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Comparison of the *Artemia salina* and *Artemia fransiscana* bioassays for toxicity of Indian medicinal plants

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the cytotoxic activity of the *A. tetracantha* and *G. asiatica* using brine shrimp lethality bioassay. The study showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to guide the fractionation process of biological extracts in order to detect antitumor compounds.
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ABSTRACT

Objective: To evaluate leaves extract of *Azima tetracantha* and *Gmelina asiatica* for lethality to brine shrimp larvae (*Artemia salina* and *Artemia fransiscana*).
Methods: The plant materials were extracted based on polarity gradients of petroleum ether, benzene, chloroform, acetone, ethanol and methanol. The extracts were investigated for their cytotoxic potential.
Results: In the brine shrimp lethality assay of all extracts, exception of acetone, ethanol and petroleum ether extracts *Gmelina asiatica* displayed 100% mortality at 1 000 μg/mL by *Artemia salina* and *Artemia fransiscana*. Chloroform extract was the most potent and presented the highest percentage of mortality with the lowest LC_{50} values by both assay too.
Conclusions: The results of the present study suggest the presence of photochemical possessing cytotoxic agents.

KEYWORDS

Artemia salina, Cytotoxicity, Brine shrimp, Indian medicinal plant

1. Introduction

Traditional medicine system is based on different plants for many years around the world and still provides remedies for mankind[1]. Active pharmaceutical compounds can be isolated from these plants which have good medicinal properties[2]. According to World Health Organization (WHO, 1993) from 119 plants derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlate directly with their traditional uses as plant medicine[3]. Many plant derived natural products are also used as anti-cancer agents like vincristine and vinblastine. The use of natural products to prevent cancer is becoming increasingly popular.

Azima tetracantha (*A. tetracantha*) Lam. (Family: Salvadoraceae) locally known as "Mulsangu", is a rambling spinous shrub flowering throughout the year found in Peninsular India, West Bengal, Orissa, African countries

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and extends through Arabia to tropical Asia. The juice of the leaves is said to relieve the cough phthisis and asthma. In Western India, juice of the leaves is applied as eardrops against earache and crushed leaves are placed on painful teeth. In India and Sri Lanka, the root, root bark and leaves are administered with food as a remedy for rheumatism[4,5]. This plant is considered as a powerful diuretic and is also used to treat rheumatism, dropsy, dyspepsia, chronic diarrhoea and as a stimulant tonic for women after confinement[6].

This plant has been reported to possess different biological activities such as anti-inflammatory, wound-healing, diuretic and analgesic activities[7,8]. Furthermore, many plants have been investigated in order to identify new and effective safe anticancer compounds, as well as to elucidate the mode of action of cancer inhibition[9]. Verbenaceae is a family of mainly tropical flowering plants; it includes some 35 genera and 1 200 species. The phenolic compounds[10] and iridoids[11] were isolated from different members of the family. *Gmelina arborea* (Roxb.) (Family: Verbenaceae) is locally known as 'Gambhari'. In English, it is known as the 'Candahar tree' or 'White teak', and it is a fast growing deciduous tree occurring naturally throughout greater part of India.

The brine shrimp lethality test is considered to be very useful in determining various biological activities such as cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities[12-16]. The assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties. The brine shrimp lethality assay is based on the ability of the extract to show lethality in laboratory cultured *Artemia nauplii* (*A. nauplii*) brine shrimp. It is considered as useful tool for preliminary assessment of toxicity. It has also been suggested for screening pharmacological activities of plant extracts[17]. The present study concentrates on the cytotoxic activity of the *A. tetracantha* and *Gmelina asiatica* (*G. asiatica*) using brine shrimp lethality bioassay which is based on the ability to kill laboratory cultured brine shrimp (*A. nauplii*).

2. Materials and methods

2.1. Plant material

The leaves of *A. tetracantha* and *G. asiatica* obtained from the foot hills of Papanasam of Tamil Nadu were used for the investigation. The plant was authenticated by Dr. V. Chelladurai, Retired Research Officer, Central Council for Research in Ayurvedha and Siddha, Government of India. The material was brought to the laboratory and air dried at room temperature (25±2) °C. The dried samples were powdered using grinder mill and stored in desiccators.

2.2. Preparation of plant extracts

About 50 g leaf powder of *A. tetracantha* and *G. asiatica* was placed in Soxhlet extractor separately. It was extracted with petroleum ether, benzene, chloroform, acetone, ethanol and methanol for approximately 72 h. After this extraction period, the solvents collected from the extractor were evaporated using flash evaporator and further the plant extracts were concentrated by placing it in an incubator at 37 °C for 24 h and then stored in desiccators for subsequent experiments.

2.3. Toxicity testing against the brine shrimp

2.3.1. Hatching shrimp

The artemia brine shrimp eggs were kept in a special conical shaped container known as a hatching chamber (1 L) filled with artificial sea water which was prepared by dissolving 30 g of sea water in 1 L of distilled water at 27-29 °C. Regular air flow with average pressure and proper light was supplied for 48 h. The pH of the environment was adjusted to 9.0 to prevent the risk of death to the *A. nauplii* due to a drop in the pH during development[18]. After hatching, the active nauplii were collected with a plastic pipette for study.

2.3.2. Brine shrimp assay

All the experiments were conducted in glass Petri dishes (60 mm diameter and 12 mm height). The containers were filled with 0.5 mL herbal extract diluted with different concentrations (10-1 000 μg/mL) of dimethyl sulfoxide and then 4.5 mL of the brine shrimp solution was added to the Petri dishes. Ten brine shrimp larvae (nauplii) from each *Artemia* species which had developed for 48 h were added to each Petri dish. For each concentration of plant sample, one control group was conducted which included 0.5 mL (vehicle treated, dimethyl sulfoxide) with 4.5 mL of brine shrimp solution without extract.

This study was performed in three replicates for each concentration. The Petri dishes were covered with their lids in the darkness at room temperature for 24 h. Feeding and air were not required during the study. In each plate, the number of dead and surviving nauplii was counted and the LC_{50} was calculated. Nauplii that did not show any movement within 10 seconds were defined as dead.

The toxicity rate of extracts was estimated on the basis of the number of dead nauplii or the mortality rate that was estimated using the following equation.

$$\text{Mortality of death rate (\%)} = \frac{\text{d test} - \text{d control}}{\text{A control}} \times 100$$

Where, d test is the average number of dead nauplii in the experiment groups; d control is the average number of dead nauplii in the control group; A control is the average number

of living nauplii in the control group.

2.4. Statistical analysis

The results are expressed as means. LC_{50} values with 95% confidence intervals were determined by the probit analysis method.

3. Results

The Soxhlet extraction of leaves of *A. tetracantha* and *G. asiatica* were employed in order to extract substances of low and medium volatility as well as thermally stable constituents based on polarity gradient of petroleum ether, benzene, chloroform, acetone, ethanol and methanol.

The bioactivities of different plant extracts against *Artemia fransiscana* (*A. fransiscana*) and *Artemia salina* (*A. salina*) are shown in Tables 1 and 2, respectively. For each plant, extracts were tested at four concentrations (10, 100, 500, 1 000 μg/mL). The LC_{50} value and 95% confidence intervals were recorded for each extract concentration by the brine shrimp lethality test (BSLT) (Tables 1 and 2). Increased extract concentrations were associated with increased mortality rates. All extracts showed 100% mortality at 1 000 μg/mL concentration by *A. salina* and *A. fransiscana* with the exception of acetone, ethanol and petroleum ether extracts of *G. asiatica* (Tables 1 and 2). The other extract concentrations demonstrated 10%-72% mortality. Chloroform extract was the most potent and showed the highest percentage of mortality with the lowest LC_{50} values (187.6-234.4 mg/L and 206.5-262.0 mg/L for *A. salina* and *A. fransiscana*, respectively). After chloroform, benzene extract presented the highest toxicity with LC_{50} value of (207.9-262.7 mg/L and 217.7-268.3 mg/L). The acetone, ethanol and petroleum ether extracts of *G. asiatica* showed the lowest mortality with the highest LC_{50} values (977.5-1 107.7 mg/L, 545.6-1 027.0 mg/L and 321.6-1 144.6 mg/L) respectively.

According to the BSLT results, the rate of extracts toxicity was as follows *A. tetracantha*>*G. asiatica*. Plant toxicity rate was more in *A. salina* when compared with *A. fransiscana*. Therefore, no significant difference was reported in LC_{50} of *A. salina* and *A. fransiscana* in plant extracts. The medicinal plants used in this research were selected on the basis of their uses in traditional medicine. One of the easiest assays for screening plant toxicity is the BSLT. According to recent results, *A. nauplii* from the different populations, *A. salina* and *A. fransiscana* showed insignificantly different sensitivities to the same toxicant according to their LC_{50} values $P>0.05$. Plant extracts toxicity differed from each other ($P<0.01$) in *A. salina* and *A. fransiscana* systems which may be due to differences in the amounts and types of cytotoxic compounds.

Table 1
Cytotoxic results of plant extracts by *A. fransiscana* brine shrimp lethality assay.

Plants	Extracts	Percentage mortality at different concentration (μg/mL)				LC_{50} 24 h (μg/mL)	95% Confidence interval
		10	100	500	1 000		
A. tetracantha	Benzene	32	48	20	100	207.9	154.40-261.50
	Chloroform	26	49	76	100	187.6	135.40-239.70
	Methanol	21	32	63	100	303.7	251.60-355.70
	Acetone	20	31	65	100	301.2	251.60-350.70
	Ethanol	19	20	61	100	353.7	303.20-404.10
	Petroleum ether	20	24	60	400	343.7	290.90-396.60
G. asiatica	Benzene	28	30	72	100	262.7	211.00-314.50
	Chloroform	20	45	72	100	234.4	165.60-269.70
	Methanol	15	20	55	100	388.0	337.00-439.00
	Acetone	10	25	31	35	1 107.1	922.50-1293.80
	Ethanol	23	30	45	83	545.6	415.80-675.40
	Petroleum ether	7	20	30	40	1 144.6	954.90-1334.60

Table 2
Cytotoxic results of plant extracts by *A. salina* brine shrimp lethality assay.

Plants	Extracts	Percentage mortality at different concentration (μg/mL)				LC_{50} 24 h (μg/mL)	95% Confidence interval
		10	100	500	1 000		
A. tetracantha	Benzene	30	42	70	100	217.5	182.3-286.00
	Chloroform	26	34	69	100	206.5	206.5-314.50
	Methanol	16	40	65	100	290.0	233.1-346.90
	Acetone	16	24	66	100	333.2	283.8-382.60
	Ethanol	19	32	48	100	373.5	258.5-488.00
	Petroleum ether	13	55	53	400	392.6	342.5-442.70
G. asiatica	Benzene	23	34	71	100	268.3	220.6-442.70
	Chloroform	19	45	71	100	262.0	204.5-320.80
	Methanol	20	24	64	100	326.2	271.6-380.80
	Acetone	11	28	30	31	977.5	829.2-1125.7
	Ethanol	10	25	31	36	127.0	868.6-1175.0
	Petroleum ether	17	35	60	100	321.6	264.7-378.50

4. Discussion

The brine shrimp bioassay is a simple and low cost technique for predicting the toxicity of the plant extract in order to consider the safety of the therapeutic agents as well as correlation with other biological models[19]. Criterion of brine shrimp toxicity for fraction, compound or plant extract was established according as LC_{50} values above 1 000 μg/mL are non toxic, between 500 and 1 000 μg/mL are weak toxic, and that below 500 μg/mL are toxic[20]. The present study showed that benzene (207.9 μg/mL), chloroform (187.6 μg/mL), methanol (303.7 μg/mL), acetone (301.2 μg/mL), ethanol (353.7 μg/mL) and petroleum ether (343.7 μg/mL) leaves extracts of *A. tetracantha* and benzene (262.7 μg/mL), chloroform (234.4 μg/mL), methanol (388.0 μg/mL) extracts of *G. asiatica* were toxic to *A. fransiscana*. While the other extracts of ethanol (545.6 μg/mL), acetone (1107.1 μg/mL) and petroleum ether (1 144.6 μg/mL) of *G. asiatica* were found to be weak toxic and non-toxic to the larvae of *A. fransiscana*. The bioactivities of all plant extracts (*A. tetracantha* and *G. asiatica*) showed toxic except acetone (977.5 μg/mL) and ethanol (1 027.0 μg/mL) extracts of *G. asiatica* against *A. salina*. The significant lethality of brine shrimp due to extracts of *A. tetracantha* and *G. asiatica* is an indicative of the presence of potent cytotoxic components which warrant further investiation[21]. Several studies have been carried on brine shrimp lethality of extracts from natural sources. Raghavendra *et al.* showed cytotoxic activity of methanol extract of *Putranjiva roxburghii* Wall. (Euphorbiaceae) seeds[22]. The extract was found to be toxic with LC_{50} of 427.74 μg/mL. The cytotoxicity of methanol extract of leaves of *Abrus pulchellus* Wall. (Fabaceae) using brine shrimp lethality bioassay revealed dose dependent activity with LC_{50} of 281.70 μg/mL[23]. Kekuda *et al.* found cytotoxic nature of extract of a macrolichen *Everniastrum cirrhatum* with LC_{50} value of 474.06 μg/mL[24]. In this study, the extract was found to cause mortality of brine shrimps in a dose dependent manner. The lethal nature of the extract could be attributed to the presence of secondary metabolites present in the extract.

Pervious phytochemical studies on different parts of *G. asiatica* showed that different classes of secondary metabolites were identified such as flavonoids[25,26], phenolics[27] and iridoids[28,29]. It is reported to contain alkaloid, glycoside, lignan derivatives and sesquiterpenoid; furthermore, phytochemical screening analysis reveals the presence of carbohydrates, saponins, tannins, anthraquinones and cardiac glycosides[26]. The root, leaf and bark part of *G. asiatica* possess cytotoxic activity[30]. Nargis Begum *et al*. reported ethanolic leaf extract of *A. tetracantha* had cytotoxicity effect of Ehrlich ascites carcinoma cells by reducing tumor volume, viable tumor cell count and increasing non viable tumor cell count[31]. The ethanolic leaf extract of *A. tetracantha* is rich in flavonoids, alkaloids and terpenoids. The cytotoxic and antitumor properties of the extract may be due to these compounds. Flavonoids have been shown to possess antimutagenic and antimalignant effect[32,33]. Moreover, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation[34] and angiogenesis[35].

The extracts studied in this work showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to guide the fractionation process of biological extracts in order to detect antitumor compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The brine shrimp bioassay is a simple and low cost technique for predicting the toxicity of the plant extract in order to consider the safety of the therapeutic agents as well as correlation with other biological models.

Research frontiers

This work determined the plant extracts toxicity differed from each other in *A. salina* and *A. fransiscana* systems.

Related reports

Many plants have been investigated in order to identify new and effective safe anticancer compounds, as well as to elucidate the mode of action of cancer inhibition.

Innovations and breakthroughs

This study showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to guide the fractionation process of biological extracts in order to detect antitumor compounds.

Applications

The assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties.

Peer review

This is a valuable research work in which authors have demonstrated the cytotoxic activity of the *A. tetracantha* and *G. asiatica* using brine shrimp lethality bioassay. The study showed significant lethality against brine shrimp, which has been successfully used as a simple biological test to guide the fractionation process of biological extracts in order to detect antitumor compounds.

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