Journal of Pharmaceutical Research International

Volume 35, Issue 21, Page 47-53, 2023; Article no.JPRI.104794
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Comparative Study of *In vitro* Cytotoxic Effect of Leaves and Stems Extracts of *Clitoria ternatea* by Brine Shrimp Lethality Assay

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2023/v35i217411

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/104794

Original Research Article

Received: 05/06/2023
Accepted: 08/08/2023
Published: 14/08/2023

ABSTRACT

Aim: The present study aims at the comparison of cytotoxic effect of methanolic extracts of leaves and stems of *Clitoria ternatea* (Fabaceae) by brine shrimp lethality assay.
Methodology: Dried leaves and stems were macerated with methanol separately and preliminary phytochemical screenings were carried out. Hatched brine shrimp nauplii were chosen for the assay. The effect was assessed by calculating % mortality of nauplii with different concentrations of the test extracts (1, 10, 25, 50, 100, 500 and 1000 µg/ml) and standard vincristine sulphate. All sets were performed in triplicate.
Results: The extracts of leaves and stems revealed to possess chiefly alkaloids, phenols and flavonoids. Both the extracts exhibited promising outcomes in dose dependant manner while

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significant responses in most of the doses were also calculated. Leaves were found to be more potent (LC_{50} value of 276.29 µg/ml.) compared to the stems (LC_{50} value of 322.95 µg/ml.). All the doses of the standard Vincristine sulphate were found to display significant activity compared to the control and was calculated to have LC_{50} value of 11.75 µg/ml.

Conclusion: The methanolic extracts of leaves and stems have shown potential cytotoxicity against brine shrimp which were highly comparable with standard. Further work on isolation, characterization and tests on cell lines may lead to identification of active principles.

Keywords: Clitoria ternatea; brine shrimp lethality assay; maceration; cytotoxicity; $LC_{50.}$

1. INTRODUCTION

One of the main maladies that kill people worldwide is cancer. It is the unregulated progression of cells in any area of the body that ends up resulting in organ enlargement or the emergence of tumours [1]. Because most established regimens have negative consequences and because different tumours respond differently to varying treatments, new tactics or substances must be found.

The promise of remedies made from plants as the mainstay of chemotherapeutic medications has long been established [2,3]. Anticancer medications have historically been derived primarily from plants and other natural items. A lot of plant-based anticancer medications are utilised extensively, including taxols (Paclitaxel), camptothecin, and vinca alkaloids (vinblastine, vincristine) [4,5].

Known by most as Aparajita, *Clitoria ternatea* (Fabaceae) is a perennial herb that grows all throughout India and has vivid blue blossoms. The leaves are pinnate with straight and flat pods having brown or black seeds inside [6,7].

The leaves and stems of the plant are reported to possess alkaloids, flavonoids, phenols etc. The whole plant and seed extracts are used for stomatitis, hematemesis, insomnia, epilepsy and as purgative or cathartic. The leaves possess strong antioxidant and free radical-scavenging properties and are used in eruptions too. The roots and their barks possess anti-inflammatory, analgesic, antipyretic, diuretic and laxative properties [8-11].

In order to limit the disruptive consequences of free radicals on the human body, antioxidants and substances that scavenge free radicals are vital. There is a relationship between the cytotoxicity to cancer cells and potential effects of scavenging free radicals [9,11]. We have previously demonstrated the plant's leaves' and stems' capacity to scavenge free radicals and to behave as antioxidants using methanol extracts [8]. Keeping with these, the goal of the current investigation was to determine the possible cytotoxic effect of the *Clitoria ternatea* extracts using a straightforward yet efficient method called as Brine Shrimp Lethality Bioassay.

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

This study relied on Merck and Loba chemie for the Solvents and chemicals. Standard was procured from Sigma Aldrich and Brine shrimp eggs (*Artemia salina*) from Megha aquafarm, Kerala, India.

2.2 Collection and Identification of Plant Material

Leaves and stems of *Clitoria ternatea* (Fabaceae), variety of blue flowers, were collected from local area of Ashokenagar, N-24 Pgs, West Bengal, India in the month of December, 2020 and identified and authenticated from BSI, Howrah, India. A sample specimen is preserved in the laboratory for future reference.

2.3 Extraction of Plant Material and Phytochemical Screening

The plant materials were shade dried and coarsely powdered. 200 g of each powdered material was extracted using methanol by maceration method for 3 days with occasional stirring. The extracts were filtered and dried under reduced pressure to get the concentrated extracts. These were kept in desiccator for seven days in vacuum to remove traces of methanol completely. Further phytochemical screening and the assay were performed using these extracts and they were termed as test extracts [8,12].

2.4 Hatching the Brine Shrimp

A specially designed tank with two unequal compartments was used for this purpose which was filled with simulated seawater maintained at

28-30°C. Arrangements were made to ensure constant oxygen supply throughout the experiment. The shrimp eggs, *Artemia salina*, were added to one side of the tank and covered. A lamp was placed above the open side of the other compartment to attract the hatched shrimps. Hatching and maturation of shrimps to nauplii was allowed for two days. These were attracted to the light (phototaxis) and so nauplii free from egg shells were collected for the further study from the illuminated part of the tank [13-15].

2.5 Preparation of Test and Standard Solutions

100 mg of leaves and stems extracts were dissolved separately in 100 ml of sterilized water. An ultrasonicator was employed to facilitate the process. From this stock solution further dilutions were prepared to get different concentrations (1 µg/ ml, 10 µg/ ml, 25 µg/ ml, 50 µg/ ml, 100 µg/ ml, 500 µg/ ml and 1000 µg/ ml) which were termed as test solutions. Similarly standard vincristine sulphate solutions were also prepared.

2.6 Bioassay

Each of 1 ml of different concentrations of tests and standard, as noted above, were then added to the pre-marked test tubes holding 10 live nauplii in simulated seawater (5 ml). Following a 24-hour period, the tubes were examined through a magnifying glass, and the quantity of living and dead nauplii within each tube was tallied. The inability to move forward for 30 seconds of surveillance was considered as the death endpoint [16]. Additionally, a blank run without the tests or standard was conducted. Three replications of the entire set were carried out.

The percentage of mortality was determined using the following equation and the median lethal concentration (LC_{50}) values were calculated by using the regression line obtained by plotting the concentration against the percentage of mortality [16-19].

% mortality = (Number of dead nauplii / Initial number of live nauplli) X 100

2.7 Statistical Analysis

One way ANOVA and Tukey test was performed to establish any possible significance between various groups and the *P* values were noted.

3. RESULTS AND DISCUSSION

Extracts from leaves and stems were revealed to be rich in flavonoids, phenols, and alkaloids while moderate concentrations of terpenoids, tannins carbohydrates were detected. Table 1 displays the outcomes of the phytochemical screening.

A Preliminary, straightforward, high throughput test for the assessment of cytotoxic behaviour of bioactive substances is the brine shrimp lethality bioassay. It is predicated on test substances' capacity to kill the lab-cultured brine shrimp *Artemia salina*, a basic zoological organism [20]. Following its first proposal by Michael *et al*., several other groups worked to further enhance this assay [21-23].

However, additional tests based on the prevention of cyst hatching have also been used, even though the majority of studies have employed hatched nauplii [24].

Table 1. Qualitative phytochemical screening of methanolic extract of leaves and stems of *Clitorea ternatea*

Secondary Metabolites	Leaves extract	Stems extract
Alkaloids	+++	+++
Phenol	+++	+++
Flavonoids	+++	+++
Carbohydrates	++	+
Tannins	++	+
Terpenoids	+	+
Amino Acids & Proteins	+	-
Resin	-	-
Glycosides	-	-
Saponins	-	-

(+, ++, +++ represent degree of intensity of colour change i.e., presence of phytochemical groups and - represents absence of phytochemical groups)

Table 2. Effects of different extracts of *Clitoria ternatea* and vincristine sulphate on the shrimp nauplii by brine shrimp lethality bioassay

Treatment	Concentration (µg/ml)	% Mortality	*P* value	LC_{50} (µg/ml)
Control (Distilled water)	Blank	3.33 ± 5.77		-
Standard (*Vincristine sulphate*)	1	13.33 ± 5.77	.049	
	10	60 ± 0.00	.001	
	25	80 ± 0.00	.001	
	50	93.33 ± 5.77	.001	9.39
	100	100 ± 0.00	.001	
	500	100 ± 0.00	.001	
	1000	100 ± 0.00	.001	
Clitoria ternatea (leaves extract)	1	0.0 ± 0.00	.89	
	10	20 ± 10	.11	
	25	33.3 ± 5.77	.001	
	50	40 ± 0.00	.001	276.29
	100	53.33 ± 11.55	.001	
	500	86.67 ± 5.77	.001	
	1000	96.67 ± 5.77	.001	
Clitoria ternatea (stem extract)	1	0.0 ± 0.00	.89	
	10	23.33 ± 5.77	.003	
	25	36.67 ± 5.77	.001	
	50	50.00 ± 0.00	.001	322.95
	100	56.67 ± 5.77	.001	
	500	66.67 ± 5.77	.001	
	1000	83.33 ± 5.77	.001	

% Mortality = Mean ± SD, (n=3)

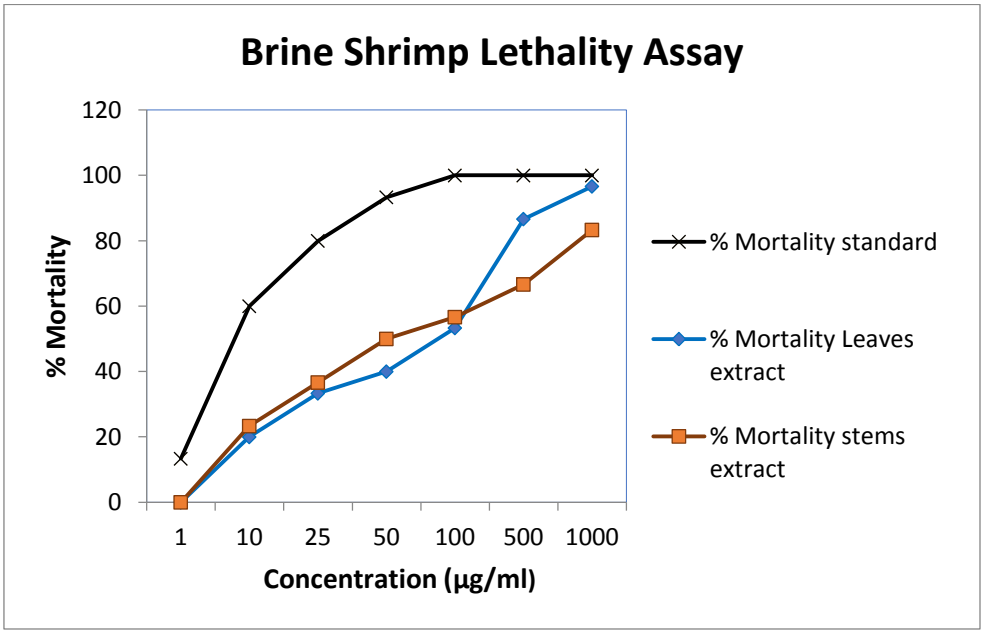

Fig. 1. Lethality effects of different extracts and standard on brine shrimp

Beside its conventional applications, many other, such as heavy metals, Fungal toxins, pesticides cytotoxicity testing of dental materials and plant extract toxicity have all been detected using it [17,25].

The lethality of several extracts of *Clitoria ternatea* (Fabaceae) against Brine Shrimp nauplii are displayed in Table 2 and Fig. 1. It was discovered that the extracts' lethality was directly correlated with their concentration, indicating the prevalence of cytotoxic components in the extracts. After 24 hours of observation, most of the shrimps were found survived in the control. All the doses of standard (Vincristine sulphate) exhibited significant response while different levels of significance were noted in leaves (25 µg/ml and higher doses) and stems extract (10 µg/ml and higher doses) when compared with the control. The LC_{50} values of leaves and stems extracts were calculated to be 276.29 µg/ml and 322.95 µg/ml respectively. However, the median lethality value for the standard vincristine sulphate was computed to be 9.39 µg/ml.

A preliminary analysis of the phytochemical composition of the test extracts of *Clitoria ternatea* demonstrated that terpenoids, alkaloids, phenols, and flavonoids were present in high concentrations which can also be correlated with the previous finding [8,26]. There are reports on these phytochemicals' contribution to plant extracts' cytotoxic effects [16,17,25,27-29]. Therefore, the cytotoxic impact seen in this investigation could also be attributed to the presence of these chemicals.

The *Artemia* nauplii have been utilised in ecotoxicology, teratology screens, and general toxicity for the past few decades. Pharmacologically strong correlation has been set between the antitumor chemicals detected in numerous plant extracts and the brine shrimp fatality test [17,18,25].

The National Cancer Institute, USA has also shown that the assay is connected with the retardation of *in vitro* proliferation of human solid carcinoma cell lines, once more, indicating its significance as a pre-screening tool for anticancer drug development. It is also said that substances with LC_{50} outcomes less than 1000 µg/ml, in this assay, may be further tested in cell lines to establish the anticancer or anti-tumor property [17-18] which is highly encouraging as the LC_{50} values obtained in the present study are far below this said level (276.29 µg/ml and 322.95 µg/ml respectively for leaves and stems extracts).

4. CONCLUSION

Leaves and stems extracts of *Clitoria ternatea* exhibited cytotoxic activity against the brine

shrimps and considered as containing bioactive components. The outcomes may hold particular significance for the identification and elucidation of the active principles accountable for its unexplored value. Additionally, they may provide more insight into the intricate molecular mechanisms underlying cell death, thereby serving as a possible reserve for the development of chemically intriguing and biologically significant therapeutic candidates.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

The authors express their sincere gratitude to the authority of BCDA College of Pharmacy & Technology, Hridaypur, Kolkata, West Bengal, India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/104794