paniculata*: *Andrographis paniculata, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit

Table 6: LC of hexane extract of *A. mexicana* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	4.8					
25	14.6					
50	36.3	96.64	550.83	81.16-118.71	377.96-941.87	3.547
100	54.9					
200	65.5					
Control	0.0					

LC: Lethal concentration, *A. mexicana*: *Argemone mexicana, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit

Table 7: LC of ethyl acetate extract of *A. mexicana* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	7.4					
25	21.5					
50	37.9	115.83	268.28	47.16-530.73	168.14-2243.65	21.379*
100	56.7					
200	69.1					
Control	0.0					

*Significant at $P<0.05$. LC: Lethal concentration, *A. mexicana*: *Argemone mexicana, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit

with 65.5% and 69.1% mortality. Their LC_{50} and LC_{90} values were 96.64, 115.83 and 550.83 and 268 ppm, respectively.

The brine shrimp mortality recorded with the hexane, ethyl acetate, acetone, and methanol extracts of *V. negundo* are presented in Tables 10-13. The methanol extract at 200 ppm showed 100% mortality with an LC_{50} and LC_{90} values of 34.22 and 144.87 ppm, respectively. The cytotoxic activity for other extracts was in the order of ethyl acetate (93.5% mortality at 200 ppm, LC_{50} = 39.27 and LC_{90} = 178.33 ppm), acetone (85.4% mortality at 200 ppm, LC_{50} = 56.41 and LC_{90} = 309.27 ppm), and hexane (79.2% mortality at 200 ppm, LC_{50} = 71.71 and LC_{90} = 377.17 ppm).

In our study, a dose-dependent result was recorded. The HI of *A. salina* cysts was increased with increasing concentration of plant extracts. Similar observation was made by Sabai *et al.* (2001) and Otang *et al.* (2013).

Table 8: LC of acetone extract of *A. mexicana* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	16.6					
25	31.4					
50	44.2	81.13	213.29	48.32-120.63	159.48361-28	7.24
100	61.5					
200	84.7					
Control	0.0					

LC: Lethal concentration, *A. mexicana*: *Argemone mexicana, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit

Table 9: LC of methanol extract of *A. mexicana* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	24.6					
25	41.4					
50	52.2	33.90	152.19	15.21-60.37	78.68-1391.37	14.70
100	78.1					
200	100					
Control	0.0					

*Significant at $P<0.05$. LC: Lethal concentration, *A. mexicana*: *Argemone mexicana, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit

Table 10: LC of hexane extract of *V. negundo* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	8.0					
25	22.8					
50	38.6	71.71	377.17	61.17-85.31	274.14-585.95	0.443
100	58.8					
200	79.2					
Control	0.0					

V. negundo: *Vitex negundo, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit, LC: Lethal concentration

Table 11: Lethal concentration of ethyl acetate extract of *V. negundo* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	19.6					
25	33.0					
50	54.7	39.27	178.33	33.68-45.47	140.29-245.12	1.785
100	78.4					
200	93.5					
Control	0.0					

V. negundo: *Vitex negundo, A. salina*: *Artemia salina,* UCL: Upper confidence limit, LCL: Lower confidence limit, LC: Lethal concentration

The results of the present research showed much potent cytotoxic studies represented by all the three plant species selected. Musa (2012) reported that the cytotoxic activity of *Cochlospermum tinctorium* by 80% acetone extract showed LC_{50} value of 240 µg/ml and n-butanol extract with LC_{50} value of 437 µg/ml. Our results are comparatively better than the previous study. Kamba and Hassan (2010) found that the brine shrimp sample test with ethanol as extract ion-solvent, recorded mortality of 100%, 93%, and 83% for various concentrations prepared such as 1000, 100 and 10 ppm, respectively.

In a study by Sokmen (2001) in which the author conducted different extracts of plant parts and callus cultures of 10 medicinal plants almost all extract showed a moderate activity (from a least 2.5 to highest brine shrimp lethality percentage 75.9% at 300 ppm after 48 h) compared to the present results with *A. paniculata, A. mexicana,* and *V. negundo.*

Arquion *et al.* (2015) studied the plant species, *Ficus nota* for brine shrimp lethality. Plant extracts were obtained through extraction of the stem samples with water and

Table 12: LC of acetone extract of *V. negundo* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	15.6					
25	24.8					
50	43.9	56.41	309.27	48.02-66.64	227.61-471.27	1.663
100	65.5					
200	85.4					
Control	0.0					

V. negundo: *Vitex negundo*, *A. salina*: *Artemia salina*, UCL: Upper confidence limit, LCL: Lower confidence limit, LC: Lethal concentration

Table 13: LC of methanol extract of *V. negundo* against brine shrimp (*A. salina*)

Concentration (ppm)	Brine shrimp mortality (%)	LC_{50} (ppm)	LC_{90} (ppm)	95% fiducial limit (ppm)		Chi-square
				LCL	UCL	
12.5	23.4					
25	38.7					
50	55.6	34.22	144.87	18.36-55.53	80.87-704.63	11.705*
100	78.4					
200	100.0					
Control	0.0					

*Significant at $P<0.05$. *V. negundo*: *Vitex negundo*, *A. salina*: *Artemia salina*, UCL: Upper confidence limit, LCL: Lower confidence limit, LC: Lethal concentration

absolute ethanol at three concentrations 100, 500, and 1000 ppm. The results showed that both decoction and ethanolic extracts were active against the brine shrimp with LC_{50} values of 991.00 and 852.22 ppm. These values are much higher than the present study.

The reported active (cytotoxic) plants in this study are worth of further pharmacological and phytochemical studies to define the exact principle compound response for cytotoxicity. Further investigations on these plants on the potent crude extract to find out the cytotoxic potential compound through a bioassay guided separation and also to find out the mechanism of action is suggested by this study.

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