Comparison of the Artemia salina and Artemia uramiana bioassays for toxicity of 4 Iranian medicinal plants

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Abstract

For evaluation of 4 Iranian medicinal plant toxicity Brine shrimp lethality assays Artemia salina and Artemia uramiana bioassay were used. A. urmiana and A. salina eggs were purchased and kept in a hatching chamber that containing artificial sea water for preparation of nauplii. The active nauplii were collected for study after 48 h. The Plantago major, Artemisia maritime, Mentha piperita and Borago officinalis were prepared in Yasuj, Iran and individually extracted with methanol, hexane, and ethyl acetate by Soxhlet apparatus. The toxicity rate of extracts was estimated on the basis of the number of dead nauplii, or mortality rate by Artemia salina and Artemia uramiana. LC50 values with 95% confidence intervals were determined by the probit analysis. All extracts, except of B. officinalis displayed 100.0% mortality at 1000 µg/ml by A. urmiana and A. salina. Ethyl acetate extract was the most potent and presented the highest percentage of mortality with the lowest LC50 values by both assays too. After ethyl acetate, hexane extract showed the highest toxicity, however, The methanol extract exhibited the lowest mortality. According to A. urmiana and A. salina toxicity results, trend of the extracts P. major > A. maritima > M. piperita > B. officinalis was reported. There was a positive correlation between the results from A. urmiana and A. salina, for detecting plants toxicity with a Pearson correlation of R² = 0.989. According to present results A. uramiana assay was valuable for the screening of plant extracts to detect of toxicity.

Keywords: Artemia salina, Artemia uramiana, medicinal plant, toxicity, Plantago major.

Introduction

Man has used herbs as medicine since the Earlier Stone Age. Plants are rich in various bioactive compounds in the name of photochemical that include total phenols, flavonoids, alkaloids, steroids, terpenoids and tannins.

The over consumption of medicinal plants can lead to excessive accumulation of herbs in the body, which may cause toxicity. Recent studies indicate that a large number of people in industrial societies use medicinal herbs for their health requirements. Therefore, medicinal plants play a significant role in the healthcare system of a large percentage of the world’s population. Over 80% of the world’s people is dependent on the herbal traditional system for their major health care. Although plants have valuable effects, they can be toxic to humans. Therefore, numerous research studies have recently focused on the pharmacology and toxicity of medicinal plants that used by human. Recent studies indicate that although, numerous plants are used as food sources and some of them may have mutagenic or genotoxic potential.

For toxicity evaluation of medicinal plants in this study brine shrimp lethality test (BSLT) with two Artemia species was designated. Artemia salina (A. salina) and Artemia uramiana (A. uramiana). Artemia (Artemiidae) are a type of salt-water shrimp invertebrate, which are found in almost 500 salty lakes. A. uramiana is found in Urmia Lake, which is one of the largest natural environments of Artemia spp. and is the only source of A. uramiana in the world. Most plant toxicity studies and research have been carried out by A. salina, but this is the first plant toxicity study in A. uramiana system.

The BSLT is used for screening of chemical and natural products toxicity and isolation of active compounds in herbal extracts. The BSLT is also useful animal method for evaluating toxicology studies because, their eggs or cysts are abundant, follow a simple transformation from hatching into the larvae cycle, and are easily maintaining a population in laboratory environment. The BSLT is simple, portable, reliable, rapidly conducted and its results significantly correlate with the state of toxicity and therefore, BSLT is a reliable answer to routine requests for toxicity screening.

Among the herbal plants of the Iranian flora Plantago major, Artemisia maritima, Mentha piperita and Borago officinalis were collected from Yasuj, the Southern of Iran. These plants have potent pharmacological potentials and used broadly in the traditional medicine by the Iranian people.

The toxicological evaluation of medicinal plants performed routinely in mice which is costly and the animals suffering caused by these procedures. Therefore, there is recently a tendency to replace the utilize of laboratory animals in toxicological procedures. Therefor in this research BSLT instead of animal toxicological assay for plant toxicity was designated.
Plantago major L. (English name: larger plantain) is used as an anti-inflammatory, anthelmintic, antiviral, analgesic, antiasthmatic, antitumor, and hypotensive agent in traditional medicine\textsuperscript{13}. Artemisia maritima (English name: Worm seed) is a medicinal plant with potent antioxidant, anthelmintic, antimicrobial, febrifuge, and tonic activities\textsuperscript{2} . It contains total phenols and flavonoids\textsuperscript{14}. Mentha piperita (English name: peppermint) is a member of the Lamiaceae family and used for the treatment of headaches, coughs, congestion, and intestinal disorders. Its active compounds include flavonoids, tannins, caffeic acid, volatile oils, and carotenes\textsuperscript{15}. Borago officinalis L. (English name: borage) is a medicinally important plant that used as a diuretic, expectorant, bronchodilator, antispasmodic, and vasodilator. Its chemical compounds contain total phenols, alkaloids, tannins, and linolenic acid\textsuperscript{16,17}.

In this study the efficiency of A. urmiana for predicting the toxicity of plant extracts was evaluated by comparison with A. salina as the golden standard.

Material and Methods

Plant collection: Aerial plant parts were collected in 2010 in various areas of Yasuj, Iran. Botanical identification was conducted for each sample and the voucher samples were kept at the herbarium of the Medicinal Herb Research Center.

Plant extraction: The plants were dried in the shade and ground using a mill (Restsch Ultra Centrifugal Mill and Sieving Machine, Haan, Germany). The ground materials were individually extracted with methanol, hexane, and ethyl acetate by Soxhlet apparatus for 6 hours and filtered through Whatman No.1 filter paper. The extracts were collected and concentrated using a rotary evaporator (Heidolph Laborota, model 4000; Germany) and remained frozen prior to the study.

The hatching larvae: A. urmiana and A. salina eggs were purchased from the Artemia Research Center of Urmia, Iran. 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 (ASW), which was prepared by dissolving 30 g of sea salt in 1 L of distilled water at 27–29°C. Regular air flow with average pressure and proper light was supplied for 48 hours. The pH of the environment was adjusted to 9.0 to prevent the risk of death to the Artemia nauplii due to a drop in the pH during development\textsuperscript{18}. After hatching, the active nauplii were collected with a plastic pipette for study.

The brine shrimp lethality test: All 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 (10–1000 µg/mL) of dimethyl sulfoxide (DMSO), 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 hours, were added to each petri dish. For each concentration of plant sample, one control group was conducted which included 0.5 mL (vehicle treated, DMSO) with 4.5 mL of brine shrimp solution without extract.

This study was performed in 3 replicates for each concentration. The petri dishes were kept covered with their lids in the darkness at room temperature for 24 hours. Feeding and air were not required during the study. In each plate, the numbers of dead and surviving nauplii were counted and the LC\textsubscript{50} was calculated. Nauplii that did not show any movement within 10 seconds were defined as dead. In this bioassay experiment, Thymol was used as a positive control\textsuperscript{19}.

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:

\[ \% \text{mortality or death rate} = \frac{d \; \text{test} - d \; \text{control} \times 100}{A \; \text{control} \times 100} \]

in which \(d \; \text{test}\) = the average number of dead nauplii in the experimental groups, \(d \; \text{control}\) = the average number of dead nauplii in the control group, and \(A \; \text{control}\) = the average number of living nauplii in the control group.

Statistical analysis: The results are expressed as means. LC\textsubscript{50} values with 95% confidence intervals were determined by the probit analysis method. For A. urmiana and A. salina comparison t-student analysis was managed.

Results and Discussion

The hatched nauplius of Artemia urmiana. (A) and 48 growth of nauplius(B) was demonstrated in (figure 1). For each plant, 3 extracts were tested at 4 concentrations (10, 100, 500, and 1000 µg/mL). The LC\textsubscript{50} value and 95% confidence intervals were recorded for each extract concentration by the BSLT (tables 1 and 2). The LC\textsubscript{50} values of A. urmiana revealed the highest toxicity with the lowest LC\textsubscript{50} value of 236.4–977.5 and 290–1107.7 mg/mL concentration. The other extract concentrations demonstrated a 10–72% mortality.

Ethyl acetate extract was the most potent and showed the highest percentage of mortality with the lowest LC\textsubscript{50} values (187.6–373.5 and 216.5–545.6 for A. urmiana and A. salina, respectively). After ethyl acetate, hexane extract presented the highest toxicity and showed the lowest LC\textsubscript{50} values of 236.4–977.5 and 290–1107.7 mg/mL concentration. The methanol extract showed the lowest mortality with the highest LC\textsubscript{50} values (303.7–1027 and 321.6–1144.6 mg/mL for A. urmiana and A. salina, respectively) at all concentrations.

According to the BSLT results, the rate of extracts toxicity were as follow: P. major > A. maritima > M. piperita > B. officinalis by both assay systems.
In present study the average of LC$_{50}$ by A. urmiana in extracts 14% was less than A. salina, this mean that plant toxicity rate was more in A. urmiana when compared with A. salina. Therefore, no significant difference was reported in LC$_{50}$ of A. urmiana and A. salina in plant extracts (figure 2).

There was a positive correlation between the results of A. urmiana and A. salina for detecting toxic compounds in plant extracts with a Pearson correlation of $R^2 = 0.978$ (figure 3).

Approximately 50% of clinically used drugs are derived from plant products, which play a key role in the treatment of inflammation, cancer and infectious diseases. 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. In this study, it used A. urmiana for the first time to detect toxicity in 4 medicinal plants.

According to recent results, Artemia nauplii from the different populations A. urmiana and A. salina showed insignificantly different sensitivities to the same toxicant according to their LC$_{50}$ values $p > 0.05$. Plant extracts toxicity differed from each other ($p < 0.001$) in A. urmiana and A. salina systems which may be due to differences in the amounts and types of cytotoxic compounds. These compounds contain total phenols, flavonoids, coumarins, triterpenoids, and tannins, which exist in the plant extracts. In this study, there was a direct correlation between mortality rate and concentration level. The maximum and minimum mortality dose rates were 1000 and 10 µg/mL, respectively.

Compounds and extracts with LC$_{50}$ values less than 1000 µg/mL were considered toxic. Therefore most of the plant extracts in this study had biological potential or toxic properties.

The LC$_{50}$ values of A. salina ranged from 11 to 1000 µg/mL in Nigerian traditional medicinal plants and ranged from 15.35 to 374 µg/mL in Tanzanian traditional medicinal plants, suggesting the presence of cytotoxic compounds. However, the LC$_{50}$ values for ethanol extracts of several plants in the latter study were more than 1000 µg/mL. LC$_{50}$ values in Philippine medicinal plants, in A. salina ranged from 37.7 to 89.5 µg/mL. LC$_{50}$ values in the Euphorbia kamerunica Pax plant, for different concentrations and extracts ranged from 0 in a hexane extract to 13.87 in an aqueous extract. A low LC$_{50}$ indicates that the extract is highly lethal and that the plant extract may be contains potent cytotoxic compounds.

The results of the first study to evaluate food additive toxicity by A. urmiana was not comparable to Gold standard method. However, in present study, the results were comparable and parallel with A. salina for evaluation of toxicity. The results from the cytotoxic assays of the plants by A. urmiana and A. salina revealed that M. piperita and A. maritima had mild cytotoxic potential whereas B. officinalis did not have a cytotoxic effect.

The strongest cytotoxic extract in the present screening was the ethyl acetate extract of the P. major plant. The low LC$_{50}$ levels obtained from the different extracts of P. major is associated with its traditional medicinal antitumor, anthelmintic, antiviral, and antirheumatic uses. These low LC$_{50}$ values are related to toxic substances such as flavonoids, flavone, and luteolin, which are present in this plant.

P. major has demonstrated growth inhibitory and cytotoxic properties on different malignant tumors, acute myelocytic leukemia, and mammary adenocarcinoma. Furthermore, this plant has displayed antibacterial activity.

The Santonin drug is derived from the A. maritima plant and is useful for Ascaris lumbricoides treatment. The anti-helminthes activity suggests that a toxic substance is present in A. maritima, which is consistent with its low LC$_{50}$ in this study.

M. piperita has potent antioxidant and free radical scavenger activities; however, its oil had a modest toxic effect in the rat cerebellum. Water extracts of M. piperita are safe for use in cosmetic preparations. These observations are compatible with our research results.

B. officinalis is a medicinally important plant that has been used against prostate and liver cancer cells. Furthermore, this plant is useful for decreasing blood pressure, anti-inflammatory effects, and immunity control. This potential may be due to presence of total phenols linoleic acid, and alkaloids derivative.

B. officinalis was the safest plant according to the studies presented herein; however, it has reported anticancer activities. The differences in our results and those reported in the literature may be due to the type of extraction and the plant species. This is maybe a limitation of detecting plant properties by Artemia toxicity.

Our results suggest that the BLST is a useful screening system in medicinal plants for toxicity and may be discovering new bioactive compounds with various activities.

**Conclusion**

In this study, the brine shrimp toxicity results suggest that the extracts of the 4 medicinal plants do not have high toxicity compared to the Thymol standard with LC$_{50}$ of 7.2 µg/mL. The present data suggest that the ethyl acetate extract obtained from P. major was toxic with both system assays and can use for further study. Furthermore, a positive correlation was reported between A. salina and A. urmiana for detecting toxic compounds in plants. Thus, the A. urmiana assay is valuable for the screening of plant extracts to detect of toxicity.

**Acknowledgments**

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Table 1

<table>
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<tr>
<th>extracts</th>
<th>% mortality at different Concentration (µg/ml)</th>
<th>LC50 24h µg/ml</th>
<th>95% Confidence interval</th>
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Table 2

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Recently hatched nauplius of *Artemia uramiana*. (A) and 48 growth (B)

**Figure-1**

Comparison of *A. salina* and *A. urmiana* systems by LC\(_{50}\) of different plant extracts

Three Arabic numbers in X axis belong to each plant species (Plantago major (1-3), Artemisia maritime(4-6), Mentha piperata(7-9) and Borrago officianalis(10-12) and the first, the second and the third number of each plant species belong to Hexan, Ethyl acetate and Methanol extracts respectively.

**Figure-2**

Relationship between medium lethal concentration (LC\(_{50}\)) in *Artemia salina* and *Artemia uramiana* brine shrimp Lethality assay in different plant extracts

\[ y = 1.116x + 6.7156 \]

\[ R^2 = 0.9781 \]
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