Abstract: This is to determine whether Artemia franciscana can be used to test toxicity of plants extract. Brine shrimp lethality assay was performed with aqueous, methanol, hexane and chloroform extracts of 16 medicinal plants frequently used in the Siddha and Ayurveda systems of traditional medicine. Hexane extracts of these plants were most toxic to the Brine Shrimp nauplii, followed by methanol extract, chloroform extract and aqueous extract regarding the lethal dose LC$_{50}$ of Artemia. All these extracts gave positive results in the brine shrimp lethality test and the shrimp mortality increased with the concentration of extract in the test samples. The LC$_{50}$ values of Andrographis paniculata, Andrographis lanceolata, Adhatoda vasica, Acalypha indica, Phyllanthus amarus, Vigna triglobata, Indigofera tinctoria, Leucas aspera, Ocimum basilicum. Evolvulus alsinoides were greater than 1000µg/ml for Artemia franciscana and A. salina. The LC$_{50}$ values of Annona squamosa, Centella asiatica, Gymnema sylvestre, Tylophora indica and Ricinus communis were between 500µg and 1000µg/ml while those of Vinca rosea and Ricinus communis were less than 500µg/ml but not less than 100µg/ml. Therefore, all these plants can be used in the preparation of medicines without fear of acute toxicity about these medicinal plants. The correlation coefficient between A. franciscana and A. salina was only ±0.02 so that both species may be employed in the brine shrimp lethality test for plant extracts. This investigation recommends that Artemia franciscana found in Kanyakumari district is suitable animal for the screening of phytochemicals in plants for certain physiological activities and pharmacological potentials.

Keywords: Artemia franciscana, brine shrimp lethality, plant extract, LC$_{50}$, mortality.

1. Introduction

In recent years, there has been an alternative trend to use lower animals as substitutes for laboratory animals such as mice and rats in toxicological tests because of their high cost and sufferings from the impact of the tests. These alternative methods replace the experiments that use large laboratory animals, reduce the number of animals used in the tests and refine the existing techniques for reducing pain and stress, which are the three main goals of Animal Welfare and Ethics (Gadir, 2012). The brine shrimp Artemia is one of the simple marine organisms that can be bred easily under laboratory conditions (Michael, et al., 1956) and used to test the toxicity conditions since it has effective range of tolerance in most toxicity testing (Hossain, et al., 2009).

The brine shrimp, Artemia is a crustacean closely related to shrimp belonging to the family Artemiidae of the phylum Arthropoda. Adult Artemia measures the average length of 8mm, but it can reach lengths up to 20mm under optimum environmental conditions and sufficient nutritional supply. The male Artemia possesses a paired penis in the posterior part of its trunk and a pair of claspers at the anterior end while the female Artemia has brood pouch in the posterior part of the trunk, but no claspers. This is the rule in all the sexually reproducing species of Artemia, but in parthenogenetic species all individuals are female and resemble the female Artemia of sexual species. Based upon a complex pattern of microspeciation, A. franciscana , A. monica, A. persimilis, A. funicina, A. salina and A. urmiana are sexual species of Artemia found in different brackish water areas of the world, and all the strains of parthenogenetic species are together called A. parthenogenetica (Abreu-Grobois and Beardmore, 1980).

The hatching rate of Artemia cysts after exposure to pesticides, petroleum products, carcinogens and other contaminants has been used to determine the toxicity level in samples (Anubha, 2007). Artemia Lethality Assay has also been applied to screen the toxicity of plant extracts (Meyer et al., 1982; McLaughlin et al., 1998a; Moshi et al., 2010; Ogugu et al., 2012; Gadir, 2012; Solanki and Selvanayagam, 2013; Sharma et al., 2013); toxicity of heavy metals (Sleet and Brendel, 1985; Martínez et al., 1999) and metal ions (Kokkali et al., 2011); toxicity of cyanobacteria (Jaki et al., 1999) and algae (Mayorga et al., 2010), cytotoxicity of dental materials (Pelka et al., 2000), toxicity of nanoparticles (Maurer- Jones et al., 2013), and screening of some natural products (Carballo et al., 2002). Although all stages in the lifecycle of Artemia are suitable for toxicity testing, nauplii after 48 hours of hatching are suitable for bioassay (Novakova et al., 2008). Lethal concentration of toxic substances for 50% mortality of Artemia after 6 hours of exposure is considered to be the lethal dose (acute LD$_{50}$) and this concentration is calculated for the total fluid content of humans to determine the LD$_{50}$ value of the substance for humans (Novakova et al., 2008).

Andrographis paniculata (Acanthaceae) is astrigent, anodyne and alexipharmic, used in the treatment of cholera, diabetes, influenza, bronchitis, itches, piles, jaundice, and helminthes infestations (Farnsworth and Soejarto, 1991). More like this, Andrographis lanceolata is also employed in the treatment of cholera, diabetes, influenza, bronchitis, itches, piles, jaundice, and helminthes infestations. Adhatoda vasica (Acanthaceae) is rich in the active principle vascine, which is useful in the treatment of bronchitis, diarrhea, hemorrhage, dysentery and glandular tumors (Kamala...
Ambasta, 1992). *Acalypha indica* (Euphorbiaceae) is a laxative containing a cytoprotective glucoside and alkaloidal acalypnine, which is used in the ailment of cough, gastrointestinal irritations, bleeding and skin troubles of different kinds (Farnsworth and Soejarto, 1991). *Annona squamosa* (Annonaceae) is an abortifacient and insect repellent to expel mosquitoes. *Centella asiatica* (Apiceae) is a diuretic herb that is used in the treatment of leprosy, jaundice, low memory, poor health and staminia, which are mainly due to the presence of the glucoside asiatoside. *Evoulvulus alsinoides* (Convolvulaceae) is a good febrifuge and vermifuge included in many medicinal preparations being used to cure human diseases. *Gymnema sylvestre* (Asclepiadaceae) contains the alkaloid gymnemic which is the active principle curing diabetes, dysentery and stomach disorders. *Indigofera tinctoria* (Fabaceae) is used in the treatment of epilepsy, nervous disorders, sores, ulcers, urinary complaints, hepatitis and breeding. *Leucas aspera* (Lamiaceae) is given for psoriasis, skin eruptions, cough and colds (Kamala Ambasta, 1992). *Ocimum basilicum* (Lamiaceae) is found to have stomachic, alexipharmic, antipyreptic, diaphoretic, expectorant, carminative, stimulant and anthelmintic properties, for which it is used in medicinal preparations for ringworms, sinuses, cough and respiratory problems. *Phyllanthus amarus* (Euphorbiaceae) has astringent, febrifuge, stomachic, diuretic and febrifuge properties, for which it is employed in the treatment of diarrhea, dysentery, colic, dropsy, sores, jaundice and diseases of urinary-genital system. *Ricinus communis* (Euphorbiaceae) contains ricinoleic acid that has medicinal value in the treatment of boils and sores. *Tylophora indica* (Asclepiadaceae) is stimulant, emetic, cathartic, expectorant, stomachic and diaphoretic and hence useful in asthma, bronchitis, whooping cough, dysentery, diarrhea, rheumatism and gouty pains; leaves of this plant contain the alkaloids tylophorine and tylophorine. *Vigna trilobata* (Fabaceae) is used in irregular fever (Kamala Ambasta, 1992). *Vinca rosea* has carminative, vomitive, hemostatic, depurative, hypotensive, astringent and diuretic properties, for which it is used in the treatment of hypertension, diarrhea, dysentery and phthisis, which are mainly due to vincamine alkaloid (Kamala Ambasta, 1992).

*Artemia salina* has been the potential animals for such assays but *Artemia franciscana* has only been used in the toxicity testing of radiations and some pollutants in the water bodies. The present study attempts to use *Artemia franciscana* to determine whether it is suitable to test the toxicity of Indian plants as *A. salina* can be used.

2. Materials and Methods

Leaves of test plants were collected from the local areas of Kanyakumari district of Tamilnadu during December 2017 and washed with clean water to remove dirt. The clean leaves were dried under shade at room temperature. The dried leaves were ground into a fine powder, the active principle of which was extracted from 25g powder using 200ml of water, chloroform, ethyl alcohol and hexane separately. The extract was filtered and the filtrate was concentrated under vacuum. 20mg of each extract was dissolved in 10 ml of pure dimethyl sulfoxide (DMSO) to get stock solutions of 2mg/ml. Experiments were conducted along with control (DMSO) and different concentrations (10µg, 100µg, 1000µg, 2000µg, 3000µg, 4000µg, 5000µg, 6000µg, and 7000µg/ml of artificial seawater medium) of the test substances.

Cysts of *Artemia franciscana* were hatched in a conical flask (1L) filled with sterile artificial seawater prepared using sea salt 38 g/L (pH 8.5) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each test tubes containing 5ml of test solutions. The tubes were maintained at room temperature for 24 hours under the light. The experiment was done in triplicate. The test tubes were inspected using a magnifying glass against a black background and the number of survived nauplii in each tube was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration and the observed mortality percentage was plotted against the concentration in a graph from which the median lethal concentration (LC50) was estimated. The percentage lethality was calculated from the mean survival larvae of extracts treated tubes and control. LC50 values were obtained by best-fit line method.

3. Results and Discussion

Hexane extracts of these plants were most toxic to the Brine Shrimp nauplii, followed by methanol extract, chloroform extract and aqueous extract regarding the lethal dose LC50 of *Artemia* (Table 1 and 2). In the LC50 bioassay, it is established that LC50 values >1000µg/ml are non toxic, that LC50 values ≥ 500 ≤ 1000 µg/ml are weakly toxic, and that LC50 values < 500 are toxic (Deciga-Campos et al. 2007; Bastos et al., 2009). Aqueous extracts of *Andrographis paniculata*, *Andrographis lanceolata*, *Adhatoda vasica*, *Acalypha indica*, *Phyllanthus amarus*, *Vigna trilobata*, *Indigofera tinctoria*, *Leucas aspera*, *Ocimum basilicum*. *Evoulvulus alsinoides* had the LC50 values greater than 1000µg/ml for *Artemia franciscana* and *A. salina*. On the other hand, extracts of *Annona squamosa*, *Centella asiatica*, *Gymnema sylvestre*, *Tylophora indica* and *Ricinus communis* had LC50 values between 500µg and 1000µg/ml, which imply that these were weakly toxic to both *Artemia salina* and *A. franciscana*. Extracts of *Vinca rosea* and *Ricinus communis* had LC50 values less than 500µg/ml for both these experimental animals and hence they are toxic materials which have to be used in low quantity in medicinal preparations. Methanol extracts of *Andrographis paniculata*, *Andrographis lanceolata*, *Adhatoda vasica*, *Acalypha indica*, *Phyllanthus amarus*, *Vigna trilobata*, *Indigofera tinctoria*, *Leucas aspera*, *Ocimum basilicum*. *Evoulvulus alsinoides* had the LC50 values greater than 1000µg/ml for *Artemia franciscana* and *A. salina*. Methanol extracts of *Annona squamosa* and *Centella asiatica* had LC50 values between 500µg and 1000µg/ml, which imply that these were weakly toxic to both *Artemia salina* and *A. franciscana*. Extracts of *Vinca rosea*, *Gymnema sylvestre*, *Tylophora indica* and *Ricinus communis* and *Ricinus communis* had LC50 values less than 500µg/ml for both these experimental animals.
Chloroform extracts of Andrographis paniculata, Andrographis lanceolata, Adhatoda vasica, Acalypha indica, Phyllanthus amarus, Vigna trilobata, Indigofera tinctoria, Leucas aspera, Ocimum basilicum, Evolvulus alsinoides had the LC50 values greater than 1000µg/ml for Artemia franciscana and A. salina; extracts of Annona squamosa and Centella asiatica had LC50 values between 500µg and 1000µg/ml; extracts of Vinca rosea, Gymnema sylvestre, Tylophora indica and Ricinus communis had LC50 values less than 500µg/ml for both these experimental animals. Hexane extracts of Andrographis paniculata, Andrographis lanceolata, Adhatoda vasica, Acalypha indica, Phyllanthus amarus, Vigna trilobata, Indigofera tinctoria, Leucas aspera, Ocimum basilicum, Evolvulus alsinoides had the LC50 values greater than 1000µg/ml for Artemia franciscana and A. salina; extracts of Annona squamosa and Centella asiatica had LC50 values between 500µg and 1000µg/ml; the extracts of Vinca rosea, Gymnema sylvestre, Tylophora indica and Ricinus communis had LC50 values less than 500µg/ml for both these experimental animals.

Table 1: Concentration of plant extract (µg) leading to 50% mortality of Artemia franciscana

<table>
<thead>
<tr>
<th>Plant source</th>
<th>Concentration of plant extract (µg) causing 50% mortality of Artemia franciscana (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Aqueous extract</td>
</tr>
<tr>
<td>Andrographis paniculata</td>
<td>3620</td>
</tr>
<tr>
<td>Andrographis lanceolata</td>
<td>3710</td>
</tr>
<tr>
<td>Adhatoda vasica</td>
<td>5101</td>
</tr>
<tr>
<td>Annona squamosa</td>
<td>827</td>
</tr>
<tr>
<td>Centella asiatica</td>
<td>828</td>
</tr>
<tr>
<td>Vinca rosea</td>
<td>325</td>
</tr>
<tr>
<td>Gymnema sylvestre</td>
<td>820</td>
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<tr>
<td>Tylophora indica</td>
<td>460</td>
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<tr>
<td>Ricinus communis</td>
<td>487</td>
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<tr>
<td>Acalypha indica</td>
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<tr>
<td>Phyllanthus amarus</td>
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</tr>
<tr>
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<tr>
<td>Indigofera tinctoria</td>
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</tr>
<tr>
<td>Leucas aspera</td>
<td>2100</td>
</tr>
<tr>
<td>Ocimum basilicum</td>
<td>3100</td>
</tr>
<tr>
<td>Evolvulus alsinoides</td>
<td>5354</td>
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</tbody>
</table>

The lethality of a test sample to the shrimp (Artemia salina) was utilized by Meyer et al. (1982) to demonstrate the toxicity of substances in the test samples, which has been a helpful tool to screen a wide range of chemical compounds for their bioactivities. This bioassay correlates reasonably well with cytotoxic and other biological properties (Mclaughlin et al., 1991). The brine shrimp bioassay has been established as a safe, practical and economic method for determination of bioactivities of synthetic compound (Almeida et al., 2002) as well as plant products (Meyer et al., 1982). In toxicity evaluation of plant extracts by Brine shrimp lethality bioassay LC50 values lower than 1000 µg/ml are considered bioactive (Meyer et al., 1982). Therefore, extracts of A. squamosa, C. asiatica, G. sylvestre and T. indica were found to have active principles which have medicinal values. Extracts of R. communis and V. rosea had some toxic principles that kill Artemia. Further, this bioassay also indicates antifungal effects, pesticidal effects, teratogenic effects, and toxicity to environment (Vanhaecke et al., 1981). The mortality rate due to the extracts was found to be directly proportional to the concentration of the extracts (Hossain et al., 2009).

The shrimp mortality was due to the presence of certain secondary metabolites in the plant extracts (Dhar et al., 1973; Badami et al., 2003; Krishnaraju et al., 2005). The secondary metabolites are alkaloids, glycosides, lignin derivatives, saponins, tannins, anthraquinones flavonoids, phenolic and iridoids (Shankar et al., 2009; Yadav et al., 2008; Tiwari et al., 2008; David et al., 2012). The brine shrimp lethality was maximum if the LC50 value was less than 100µg/ml. No one plant chosen for this test showed LC50 >100µg/ml. The high toxicity of methanolic extract of leaf probably attributed to the alkaloid that is confirmed in phytochemical screening.

The correlation coefficients (r) between the bioassay using Artemia franciscana and A. salina were not different significantly (p>0.05). The r values were +0.02 for Andrographis paniculata and Andrographis lanceolata, +0.03 for Adhatoda vasica, Gymnema sylvestre and Ricinus communis -0.02 for Acalypha indica and Phyllanthus amarus, +0.01 for Vigna trilobata, Indigofera tinctoria, Leucas aspera, Ocimum basilicum and Evolvulus alsinoides and -0.01 for Annona squamosa and Centella asiatica. The correlation coefficients (r) between these two species of

Table 2: Concentration of plant extract (µg) leading to 50% mortality of Artemia salina

<table>
<thead>
<tr>
<th>Plant source</th>
<th>Concentration of plant extract (µg) causing 50% mortality of Artemia salina (µg/ml)</th>
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<td>Adhatoda vasica</td>
<td>5210</td>
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<tr>
<td>Annona squamosa</td>
<td>815</td>
</tr>
</tbody>
</table>
**Artemia** were only within the range of 0.01 to 0.03, which implies that both these species can be used in the brine shrimp lethality assay of plant extracts. Works of Carbello et al. (2002), Veni and Pushpanathan (2004), Gosh et al. (2015), and Quazi Sahely Sarah et al. (2017) also agree with the present finding that both *A. franciscana* and *A. salina* behave differently in the brine shrimp lethality assays but the difference was so little as to show only minor difference in the results because of the genomic difference among the species. Zapata et al. (1990) and Agh et al. (2008) also concluded that the heterogeneity of *Artemia* species is a critical factor in determining the survival even in the same salinity condition of a natural habitat. Since *A. franciscana* and *A. salina* were quite different depending upon their difference in genetic composition, the genetic effect in controlling survival rate may be the reasons for difference in the tolerance limit of *Artemia* species (Michael Babu, 1999; Immanuel et al., 2002).

### 4. Conclusion

From these results it is clear that, more like *A. salina* being used in the brine shrimp lethality assay, *A. franciscana* can be used to test the toxicity of plant extracts, and that the lethality of shrimp is due to cytotoxicity of the active principles in the plant extracts. The LC50 value between 100µg and 1000µg showed the presence of bioactive compounds which serve as active principles in the medicinal plants and hence this test would be a useful tool to screen a wide range of chemical compounds for their various bioactivities. This assay has been utilized to screen physiologically active plant extracts having some cytotoxic and other biological properties. This work further confirms that brine shrimp bioassay is a safe, practical and economical method for determination of bioactivities of plant –based medicinal formulations. All these plants may be used in the preparation of medicines without fear of acute toxicity about these medicinal plants because of their LC50 values above 100µg/ml. If this assay is standardized as it should be, it may be adopted to test the toxicity of all the plants in the world.

### References


zinc levels in the brine shrimp Artemia parthenogenetica. Aquaculture. 172, 315-325.


