Original Article

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Evaluation and Comparison of the Toxicity of Some Iranian Native Plants and Microalgae, Using Brine Shrimp Test (BST)

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Abstract

Brine shrimp lethality test is a useful and appropriate method for toxicity identification of compounds in plants. Moreover, *Artemia* spp. are among the most common salt water organisms worldwide, which can be used in this kind of experiments. Many plants are being used for treatment of variable diseases and have many applications in pharmaceuticals and medicine. However, some of them have toxic components that limit their use and can create some risks for consumers. This study investigated the toxicity of two kinds of microalgae and three kinds of plants against larvae of *Artemia urmiana* (*A. urmiana*). This study aimed to evaluate the toxicity degree of active metabolites in some plants and microalgae against *A. urmiana* nauplii as a standard toxicity evaluating method. After 48 h of incubation, newly hatched *Artemia nauplii* were exposed to different concentrations of herbal extracts. Brine shrimp lethality test was used for investigation of samples toxicity in different doses between 100 to1000 µg/mL in time intervals. Results showed that these herbal extracts have high potential larvicidal properties on *A. urmiana*. Ephedra Intermedia had the maximum and *Dunaliella Salina* showed the minimum effects on mortality. The achieved results indicated that there were straight correlation between dose of extracts and mortality of brine shrimp nauplii. The potential toxicity of extracts was as follows:
E.intermedia>C.procera>O.persica>C.vulgaris>D.salina.

Keywords: Artemia Salina, Brine Shrimp, Lethality Assay, Toxicity Test.

1. Introduction

The use of plants for medicinal purposes is a worldwide perception (1). The World Health Organization (WHO) encourages many countries to increase their interestsin using medicinal plants and their products in treating various maladies (2). Some of these agents can help the human body in fighting against some microorganisms; others may lead to disease or might be represented as important sources of natural drugs. These plants that are found in our environment have ex-

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tensive acceptability among people and serve as inexpensive possibilities to orthodox medicine. However, many plants are known to be toxic (3). The brine shrimp cytotoxicity assay was considered as a suitable probe for the primary assessment of toxicity (3).

Brine shrimp lethality test (BSLT) is a simple, affordable, non-aseptic, and high potential cytotoxicity test for bioactive chemicals (4). This test has been suggested by Michael *et al*. (1956) (1, 5). *Artemia* is one of the aquatic crustaceans genuses known as brine shrimp, which is a genus in the Artemiidae family (3, 6). In this paper, we studied some plants (*Otostegia persica*, *Calotropis procera*, and *Ephedra intermedia*) and microalgae (*Chlorella vulgaris* and *Dunaliella salina*) toxicity. This study was performed according to the mortality of brine shrimp *Artemia urmiana* in some different concentrations of plants and microalgae extracts. Their applications were investigated in theliterature. *Otostegia persica* (*O.persica*) belongs to the *Lamiaceaeis* family, that grows in south of Iran. Akbarzadeh *et al*. (2012) have reported some applications for *O.persica*, such as antispasmodic, antihistamine, anti-diabetic, anti-arthritis properties (7). *Calotropis prosera* (*C. prosera*) is a plant that grows in south of Asia and Africa with antimicrobial, anti-inflammatory, anticancer, larvicidal and anti-fertility properties (8, 9). It is the member of Asclepiadaceae family (9). *Ephedra intermedia* (*E. intermedia*), a species of *Ephedra*, belongs to the Ephedraceae family, which is native to China, Pakistan, Afghanistan, Central Asia, Iran, Siberia, Mongolia, the western Himalayas, and Tibet (10). Nowadays, microalgae have many applications. They have the ability to grow in various conditions and are good candidates especially for fuel production (11). Many researches focused on the plants and microalgae that have high efficiency in medicine. In this study, we evaluated the toxicity of three kinds of plants and two kinds of microalgae on naupllii of *Artemia Urmiana* in different intervals as a standard and reliable test. The present research provides a full description to evaluate the degree of some Iranian native plants toxicity, which have been used as home remedies.

2. Materials and methods

2.1. Plant Materials

Collected plant parts (bark, leaves, branches, stipules, flowers, fruits, or whole plant) were documented by comparing with herbarium specimens. After identification, 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 the refrigerator before examination. The targeted plants (Table 1) used in this research were collected in Kazeroon, and Bushehr, two cities in Iran. The microalgae *C. vulgaris* was isolated from lakes and rice paddy fields of Sivand, in Fars province and *D. salina* was isolated from Maharlu Salt Lake, 27 kilometers southeast of Shiraz, Iran. Two kinds of microalgae were identified by 18s rRNA PCR method. Finally, both of them were cultivated in BG-11 and Johnsons medium for growth and analysis (12, 13).

2.2. Extraction of samples

For extraction of all samples, 5 g of freeze-dried powder of microalgae biomass and plants were macerated in methanol, propanol, chloroform, acetone, and a mixture of chloroform/methanol/acetone in ratio of 2.1.1 (500 mL) for 72 h. Then, the extraction of each sample was prepared by using rotary evaporator (TW-10, JAPAN) at the temperature of 45 ºC for 8 h.

Biomass of microalgae was collected by centrifugation (at 6000 rpm, 10 min). The supernatant was discarded and the biomass was stored at -21 ºC prior to analysis (14).

The freeze-drying process was performed by a laboratory-scale lyophilizer (GLD-136C-JAPAN). The temperature, chamber pressure, and operation time of the lyophilizer were -70 ºC, 1.33 Pa, and 48 h, respectively. The dried products were collected and stored in 50 mL falcon tubes.

2.3. Toxicity testing against the brine shrimp

2.3.1. Hatching brine shrimp cysts

The hatching process of brine shrimp cysts were done according to the method described by Wu (2014) and Moshi *et al*. (2010) with some modifications. After hatching, the active nauplii were collected with a plastic pipette for the

Table 1. Toxicity results of plants and microalgae using *Artemia urmiana* lethality test.

Samples	Extract	Percentage mortality (µg/mL)				Brine shrimp lethality (LC_{50}, µg/mL, 24h)
		100 µg/mL	300 µg/mL	500 µg/mL	1000 µg/mL	
Chlorella vulgaris	Methanol	23.25	32.25	58.75	100	490.6
	Acetone	25.75	24.25	44	100	608.8
	Chloroform	29.75	63.75	86.75	100	231.98
	Propoanol	24	28.5	45	75.25	551.2
	mixed of chloroform/ methanol/acetone	33.5	55.5	78	100	289.94
Dunaliella salina	Methanol	13.5	31	35.5	89	698.71
	Acetone	15.75	26.75	40.75	91	607.82
	Chloroform	30	45.25	55.5	100	481.7
	Propoanol	23.25	27.5	34.75	86	739.83
	mixed of chloroform/ methanol/acetone	26.5	39.25	57.25	100	496.95
Otostegia persica	Methanol	31.75	35.75	46.25	99	634.6
	Acetone	22	61	73.75	95	281.05
	Chloroform	57.5	78.25	85.25	100	50.9
	Propoanol	36.5	43.25	67.5	85.5	463.9
	mixed of chloroform/ methanol/acetone	41.75	62.25	88.75	100	235.4
Calotropis procera	Methanol	68.5	89	100	100	81.4.7
	Acetone	76	91.5	100	100	73.9
	Chloroform	33.5	62.75	100	100	205.8
	Propoanol	55.25	75.25	100	100	86.7
	mixed of chloroform/ methanol/acetone	87.75	99.5	100	100	39.7
Ephedra intermedia	Methanol	90.25	91	100	100	30.71
	Acetone	60.75	91.5	100	100	77.82
	Chloroform	86	62.75	100	100	33.98
	Propoanol	80	88.5	100	100	35.83
	mixed of chloroform/ methanol/acetone	83.25	99.5	100	100	33.65

study (4, 5).

2.3.2. Brine shrimp assay

All experiments were conducted in 12 well Cell Culture Plates. Each chamber was filled with 0.5 mL herbal extract with different concentrations (100-1000 µg/mL) and then 4.5 mL of the brine shrimp solution was added to each plate (5, 15). Fifteen newly hatched *Artemia urmiana* nauplii were added to each well. For each concentration of plant sample, one control group was designed, which was a combination of 0.5 mL of solvent (vehicle treated, solvent of extraction such as methanol, acetone, chloroform, propoanol, and mixed of chloroform/methanol/acetone) with 4.5 mL of brine shrimp solution sea water (34 g sea salt in 1 L deionized water) (4, 5, 15). This study was performed in four replicates for each concentration. The plates were kept in adark place at room temperature for 24 h. In some specific times, the mortality of nauplii was investigated using stereo microscope. Feeding and air was not required

during the study. In each well, the numbers of dead and surviving nauplii were counted and the LC_{50} was calculated. According to *Thangapandi veni calculations* (2, 5, 15), the toxicity properties of extracts were estimated based of the number of dead nauplii or the percentage of nauplii mortality obtained due the equation below (16):

Mortality %=(D test-D control/A control×100)

D test=the number of dead larvae in each test plate

A control=the number of live larvae on control plates

D control=the number of dead larvae in each control plate

All the experiment were done at quadruplicate (16).

2.4. Statistical analysis

LC_{50} values were determined by analyzing the data on a computer loaded with a "SPSS 16". The LC_{50} values for the brine shrimps were determined for each extract concentration (11, 17).

3. Results and discussion

The hatched nauplius of *A. urmiana* was demonstrated in Figure 1. Crude extracts of samples showed different results on mortality of brine shrimps (Table 1). Both polar and non polar solvents were used for extraction,. In extraction processes, different compounds were separated based on the polarity gradient of acetone, methanol, propanol, chloroform, and mixed of chloroform/methanol/acetone. For each sample, five extracts were tested at four concentrations (100, 300, 500 and 1000 µg/mL). LC_{50} was recorded in defined intervals in 24 h by Brine Shrimp Lethality Test (BSLT) (Table 1). According to the results, in high concentrations (1000 µg/mL), approximately all the shrimps were dead.

The *Artemia* species have been found to be useful and suitable for various toxicity evaluations for the identification of bioactive compounds in crude plant extracts (3, 16). BSLT is one of the useful and reliable and routine tests in the laboratory. This test has been used for toxicity screening of some plant extracts, pharmaceutical compounds, heavy metals, pesticides, and food additives (16, 18). Due to its simplicity, high sensitivity, and low costs, BSLT has been received great attention from many researchers (3, 16).

The natural mortalities, which were determined in blank sea water and in wells treated with positive control only, generally did not rise above 24%. This seemed to be a result of lacking oxygen since most of the shrimps did not survive during 48 h after assay (19). In this regard,factors like temperature, composition, and salinity of the medium and the age of the larvae are effective factors in natural mortality.

In this study, the maximum and minimum lethal concentrations were 1000 and 100 µg/mL, respectively. There was a direct correlation between number of deaths and plant concentrations. According to the BSLT results, the rates of extracts toxicity were as follow:

E. intermedia>C.procera>O.persica>C.vulgaris>D.salina.

Extractions using different polar and non-polar solvents, showed different results on mortal-

a

b

Figure 1. a: naplii of *Artemia urmiana*. b: hatching *Artemia* cyst.

ity percentage.

Based on the results, *E. intermedi* showed the most mortality effects compared to other herbs. *O. persica* and *C. procera* have potent antioxidant and free radical scavenging activities. Besides, *O. persica* is useful in treatment of diabetic diseases and exhibits antimicrobial activity against some pathogenic microorganisms (17, 20). However, in BSLT methods, the LC_{50} of these two extracts were between 500-1000 µg/mL, and they showed high mortality and cytotoxicity in comparison to *D. salina* extract. The weakest effect belonged to *D. Salina* extract. It is suggested that *D. salina* has the most compatibility with *A. urmiana* in comparison to other extracts. The LC_{50} of propanol extraction of *D. salina* was 739.83 µg/mL and 496.95 µg/mL for a mixture of three solvents. Next to the samples, Tween20 was used as a positive control with a 100% mortality effect on nauplii of *Artemia*.

D. salina showed significantly lower mortality compared to Tween20 according to their LC_{50} values ($p<0.05$). Conversely, *E. intermedia* showed different results ($p>0.05$). Other extracts such as *C. procera*, *O. persica*, and *C. vulgaris* showed an intermediate sensitivity in mortality of *Artemia urmiana* nauplii.

The toxicity of herbal extracts expressed as LC_{50} was evaluated by comparison to Meyer's or to Clarkson's toxicity index. According to these indexes, extracts with LC_{50} lower than 1000 µg/mL are known as toxic, while extracts with LC_{50} more than 1000 µg/mL are considered non-toxic (21). According to Clarkson's toxicity criteria, plant extracts are categorized in the following order: extracts with LC_{50} more than 1000 µg/mL are categorized as non-toxic, LC_{50} of 500-1000 µg/mL have low toxicity, extracts with LC_{50} of 100-500 µg/mL show medium toxicity, while extracts with LC_{50} of 0-100 µg/mL are highly toxic (21, 22). This significant lethality results of plant and microalgae extracts on brine shrimps can verify the presence of potent cytotoxic components, which require further investigation (23, 24). These compounds contain total phenols, flavonoids, coumarins, triterpenoids, and tannins, which exist in the plant extracts and are produced as secondary metabolites (16, 25).

5. Conclusion

In conclusion, root and stem parts of *E. intermedia* possess the most cytotoxic activity among 4 other extracts. According to this point, *E. intermedia* extract has the highest effect on mortality in BSLT. The maximum toxic effect of *E. intermedia* extract was recorded in 1000 µg/mL concentration. It shows that *E. intermedia* applications in dietary supplements, weight loss products, or hay fever treatments should be by caution. Because of this toxicity, FDA has banned the use of this herb on December 30, 2003 (26). *C. vulgaris* and *D. salina* have the minimum toxicity on BSLT, which justify their wide use in dietary supplements and foods.

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Conflict of Interest

None declared.

6. References

1. Carballo JL, Hernández-Inda ZL, Pérez P, García-Grávalos MD. A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. *BMC Biotechnol.* 2002;2:17.
2. Bucker A, Falcao-Bucker NC, Nunez CV, Pinheiro CCdS, Tadei WP. Evaluation of larvicidal activity and brine shrimp toxicity of rhizome extracts of *Zingiber zerumbet* (L.) Smith. *Rev Soc Bras Med Trop.* 2013;46:377-80.
3. Baravalia Y, Vaghasiya Y, Chanda S. Brine Shrimp cytotoxicity, anti-inflammatory and analgesic properties of Woodfordia fruticosa Kurz Flowers. *Iran J Pharm Res.* 2012;11:851.
4. Wu C. An important player in brine shrimp

lethality bioassay: The solvent. *J Adv Pharm Technol Res*. 2014;5:57.
5. Moshi M, Innocent E, Magadula J, Otieno D, Weisheit A, Mbabazi P, *et al.* Brine shrimp toxicity of some plants used as traditional medicines in Kagera region, north western Tanzania. *Tanzan J Health Res.* 2010;12:63-7.
6. Libralato G. The case of *Artemia* spp. in nanoecotoxicology. *Mar Environ Res.* 2014;101:38-43.
7. Akbarzadeh S, Bazzi P, Daneshi A, Nabipour I, Pourkhalili Kh MG, Sartavi K, *et al.* Antidiabetic effect of *Otostegia persica* extract on diabetic rats. *J Med Plant Res*. 2012;6:3176-80.
8. Kareem S, Akpan I, Ojo O. Antimicrobial activities of *Calotropis procera* on selected pathogenic microorganisms. *Afr J Biomed Res.* 2008;11:105-10.
9. Kumar V, Basu N. Anti-inflammatory activity of the latex of *Calotropis procera*. *J Ethnopharmacol.* 1994;44:123-5.
10. Yang Y. Ontogeny of triovulate cones of *Ephedra intermedia* and origin of the outer envelope ofovules of Ephedraceae. *Am J Bot.* 2004;91:361-8.
11. Zarei M, Mobasher MA, Morowvat MH, Mousavi P, Montazeri-Najafabady N, Hajighahramani N, Ghasemi Y . Effects of menthone and piperitone on growth, chlorophyll a and β-carotene production in *Dunaliella salina*. *J Appl Pharm Sci.* 2016;6:215-9.
12. Rasoul-Amini S, Ghasemi Y, Morowvat MH, Mohagheghzadeh A. PCR amplification of 18S rRNA, single cell protein production and fatty acid evaluation of some naturally isolated microalgae. *Food Chem.* 2009;116:129-36.
13. Rasoul-Amini S, Montazeri-Najafabady N, Mobasher MA, Hoseini-Alhashemi S, Ghasemi Y. *Chlorella* sp.: A new strain with highly saturated fatty acids for biodiesel production in bubble-column photobioreactor. *Appl Energy*. 2011;88:3354-6.
14. Rasoul-Amini S, Mousavi P, Montazeri-Najafabady N, Mobasher MA, Mousavi SB, Vosough F, *et al.* Biodiesel properties of native strain of *Dunaliella salina*. *Renew Energy*. 2014;4:39-41.
15. Tan DC, Flematti GR, Ghisalberti EL, Sivasithamparam K, Barbetti MJ. Toxigenicity of enniatins from Western Australian *Fusarium* species to brine shrimp (*Artemia franciscana*). *Toxicon*. 2011;57:817-25.
16. Mirzaei A, Mirzaei N, Ghavamizadeh M. Antioxidant Activity and Cytotoxicity of *Dorema aucheri* by *Artemia urmiana*: a Brine Shrimp Lethality Test. *Life Sci J.* 2013;10:8-12.
17. Brand-Williams W, Cuvelier M, Berset C. Use of a free radical method to evaluate antioxidant activity. *Food Sci Technol.* 1995;28:25-30.
18. Balali-Mood M, Saber H. Recent Advances in the Treatment of *Organo phosphorous* Poisonings. *Iran J Med Sci.* 2012;37:74-91.
19. Hartl M, Humpf H-U. Toxicity assessment of fumonisins using the brine shrimp (*Artemia salina*) bioassay. *Food chem toxicol.* 2000;38:1097-102.
20. Asghari G, Nourallahi H, Havaie S, Issa L. Antimicrobial activity of *Otostegia persica* Boiss. extracts. *Res Pharm Sci.* 2007;1:53-8.
21. Meyer B, Ferrigni N, Putnam J, Jacobsen L, Nichols Dj, McLaughlin J. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta med.* 1982;45:31-4.
22. Clarkson C, Maharaj VJ, Crouch NR, Grace OM, Pillay P, Matsabisa MG, *et al*. *In vitro* antiplasmodial activity of medicinal plants native to or naturalised in South Africa. *J Ethnopharmacol.* 2004;92:177-91.
23. Krishnaraju AV, Rao TV, Sundararaju D, Vanisree M, Tsay H-S, Subbaraju GV. Assessment of bioactivity of Indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. *Int J Eng Sci.* 2005;3:125-34.
24. Keshavarz M, Emamghoreishi M, Nekooeian AA, Warsh JJ, Zare HR. Increased bcl-2 protein levels in rat primary astrocyte culture following chronic lithium treatment. *Iran J Med Sci.* 2013;38:255-62.
25. Peteros NP, Uy MM. Antioxidant and cytotoxic activities and phytochemical screening of four Philippine medicinal plants .*J Med Plants Res.* 2010;4:407-14.
26. Mark B. McClellan TGT. Rules and Regulations. *Assess Health Technol Rep.* 2004;69:6788-854.