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OPTIMIZATION OF BRINE SHRIMP LETHALITY TEST FOR *IN VIVO* TOXICITY EVALUATION OF POISONOUS PLANT SPECIES COLLECTED FROM QUANG TRI PROVINCE

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ABSTRACT

Plants are natural resources providing several important bioactive compounds for human health. To discover such valuable properties, researchers need to focus on both the pharmacology and toxicity of plant materials. Preliminary toxicity assessment of plants using the Brine Shrimp Lethality Test is a convenient, simple, and effective tool. However, some environmental parameters such as light, temperature and salinity need to be optimized for our own laboratory conditions and *Artemia salina* cysts produced in Vietnam. The obtained results indicated that the continuous lighting regime, temperature of 30 ^{o}C and salinity of 30 ppt are the most suitable parameters for cyst hatching within 24 hours and development of nauplii after 24 hours of hatching. Based on the optimal cyst-hatching procedure, the potential toxicity of 26 extracts from 24 poisonous plant species collected in Quang Tri was determined with median lethal concentration (LC_{50}). The results showed that 3/26 extracts were extremely toxic with $LC_{50} \leq 10$ µg/mL; 10/26 extracts were highly toxic with LC_{50} from 10 µg/mL to 100 µg/mL; 8/26 extracts were moderately toxic with LC_{50} from 100 µg/mL to 250 µg/mL; 4/26 extracts had low toxicity with LC_{50} from 250 µg/mL to 1,000 µg/mL; and 1/26 extract was not toxic with $LC_{50} > 1{,}000$ µg/mL. The presented data could provide scientific evidence for further pharmacological and toxicological investigations of these plant species. The optimal conditions for hatching *A. salina* cysts in this study will be applied in our laboratory for *in vivo* toxicity assessment of other plant species.

Keywords: Brine shrimp, *Artemia salina*, In vivo toxicity test, Plant extracts.

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INTRODUCTION

Plant toxins represent a large group of structurally diverse small molecules and are products of secondary metabolism. Among the secondary substances toxic to other organisms, many alkaloids, terpenes, steroids and phenolic compounds have become drugs or natural sources for drug development (Anywar et al., 2020). For example, toxins affect nerve transmission or cell division, leading to the discovery of drugs to treat neurological disorders and cancers (Kasali et al., 2020). In addition to secondary metabolites, toxic proteins such as plant lectins are known as tools for disease diagnosis as well as a source for the development of anti-cancer drugs (Dang & Van Damme, 2015). Therefore, toxicity evaluation is one of the initial requirements in exploiting and applying certain plant species in life.

In order to evaluate toxicity, many biological models have been developed. *In vitro* cell culture systems are the most widely used because of their low cost and time saving. However, toxicity results at *in vitro* levels have limitations when interpreted by living organisms, including humans. Therefore, *in vivo* studies have been applied to gain more accurate predictions. Zebra fish is one of the valuable *in vivo* models to study biological responses and analyse action mechanisms of target compounds on the body. However, according to REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) of the European Union, invertebrate animals were recommended to replace vertebrates in toxicity testing due to ethical apprehensions (Dvorak et al., 2012).

Among the invertebrates used to test the possible toxicity of physical and chemical agents, brine shrimp *Artemia* spp. such as *Artemia franciscana, Artemia persimilis, Artemia salina, Artemia sinica, Artemia tibetiana, Artemia urmiana* demonstrated high sensitivity to toxicity (Van Steertegem & Persoone, 1993). *Artemia* spp. were first proposed for toxicity screening by Michael et al. (1956) and then improved by several other research groups (Van Walbeek et al., 1971; Vanhaecke et al., 1981). Since the 1970s, the US Environmental Protection Agency has recognized *Artemia* spp. as a standard model for *in vivo* experiments (Nadeau et al., 1974).

A. salina, a small brine shrimp, belonging to the *Artemia* genus, Crustacea class, Arthropoda phylum, is commonly used for toxicity screening experiments. Nauplii of *A. salina*, about 22 mm long, are large enough to observe with the naked eyes or under low magnification, and small enough to use in an extensive number of individuals for experimental scale. Therefore, it is one of the most popular approaches in toxicological studies to evaluate the toxicity of various compounds including plant extracts (Kibiti & Afolayan, 2016). In addition, some studies have shown that there is a correlation between the lethal concentration (LC_{50}) in the *A. salina* lethality test and the acute oral toxicity test in mice (Arslanyolu & Erdemgil, 2006).

Although as a common method to evaluate the toxicity of substances, the conditions for cyst hatching of *A. salina* such as temperature, lighting period and salinity vary depending on the laboratory conditions as well as the cyst sources. Therefore, in this study, a range of temperatures, lighting periods and salinity levels were examined for cyst hatching. The quality nauplii from optimized cyst hatching conditions were used to determine the toxicity of 26 methanol extracts of 24 poisonous plant species collected from the mountainous regions in Quang Tri province of Vietnam.

MATERIALS AND METHODS

Artemia salina cysts and plant extracts

A. salina cysts (embryos packed in shells) were provided by Duong Khoi Production and Trading Company Limited, Can Tho, Vietnam. The plant materials of 24 common poisonous plant species were collected in Dakrong and Huong Hoa districts, Quang Tri province in 2021–2022 as described in our previous study (Mai et al., 2023). Plant samples (~100 g dry powder) were extracted twice with 400 mL methanol in a sonicator

(Emasonic Easy 180H, Germany) for 1 hour at 40–50 ^{o}C. The extractions were filtered through filter paper, pooled, and evaporated in vacuo to obtain plant methanol extract.

Table 1. List of plant extracts used in the toxicity assessment experiments.

No.	Plant extract	Scientific name of plant species	Vietnamese name	Family	Plant materials
1	TNSV06	*Oldenlandia pilulifera* Pit.	An điền nón	Rubiaceae	Whole plants
2	TNSV08	*Acorus gramineus* Aiton	Thạch xương bồ	Acoraceae	Whole plants
3	TNSV13H	*Antheroporum harmandii* Gagnep.	Mát	Fabaceae	Seeds
4	TNSV13	*Antheroporum harmandii* Gagnep.	Mát	Fabaceae	Twigs and leaves
5	TNSV14	*Buddleja asiatica* Lour.	Bọ chó	Loganiaceae	Whole plants
6	TNSV15	*Ricinus communis* L.	Thầu dầu	Euphobiaceae	Twigs, leaves, fruits
7	TNSV17	*Strychnos vanprukii* Craib	Mã tiền cành vuông	Loganiaceae	Whole plants
8	TNSV28	*Combretum indicum* (L.) DeFilipps	Dây giun	Combretaceae	Whole plants
9	TNSV30	*Stemona tuberosa* Lour.	Bách bộ	Stemonaceae	Roots
10	TNSV40	*Euphorbia tithymaloides* L.	Thuốc dấu	Euphorbiaceae	Twigs and leaves
11	TNSV41	*Euphorbia tirucalli* L.	Xương khô, Giao	Euphorbiaceae	Twigs
12	TNSV42	*Heliotropium indicum* L.	Vòi voi	Boraginaceae	Whole plants
13	TNSV44	*Kibatalia laurifolia* (Ridl.) Woodson	Thần linh lá quế	Apocynaceae	Twigs and leaves
14	TNSV45	*Sarcodum scandens* Lour.	Muồng dây	Fabaceae	Twigs and leaves
15	TNSV46	*Gelsemium elegans* (Gardner & Champ.) Benth.	Lá ngón	Loganiaceae	Twigs and leaves
16	TNSV46H	*Gelsemium elegans* (Gardner & Champ.) Benth.	Lá ngón	Loganiaceae	Flowers
17	TNSV47	*Vernicia montana* Lour.	Trẩu lá xẻ	Euphobiaceae	Twigs and leaves
18	TNSV48	*Callicarpa kochiana* Makino	Tử châu thùy dài	Verbenaceae	Twigs and leaves
19	TNSV49	*Rauvolfia verticillata* (Lour.) Baill.	Ba gạc vòng	Apocynaceae	Whole plants
20	TNSV50	*Melia azedarach* L.	Xoan	Meliaceae	Twigs and leaves
21	TNSV51	*Millettia erythrocalyx* Gagnep.	Thàn mát lá đỏ	Fabaceae	Twigs and leaves

No.	Plant extract	Scientific name of plant species	Vietnamese name	Family	Plant materials
22	TNSV52	*Ficus hispida* L.f.	Ngái	Moraceae	Twigs and leaves, fruits
23	TNSV54	*Tabernaemontana divaricata* (L.) R.Br. ex Roem. & Schult.	Ớt rừng	Apocynaceae	Twigs and leaves
24	TNSV57	*Cryptolepis dubia* (Burm.f.) M.R.Almeida	Dây càng cua	Asclepiadaceae	Whole plants
25	TNSV58	*Zanthoxylum avicennae* (Lam.) DC.	Muồng truổng	Rutaceae	Twigs and leaves
26	TNSV100	*Catharanthus roseus* (L.) G. Don	Dừa cạn	Apocynaceae	Whole plants

Optimization of *Artemia salina* cyst hatching conditions

A. salina cyst hatching conditions were optimized based on the method of Migliore et al. (1997). The artificial seawater was prepared by adding commercial sea-salt to distilled water to get appropriate salinity (pH 8.0 ± 0.5). The mixture was then placed on a magnetic stirrer so that dissolved oxygen and salt were evenly distributed in the solution. *A. salina* cysts used in each experiment was weighed on the analytical scale (GS323, SHINKO) and added to artificial seawater solution to obtain the density of 10 cysts/mL. The cyst suspension (approximately 5 mL, containing 50 eggs) was immediately placed into a 20 mL plastic container. The tested parameters including lighting periods of 0, 8, 16 and 24 hours, temperatures of 20, 25, 30 and 35 ^{o}C and salinities of 0, 5, 10, 15, 20, 25, 30, 35 and 40 ppt were carried out in a control environment unit. The numbers of hatched cysts and live nauplii were counted after 24 hours and 48 hours under the Stemi DV4 Stereo Microscope (Zeiss, Germany). The percentage of hatching and live nauplii in each tested condition was calculated using the following formulas:

$$Hatching\,(\%) = \frac{Number\ of\ hatched\ cysts}{Total\ cysts} \times 100$$

$$Live\ nauplii\,(\%) = \frac{Number\ of\ live\ nauplii}{Total\ cysts} \times 100$$

The morphology of the *A. salina* cysts/nauplii at different stages was observed under Stemi DV4 Stereo Microscope (Zeiss, Germany) with suitable magnification levels.

***In vivo* toxicity of methanol extracts using *Artemia salina* nauplii lethality test**

The toxicity of 26 methanol extracts from plant samples (Table 1) was evaluated *via* the lethal effect on *A. salina* nauplii as described by Kibiti & Afolayan (2016) with minor modifications. Firstly, the Nunc™ 4-Well IVF Dishes containing 1.8 mL/well of artificial seawater at the optimized concentration (30 ppt, pH 8.0 ± 0.5) were prepared. *A. salina* cysts were gently released onto the surface of the wells. The dishes were kept at 30 ^{o}C and under continuous light for 24 h, and then cyst shells were removed.

Each extract stock solution was prepared by dissolving 100 mg of methanol extract residue in 1 mL DMSO. Then different extract concentrations of 2,000 µg/mL; 1,000 µg/mL;

500 µg/mL, 400 µg/mL, 200 µg/mL, 100 µg/mL, 20 µg/mL, 10 µg/mL and 2 µg/mL were obtained by diluting the stock solution in 30 ppt artificial seawater. 500 µL of each prepared concentration was added into a well, followed by 450 µL of 30 ppt artificial seawater and 50 µL of nauplii suspension (containing approximately 10–30 nauplii) to get the final concentrations of test extracts to be 1,000 µg/mL; 500 µg/mL, 400 µg/mL, 200 µg/mL, 100 µg/mL, 20 µg/mL, 10 µg/mL, 2 µg/mL and 1 µg/ mL. The negative control was 1% DMSO in 30 ppt artificial seawater, which was preliminary proved to be safe for nauplii of *A. salina*. Each treatment was repeated thrice. After 24 hours, nauplii were considered as death if no forward motion during 10 s observation under Zeiss Stemi DV4 Stereo Microscope (Germany). The lethality rate of nauplii for each concentration and control was calculated as the following formula:

$$Lethality(\%) = \frac{Number\ of\ dead\ nauplii}{Number\ of\ dead\ nauplii + Number\ of\ live\ nauplii} \times 100$$

LC_{50} values were calculated based on a correlation graph between the concentrations of the test substance and the lethality (%) using Quest Graph™ LC50 Calculator (AAT Bioquest Inc., 2023).

Data analysis

The data from three replicate experiments was analysed using a one-way ANOVA test. Differences between means were compared according to Duncan's Multiple Range Test (DUNCAN) using SPSS software (Version 16.0, SPSS Inc., Chicago, USA) with $p < 0.05$.

RESULTS AND DISCUSSION

Optimisation of *Artemia salina* cyst hatching conditions

Among tested parameters, cyst hatching ability seems to depend on photoperiod (Fig. 1A). The hatching percentage increased by increasing light duration from total darkness to constant lighting. When the photoperiod increased from 0, 8, 16 to 24 hours of illumination, the hatching rate increased from 50%, 60%, 80% to 98%. After hatching, no significant difference was found in the number of nauplii alive within treatments until 48 hours. It is obvious that *A. salina* cysts need light conditions for the hatching process. This phenomenon was not observed in *A. urmiana*; where a high percentage of the cysts hatched in both light and complete darkness condition (Asil et al., 2012).

Regarding temperature parameters, the rate of cyst hatching at 20 ^{o}C is the lowest level (43.30%). As the temperature increases, the larval hatching rate also increases and reaches the highest level of over 90% in temperatures from 30 ^{o}C to 35 ^{o}C. However, at 35 ^{o}C, the number of nauplii alive after 48 hours were significantly lower compared to the number of hatched nauplii after 24 hours (Fig. 1B). Therefore, the temperature of 30 ^{o}C was selected as the appropriate temperature for *A. salina* cyst hatching and toxicity testing.

With the salinity parameter (0–40 ppt), the cysts hardly hatched after 24 hours in no salinity condition. At low salinity (≤ 10 ppt), the hatching rate was only 60%. There was no statistical difference in the hatching percentages (72.68, 80.97 and 79.73) among tested salinities of 15, 20 and 25 ppt (Fig. 1C). The hatching rate at salinities of 40 and 35 ppt was 88.58 and 96.77 %, respectively. The highest rate of 97.73% was achieved at the salinity of 30 ppt. In most tested salinity concentrations, the rate of nauplii alive after 48 hours of hatching was as comparable as the hatching rate, except at 40 ppt where the number of live nauplii decreased significantly after 48 hours. As a result, a salinity of 30 ppt was selected for further experiments.

In Vietnam, the *A. salina* hatching procedure of cysts had been applied in testing the toxicity of extracts from *Abutilon indicum*

root (Vu et al., 2016) and sponge *Xestospongia testudinaria* (Phuong & Huong, 2022), however sea salt concentration for cyst hatching was different to be 30% and 38%, respectively. In addition, other environmental factors such as temperature and lightning have not been mentioned yet. The effects of environmental parameters on the hatching process of *Artemia* cysts have been investigated in several studies. The optimal hatching performance of *Artemia* cysts could be at the temperature of 29 ^{o}C and the salinity of 29 ppt (Kumar et al., 2015) or 27–28 ^{o}C and 35 ppt (Sharahi & Zarei, 2016) or 24 ^{o}C and 30 ppt (Hasan & Rabbane, 2018) or 30 ^{o}C and 60 ppt (Bahr et al., 2021). Variation in optimal parameters may be due to the differences in cyst sources as well as experimental setup such as experimental volume, light quality, and chemicals used.

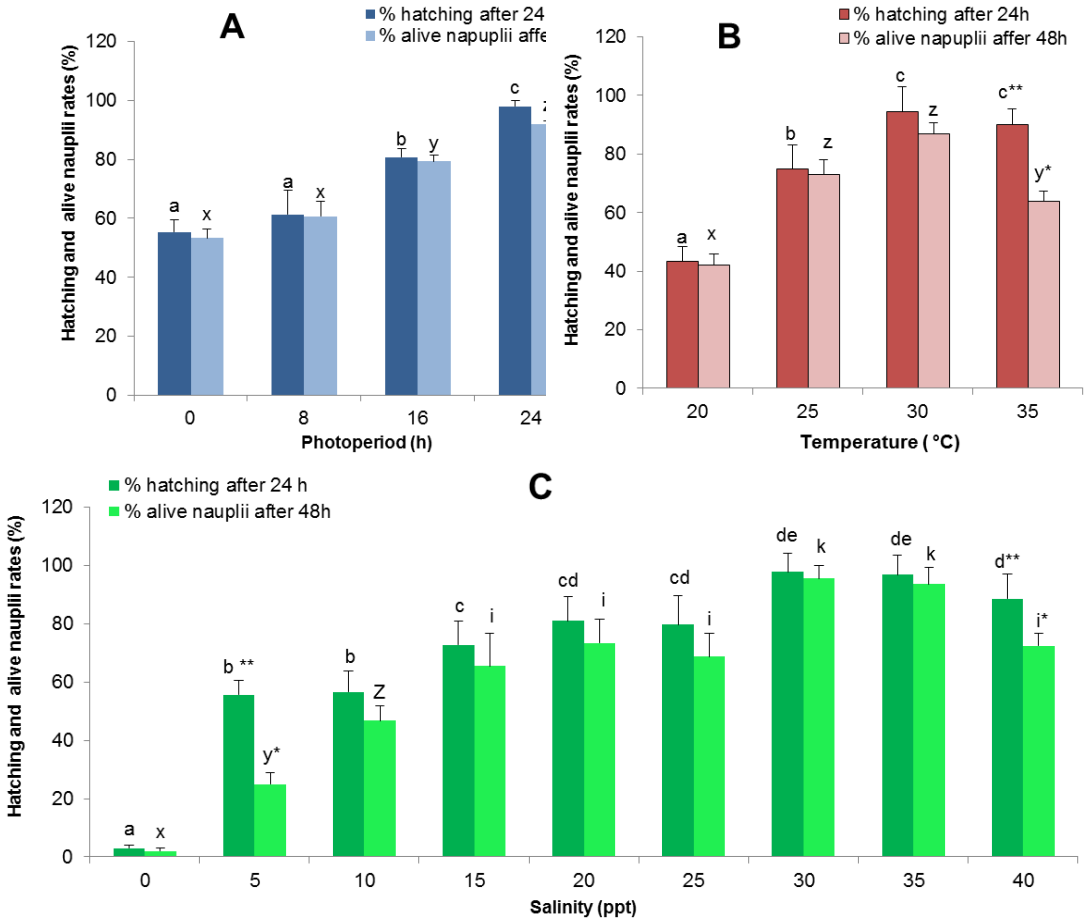

Figure 1. Effect of photoperiod, temperature, salinity conditions on *Artemia salina* cyst hatching after 24 hours and nauplii alive after 48 hours. **A:** Photoperiod; **B**: Temperature; and **C**: Salinity. Different superscript letters (a, b, c, d, e for hatching rate after 24 hours and x, y, z, i, k for live nauplii rate after 48 h) show significantly differences among means, based on Duncan test ($p < 0,05$). ** or *: refers to a significant difference between the percentage of hatching and the percentage of nauplii alive at the same temperature or salinity condition

The results clearly showed that constant lighting of 24 hours, temperature of 30 ^{o}C and salinity of 30 ppt are the optimal conditions for the hatching process of domestically produced *A. salina* cysts. In the optimal conditions, the morphology of the *A.*

salina cysts/nauplii at different stages was observed (Fig. 2). Soon after immersing the cysts in water, the hydration stage was started, in which the cysts inflate and develop into a spherical shape (Fig. 2A). When most of the cysts are spherical in shape, the differentiation stage commences (Fig. 2B). In the third stage (emergence), the cyst coat starts to fracture resulting from an increased amount of glycerol, and the embryos come out from the broken cysts (Fig. 2C). Finally, at the last stage (hatching), the embryos leave the cyst and begin to swim (Fig. 2D).

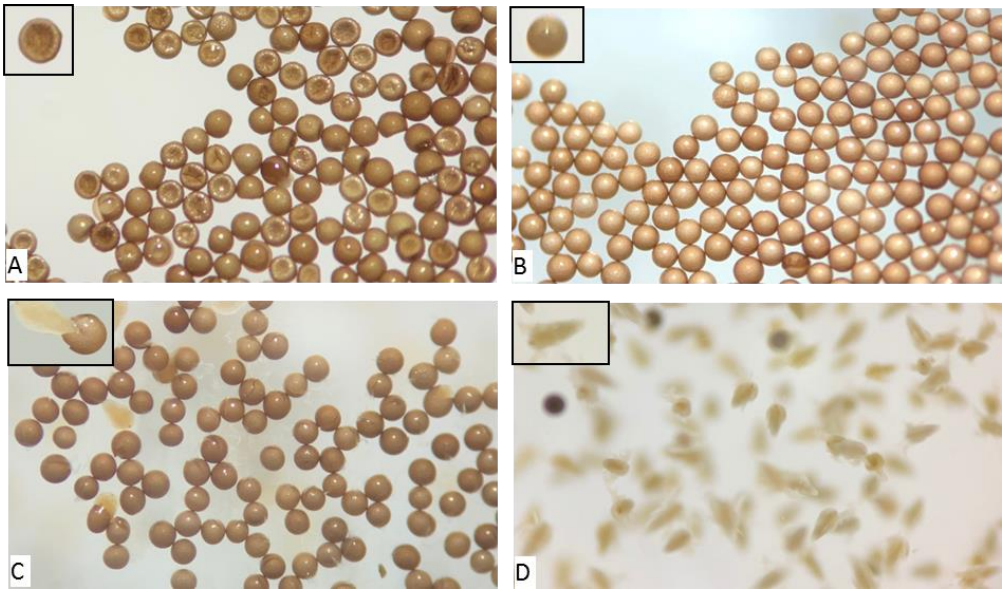

Figure 2. Morphology of the cysts/nauplii of *Artemia salina* at different stages in the optimized hatching conditions. **A**. Hydration; **B.** Differentiation; **C**. Emergence; **D**. Hatching (32X magnification)

***In vivo* toxicity of methanol extracts using *Artemia salina* nauplii lethality test**

The present study shows that hatched nauplii of *A. salina* from optimized hatching conditions is effective for the preliminary toxicity evaluation. After 24 hours treated with plant extracts, most nauplii survived in the control wells. At the highest concentration of 1,000 µg/mL, nauplii lethality rates were 100% in most tested cases, except for extracts of *Heliotropium indicum* (TNSV42) and *Buddleja asiatica* (TNSV14) which caused the death of 44.66% and 72.94% treated nauplii, respectively (Table 2). At the lowest concentration of 1.0 µg/mL, several extracts still were the reason for the lethal of *A. salina* nauplii, such as the extracts of *Gelsemium elegans* twigs-leaves and flowers (TNSV46 - 5.59% and TNSV46H - 23.78%), *Callicarpa kochiana* (TNSV48 - 4.95%), *Rauvolfia verticillata* (TNSV49 - 2.38%), *Melia azedarach* (TNSV50 - 4.79), *Ficus hispida* (TNSV52 - 2.38%), *Tabernaemontana divaricata* (TNSV54 - 6.06%), *Zanthoxylum avicennae* (TNSV58 - 5.56%); *Catharanthus roseus* (TNSV100 - 9.85%), twigs-leaves and seeds of *Antheroporum harmandii* (TNSV13 - 22.5% and TNSV13H - 48.65%).

In addition to the lethality rate, the toxicity of substances is often expressed in the form of LD_{50}. According to Rieser et al. (1996), plant extracts with LC_{50} values less than 250 µg/mL are considered highly toxic and potential for exploitation; and those greater than 1,000 µg/mL are non-toxic. In this study, LC_{50} values of 26 plant extracts shown in Table 2 indicated that plant extracts had different toxicity levels against *A. salina*

nauplii. Based on LC_{50} values, the toxicity of the tested plant extracts can be classified into 5 groups as follows:

i) Extremely toxic with $LC_{50} \leq 10$ µg/mL including methanol extracts of *A. harmandii* seeds (TNSV13H), *A. harmandii* twigs and leaves (TNSV13), and G. *elegans* flowers (TNSV46H)

ii) Highly toxic with LC_{50} from 10 to 100 µg/mL including methanol extracts of *C. roseus* (TNSV100), *Euphorbia tithymaloides (*TNSV40), *Stemona tuberosa (*TNSV30), *G. elegans* (TNSV46), *C. kochiana* (TNSV48), *R. verticillata* (TNSV49), *M. azedarach* (TNSV50), *Ricinus communis* (TNSV15), *Combretum indicum* (TNSV28), and *Kibatalia laurifolia* (TNSV44)

iii) Moderately toxic with LC_{50} from 100 to 250 µg/mL including methanol extracts of *Z. avicennae* (TNSV58), *Cryptolepis dubia* (TNSV57), *Euphorbia tirucalli* (TNSV41), *Sarcodum scandens* (TNSV45), *F. hispida* (TNSV52), *T. divaricata* (TNSV54), *Oldenlandia pilulifera* (TNSV06), and *Acorus gramineus* (TNSV08)

iv) Slightly toxic with LC_{50} from 250 to 1,000 µg/mL including methanol extracts of *Millettia erythrocalyx* (TNSV51), *Vernicia montana* (TNSV47), *B. asiatica* (TNSV14), and *Strychnos vanprukii* (TNSV17)

v) Non-toxic with $LC_{50} \geq 1000$ µg/mL including only extract of *H. indicum* (TNSV42).

Here, the obtained toxicity results have a good correlation with previously reported toxicity. Among 26 tested extracts, only *H. indicum* extract is non-toxic and the 25 others all gave certain toxicity to *A. salina* nauplii. In the extremely toxic group, the extracts of *A. harmandii* seeds and twigs-leaves could kill 50% of shrimp nauplii even at the concentration of 1 µg/mL and 5 µg/mL, respectively. While the toxicity of *A. harmandii* has not been studied, *G. elegans* is widely known to be a popular poisonous plant in Asia (Lin et al., 2021). All parts of the studied plant are strongly toxic, especially roots and young leaf tissues, causing poisoning to humans and animals if orally taken (Qu et al., 2021). The indole alkaloids, the main toxic components of this plant were known to have many pharmacological and biological properties such as anti-inflammation, pain relief, insecticide, and itching reduction as well as anti-cancer and immune regulation (Wang et al., 2017).

In the highly toxic group, *C. roseus* is a poisonous plant, which can cause poisoning and death for humans and animals if admitted a large amount. Previous studies had indicated that the methanol extract of *C. roseus* was directly toxic to the cell division and impacted nerve functions controlling digestive, cardiovascular, and reproductive systems (Lobert et al., 1998). At low doses, *C. roseus* extract has been used in many countries to treat malaria, diabetes, insomnia, leukemia, and Hodgkin's lymphoma (Das et al., 2020). Other plants in this group are also known as poisonous species, such as *S. tuberosa* causing toxic to insects and microorganisms (Petcharawan et al., 2016); *E. tithymaloides* triggering skin irritation, inflammation, and even blisters (Salehi et al., 2019); *C. kochiana* producing toxic to the nervous system (Tu et al., 2013); tetranortriterpene of *M. azedarach* eliciting neurotoxicity. *R. communis* containing a toxic compound called ricin can inhibit intestinal protein synthesis and damage the intestinal mucosa. *C. indicum* causes intestinal swelling, stomach swelling, dysentery, constipation, vomiting and headaches; while *K. laurifolia* is cytotoxic (Dao et al., 2011).

Most plant species in the moderately toxic group have been seen in folk medicine; even though there are obvious evidences of their toxicity to a certain extent for people and animals. For example, the secreted resin of *E. tirucalli* is skin toxic and especially dangerous for the eyes (Binckley & Zahra, 2023). *F. hispida* contains more than 76 secondary compounds including sesquiterpenoids, triterpenoids, flavonoids, coumarin, phenylpropionic acid, alkaloids, steroids, glycosides, and alkanes (Cheng et al., 2020). Ethanol extract of *F. hispida* leaves has

cytotoxic impact, causing cell cycle arrested in the G0/G1 phase (Sathiyamoorthy & Sudhakar, 2018). The wild chilli *T. divaricata* contains many toxic indolic alkaloids (Ara et al., 2012). Asarones identified in *Acorus gramineus* rhizome were found to be against three coleopteran stored-product insects (Park et al., 2003).

Table 2. Lethality rates and LC_{50} values of plant extracts in toxicity assessment

No.	Extracts	Con. (µg/mL)	Lethality (%)	LC_{50} (µg/mL)	No.	Extracts	Con. (µg/mL)	Lethality (%)	LC_{50} (µg/mL)
1	TNSV06	1,000	100.00	128	14	TNSV45	1,000	100.00	144
		100	37.12 ± 0.66				100	8.93 ± 1.16	
		1	0.00				1	0.00	
2	TNSV08	1,000	100.00	238	15	TNSV46	1,000	100.00	76
		100	16.99 ± 0.57				100	56.84 ± 8.73	
		1	0.00				1	5.59 ± 1.90	
3	TNSV13	100	100.00	5	16	TNSV46H	1,000	100.00	9
		5	47.03 ± 7.54				100	71.41 ± 8.21	
		1	22.50 ± 9.42				1	23.78 ± 6.90	
4	TNSV13H	50	100.00	1	17	TNSV47	1,000	100.00	360
		5	98.15 ± 3.21				100	10.20 ± 2.64	
		1	48.65 ± 5.99				10	0.00	
5	TNSV14	1,000	72.94 ± 23.44	310	18	TNSV48	1,000	100.00	68
		100	13.93 ± 6.61				100	57.07 ± 8.62	
		1	0.00				1	4.95 ± 1.29	
6	TNSV15	1,000	100.00	78	19	TNSV49	1,000	100.00	81
		100	72.19 ± 7.40				100	70.55 ± 7.56	
		1	0.00				1	2.38 ± 1.12	
7	TNSV17	1,000	100.00	425	20	TNSV50	1,000	100.00	85
		100	9.29 ± 3.26				100	58.01 ± 7.64	
		1	0.00				1	4.79 ± 1.18	
8	TNSV28	1,000	100.00	88	21	TNSV51	1,000	100.00	332
		100	63.36 ± 14.83				100	22.69 ± 2.52	
		1	0.00				1	0.00	
9	TNSV30	100	100.00	49	22	TNSV52	1,000	100.00	221
		10	8.62 ± 0.81				100	26.04 ± 5.44	
		1	0.00				1	0.00	
10	TNSV40	100	75.24 ± 9.07	24	23	TNSV54	1,000	100.00	219
		50	21.80 ± 3.43				100	26.27 ± 4.81	
		5	0.00				1	6.06 ± 1.25	
11	TNSV41	1,000	100.00	149	24	TNSV57	1,000	100.00	129
		100	36.25 ± 4.51				100	32.48 ± 1.48	
		1	0.00				1	0.00	
12	TNSV42	1,000	45.52 ± 1.11	2.054	25	TNSV58	1,000	100.00	137
		100	15.14 ± 4.57				100	28.71 ± 2.47	
		1	0.00				1	5.56 ± 1.81	
13	TNSV44	1,000	100.00	90	26	TNSV100	1,000	100.00	99
		100	61.11 ± 9.62				100	51.11 ± 1.92	
		1	0.00				1	8.59 ± 1.72	

Notes: The mortality rate is the average percentage of shrimp nauplii that died after 24 hours of treatment with the tested extracts ± SD (Standard Deviation); Con. = concentration.

The plant species groups with LC_{50} from 250 μg/mL to 1,000 μg/mL are slightly toxic and used in many traditional remedies. Flavonoids isolated from stem barks, roots, and leaves of *M. erythrocalyx* species were found to be active against *Herpes simplex* virus; its extracts were used to treat skin rashes and allergies; bones and joint pains and hepatitis (Likhitwitayawuid et al., 2005). Oil from *V. montana* seeds treated scabies and swelling caused by poisoning; its roots are used as an anthelmintic; leaves are used to manage ulcers and rheumatoid arthritis (Wang et al., 2022). *S. vanprukii* seeds are important in the treatment of gonorrhea, leukemia, stomach diseases, bronchitis, chronic diarrhoea, dysentery, kidney stones, blisters, diabetes, conjunctivitis, scleritis, ulcers and other eye diseases (Yadav et al., 2014). While *B. asiatica* can cause abortion, the roots, stems and roots are effective in treating fever, pain, diarrhoea and rheumatism (Chen et al., 2005).

CONCLUSION

In this study, the temperature of 30 ^{o}C, salinity of 30 ppt and lighting period of 24 hours were found to be the optimal conditions for the hatching process of *A. salina* under our laboratory conditions. The toxicity evaluation of the 26 tested extracts based on the lethality of *A. salina* nauplii indicated that this toxicity assay is rapid and comprehensive, meeting the requirement for screening a large number of plant extracts in small amounts.

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