



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

Mahsa Mirzaei and Ali Mirzaei

Medicinal Plant Research Center, Yasuj University of Medical Sciences, Yasuj, IRAN

Available online at: www.isca.in

Received 26th January 2013, revised 6th February 2013, accepted 24th February 2013

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 preped 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*. LC_{50} values with 95% confidence intervals were determined by the probit analysis. All extracts, exception of *B. officinalis* displayed 100.0% mortality at 1000 $\mu\text{g/ml}$ by *A. urmiana* and *A. salina*. Ethyl acetate extract was the most potent and presented the highest percentage of mortality with the lowest LC_{50} 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. maritime* > *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 $R^2 = 0.989$. 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^{1,2}.

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 population³. Over 80% of the world's people is dependent on the herbal traditional system for their major health care^{4,5}. 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 human⁶. Recent studies indicate that although, numerous plants are used as food sources and some of them may have mutagenic or genotoxic potential⁷.

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*). Artemias (Artemiidae) are a type of salt-water shrimp invertebrate, which are found in almost 500 salty lakes⁸. *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 world⁹. 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 extracts¹⁰. 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 environment¹¹. 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 screening¹².

Among the herbal plants of the Iranian flora *Plantago major*, *Artemisia maritime*, *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 procedures⁶. Therefore 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, antihistaminic, antitumor, and hypotensive agent in traditional medicine¹³. *Artemisia maritima* (English name: Worm seed) is a medicinal plant with potent antioxidant, anthelmintic, antiseptic, antimalarial, febrifuge, and tonic activities. It contains total phenols and flavonoids¹⁴. *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 carotenes¹⁵. *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^{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 development¹⁸. 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 LC₅₀ 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 control¹⁹.

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:

$$\% \text{ mortality or death rate} = (d \text{ test} - d \text{ control} / A \text{ control}) \times 100$$

in which d test = the average number of dead nauplii in the experimental groups, d control = the average number of dead nauplii in the control group, and A control = the average number of living nauplii in the control group.

Statistical analysis: The results are expressed as means. LC₅₀ 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 LC₅₀ value and 95% confidence intervals were recorded for each extract concentration by the BSLT (tables 1 and 2, Increased extract concentrations were associated with increased mortality rates.

The bioactivities of different extracts against *A. urmiana* and *A. salina* are shown in tables 1 and 2, respectively. All extracts showed 100.0% mortality at 1000 µg/mL concentration by *A. urmiana* and *A. salina* with the exception of *B. officinalis* (tables 1 and tables 2). 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 LC₅₀ 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 with LC₅₀ value of 236.4–977.5 and 290–1107.7 for *A. urmiana* and *A. salina*, respectively. The methanol extract showed the lowest mortality with the highest LC₅₀ values (303.7–1027 and 321.6–1144.6 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 LC₅₀ by *A. urmiana* in extracts 14% was less than *A. salina*, this mean that plant toxicity rate was more in *A. urmiana* when compare with *A. salina*. Therefore, no significant difference was reported in LC₅₀ 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 R² = 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^{20,21}. 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 LC₅₀ values p>.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 extracts²². 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 LC₅₀ values less than 1000 µg/mL were considered toxic²³. Therefore most of the plant extracts in this study had biological potential or toxic properties.

The LC₅₀ values of *A. salina* ranged from 11 to 1000 µg/mL in Nigerian traditional medicinal plants²⁴ and ranged from 15.35 to 374 µg/mL in Tanzanian traditional medicinal plants, suggesting the presence of cytotoxic compounds. However, the LC₅₀ values for ethanol extracts of several plants in the latter study were more than 1000 µg/mL^{24,25}. LC₅₀ values in Philippine medicinal plants, in *A. salina* ranged from 37.7 to 89.5 µg/mL²². LC₅₀ 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 LC₅₀ indicates that the extract is highly lethal and that the plant extract may be contains potent cytotoxic compounds²⁶.

The results of the first study to evaluate food additive toxicity by *A. urmiana* was not comparable to Gold standard method¹⁰ 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 LC₅₀ levels obtained from the different extracts of *P. major* is associated with its traditional medicinal antitumor, anthelmintic, antiviral, and antirheumatic uses²⁷. These low LC₅₀ values are related to toxic substances such as flavonoids, flavone, and luteolin, which are present in this plant¹³.

P. major has demonstrated growth inhibitory and cytotoxic properties on different malignant tumors, acute myelocytic leukemia, and mammary adenocarcinoma^{28,13}. Furthermore, this plant has displayed antibacterial activity²⁹.

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 LC₅₀ in this study³⁰.

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 results³¹.

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 derivative³².

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 LC₅₀ 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. uramiana* for detecting toxic compounds in plants. Thus, the *A. uramiana* assay is valuable for the screening of plant extracts to detect of toxicity.

Acknowledgments

We are grateful to the Biochemistry Department and the Iranian Medicinal Plant Research Center for making their facilities available for this investigation.

Table-1
Cytotoxic results of plant extracts by *Artemia uramiana* a Brime Shrimp Lethality Assay

extracts	% mortality at different Concentration (µg/ml) *				LC ₅₀ 24h µg/ml	95% Confidence interval
	10	100	500	1000		
<i>Plantago major</i>						
Hexan	20	45	72	100	234.4	182.3-286
Ethyl acetate	26	49	76	100	187.6	135.4-239.7
methanolic	21	32	63	100	303.7	251.6-355.7
<i>Artemisia maritima</i>						
Hexan	26	34	69	100	260.5	206.5-314.5
Ethyl acetate	32	48	65	100	207.9	154.4-261.5
methanolic	20	26	64	100	326.2	271.6-380.8
<i>Mentha piperata</i>						
Hexan	16	24	66	100	333.2	283.8-382.6
Ethyl acetate	23	34	71	100	268.3	220.6-316
methanolic	13	22	53	100	392.6	342.5-442.7
<i>Borrago officianalis</i>						
Hexan	11	28	31	38	977.5	829.2-1125.7
Ethyl acetate	19	32	48	100	373.5	258.5-488
methanolic	10	25	31	36	1027	868.6-1175

Table -2
Cytotoxic results of plant extracts by *artemia salina* a Brime Shrimp Lethality Assay

extracts	% mortality at different Concentration (µg/ml) *				LC ₅₀ 24h µg/ml	95% Confidence interval
	10	100	500	1000		
<i>Plantago major</i>						
Hexan	16	40	65	100	290	233.1-346.9
Ethyl acetate	19	45	71	100	262	204.5-320.8
methanolic	17	35	60	100	321.6	264.7-378.5
<i>Artemisia maritima</i>						
Hexan	28	30	72	100	262.7	211-314.5
Ethyl acetate	30	42	70	100	217.5	165.6-269.5
methanolic	24	20	60	100	343.7	290.9-396.6
<i>Mentha piperata</i>						
Hexan	20	19	61	100	353.7	303.2-404.1
Ethyl acetate	20	31	65	100	301.2	251.6-350.7
methanolic	15	20	55	100	388 ±	337-439
<i>Borrago officianalis</i>						
Hexan	10	25	31	35	1107.7	922.5-1293.8
Ethyl acetate	23	30	45	83	545.6	415.8-675.4
methanolic	7	20	40	30	1144.6	954.9-1334.6

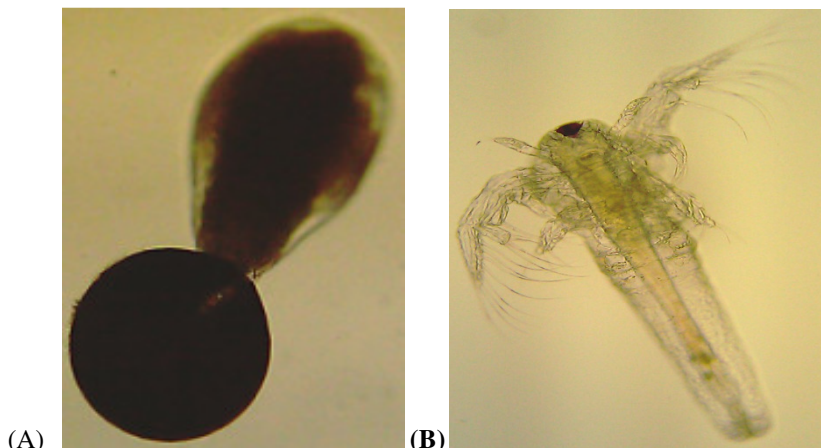


Figure-1

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

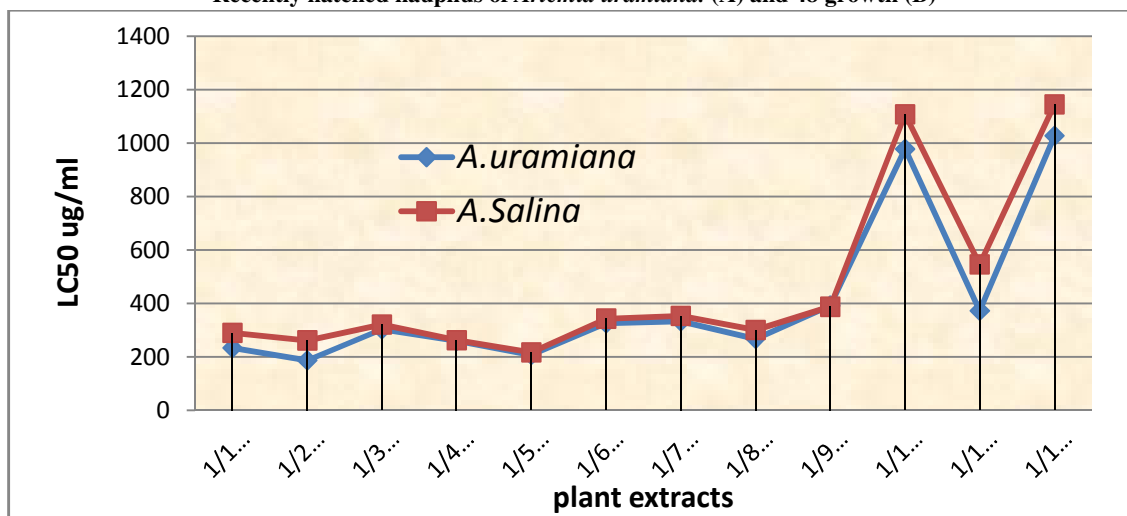


Figure-2

Comparison of *A. salina* and *A. urmiana* systems by LC₅₀ 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.

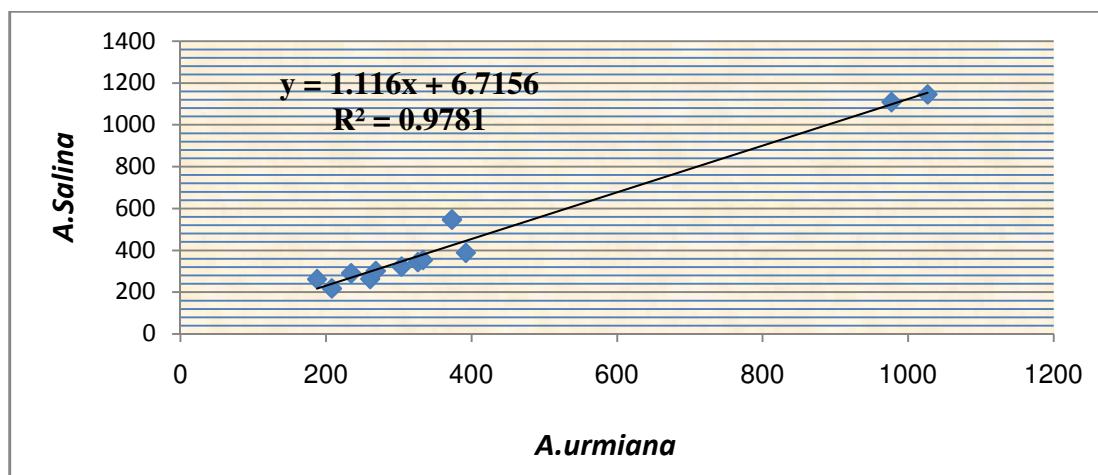


Figure-3

Relationship between medium lethal concentration (LC₅₀) in *Artemia salina* and *Artemia urmiana* brime shrimp Lethality assay in different plant extracts

References

1. Sirappuselvi S. and Chitra M. In vitro Antioxidant Activity of *Cassia tora* Lin, *Inter.Res. J. of Biolo. Sciences*, **1(6)**, 57-61 (2012)
2. Rosangkima G. and Prasad S.B., Antitumour activity of some plants from Meghalaya and Mizoram against murine ascites Dolton's lymphoma, *Indian J. Exp. Biol.*, **42**, 981-988 (2004)
3. Aweng E.R.I, Nur Hanisah1, Mohd Nawi M.A.I, Nurhanan Murni Y. and Shamsul M. Antioxidant Activity and Phenolic Compounds of *Vitex Trifolia* Var, *Simplicifolia* Associated with Anticancer, *ISCA J. of Biolo. Sciences*, **1(3)**, 65-68 (2012)
4. Madhuri S. and Pandey G., Some dietary agricultural plants with anticancer properties, *Plant Arch.*, **8**, 13-16 (2008)
5. Sivalokanathan S., Ilayaraja and M. and Balasubramaniam M.P., Efficacy of *Terminalia arjuna* (Roxb.) on N-nitrosodiethylamine induced hepatocellular carcinoma in rats, *Indian J. Exp. Biol.*, **43**, 264-267 (2005)
6. Lagarto Parra R. Silva Yhebra I. Guerra S. and Iglesias Buena L., Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD50 value) in mice, to determine oral acute toxicity of plant extracts, *Phytomedicine*, **8(5)**, 395-400 (2001)
7. Tülay A.Ç. and Özlem S.A., Cytotoxic and genotoxic effects of *Lavandula stoechas* aqueous extracts, *Biologia.*, **3**, 292-296 (2007)
8. Sorgeloos P., Lavens P., Lege P.H., Tackaert W. and Versichele D., Manual for the culture and use of the brine shrimp *Artemia* in aquaculture, Faculty of Agriculture, State University of Gent: Gent, Belgium, 319 (1986)
9. Agh N. and Sorgeloos P., INCO-DEV project on *Artemia* biodiversity international workshop. Urmia, Iran.(2004)
10. Sadighara P., Salar-Amoli J., Asadinejad S.H. and Zadehashem E., Using *Artemia urmiana* in detecting of the cytotoxicity of some selected food additives, *Biharean. Biologist*, **4(1)**, 81-82 (2010)
11. Anubha S.K., Brine shrimp *Artemia salina* -a marine animal for simple and rapid biological assays, *J. of Chinese Clinical Medicine*, **4(2)**, 1-4 (2007)
12. Abdullahi S.K. And lawal G.H., Antibacterial Screening and Brine Shrimp (*Artemia salina*) Toxicity of *Securidaca longepedunculata* (Polygalaceae) Root Bark, *African J. of Pharmaceutical Sciences and Pharmacy*, **1(1)**, 85-95 (2010)
13. Kobeasy M.I., Abdel-Fatah O.M., Abd El-Salam S.M., Zahrat M.M. and Ola E.I., Biochemical studies on *Plantago major* L. and *Cyamopsis tetragonoloba* L, *International Journal of Biodiversity and Conservation*, **3(3)**, 83-91 (2011)
14. Sushil C.S., Nitin S., Rawat U. and Sati O.P., Medicinal Plants as a Source of Antioxidants, *Research J of Phytochem.*, **4**, 213-224 (2010)
15. Rajinder S., Muftah A. and Shushni M., Antibacterial and antioxidant activities of *Mentha piperita* L. *Arabian J. of Chem.*, **1**, 744-752 (2011)
16. Naghdi Badi H. and Sorooshzadeh A., Evaluating potential of borage (*Borago officinalis* L.) in bioremediation of saline soil, *Afr. J. of Biotechnol.*, **10(2)**, 146-153 (2011)
17. Mayank G. and Swati S., *borago officinalis* linn. An Important Medicinal Plant Of Mediterranean Region, *Int. J. of Pharmaceutical Sci.Rev. and Res.*, **5(1)**, 27-34 (2010)
18. Amara A.A. and El-Masry M.H., Plant crude extracts could be the solution: extracts showing in vivo antitumorigenic activity, *Pak. J. Pharm. Sci.*, **21(2)**, 159-171 (2008)
19. Sam T.W., Toxicity testing using the brine shrimp: *Artemia salina*, In: Colegate, S.M., and Molyneux, R.J. (Ed), *Bioactive Natural Products Detection, Isolation, and Structural Determination*, CRC Press, Boca Raton FL., 442-456 (1993)
20. Koehn F.E. and Carter G.T., The evolving role of natural products drug discovery, *Nat. Rev.*, **4**, 206-22 (2005)
21. Fabricant D.S. and Farnsworth N.R., The Value of Plants Used in Traditional Medicine for Drug Discovery, *Environ. Health Perspect.*, **109**, 69-75 (2001)
22. Nonita P.P. and Mylene M., Antioxidant and cytotoxic activities and phytochemical screening of four Philippine medicinal plants, *J. of Medicinal Plants Research*, **4(5)**, 407-414 (2010)
23. Meyer B.N., Ferrigni N.R., Putnam J.E., Jacobsen L.B., Nichols D.E. and Mc Laughlin J.L., Brine shrimp: a convenient general bioassay for active plant constituents, *Planta Medica.*, **45**, 31-4 (1982)
24. Oyewale A.O., Audul O.T., Ayo R.G. and Amupitan J.O., Cytotoxic correlation of some traditional medicinal plants using brine shrimp lethality Test, *Chemclass J.*, **1**, 110-112 (2004)
25. Moshi M.J., Innocent E., Masimba P.J., Otieno D.F., Weisheit A.P. and Mbabazip P., Antimicrobial and brine shrimp toxicity of some plants used in traditional medicine in Bukoba District, north-western Tanzania), *Tanzan J. Health Res.*, **11(1)**, 23-8 (2009)
26. Ogunnusi T.A. and Dosumu O.O., Bioactivity of crude extracts of *Euphorbia kamerunica* Pax using brine shrimp (*Artemia salina*) lethality assay, *J. of Medicinal Plants Research*, **2(12)**, 370-373 (2008)
27. Franca F., Lago E.L. and Marsden P.D., Plants used in the treatment of leishmanial ulcers due to *Leishmania (Viannia) braziliensis* in an endemic area of Bahia, Brazil, *Rev. Soc. Bras. Med. Trop.*, **29**, 229-232 (1996)
28. Gálvez M., Martín-Cordero C., López-Lazaro M., Cortes F. and Ayusa M.J., Cytotoxic effect of *Plantago* spp. on cancer cell lines, *J. Ethnopharm.*, **88**, 125-130 (2003)
29. Gomez F.R., Calderon C.L., Scheibel L.W., Tamez-Guerra P., Rodriguez-Padilla C. and Tamez- Guerra R., Immunoenhancing properties of *plantago major* leaf extract, *Phytother. Res.*, **14**, 617-622 (2000)
30. Daniell S.F., Norman R.F., Fabricant D.S. and Farnsworth N.R., The Value of Plants Used in Traditional Medicine for Drug Discovery, *Environ. Health Perspect.*, **109**, 69-75 (2001)
31. Nair B., Final report on the safety assessment of *Mentha piperita* (Peppermint) Oil, *Mentha piperita* (Peppermint) Leaf Extract, *Mentha piperita* (Peppermint) Leaf, and *Mentha piperita* (Peppermint) Leaf Water, *Int. J. Toxicol.*, **20**, 61-73 (2001)
32. Lin L.T., Liu L.T., Chiang L.C. and Lin C.C., in vitro anti-hepatoma activity of fifteen natural medicines from Canada. *phytother. res.*, **16**, 440-4 (2002)