Toxicological evaluation of the plant products using Brine Shrimp (*Artemia salina* L.) model

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Abstract

Many natural products could serve as the starting point in the development of modern medicines because of their numerous biological and pharmacological activities. However, some of them are known to carry toxicological properties as well. In order to achieve a safe treatment with plant products, numerous research studies have recently been focused on both pharmacology and toxicity of medicinal plants. Moreover, these studies employed efforts for alternative biological assays. Brine Shrimp Lethality Assay is the most convenient system for monitoring biological activities of various plant species. This method is very useful for preliminary assessment of toxicity of the plant extracts. Rapidness, simplicity and low requirements are several advantages of this assay. However, several conditions need to be completed, especially in the means of standardized experimental conditions (temperature, pH of the medium, salinity, aeration and light). The toxicity of herbal extracts using this assay has been determined in a concentration range of 10, 100 and 1000 µg/ml of the examined herbal extract. Most toxicity studies which use the Brine Shrimp Lethality Assay determine the toxicity after 24 hours of exposure to the tested sample. The median lethal concentration (LC_{50}) of the test samples is obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration. LC_{50} values are estimated using a probit regression analysis and compared with either Meyer's or Clarkson's toxicity criteria. Furthermore, the positive correlation between Meyer's toxicity scale for *Artemia salina* and Gosselin, Smith and Hodge's toxicity scale for higher animal models confirmed that the Brine Shrimp Lethality Assay is an excellent predictive tool for the toxic potential of plant extracts in humans.

Keywords: brine shrimp lethality assay; toxicity testing; plant extracts; probit analysis; LC_{50}

Introduction

The search for new drugs which are plant-derived has been receiving renewed interest among researchers throughout the world in view of discovering new drugs that possess potency to combat the menace of drug resistant pathogenic microorganisms, antitumor and anticancer agents (Mirzaei and Mirzaei, 2013; Santos Pimenta et al., 2003).

Plants can be useful either in their crude or advanced forms, offering a source of drugs in their pure state (Farnsworth and Soejarto, 2009). According to the World Health Organization's questionnaire, it is announced that 70-80% of the population in the world are relying on unconventional medicine, mainly in plant sources, in the primary health protection (WHO, 2007). Recognized for their ability to produce a wealth of secondary metabolites, many of these natural products have been shown to present interesting biological and pharmacological activities, which could serve as the starting point in the development of modern medicines (Abubakar et al., 2010)**.**

Well-known drugs which were developed from plant species are Vinblastine and Vincristine (first cures in human cancer) from *Catharanthus roseus*, Quinine (anti-malarial agent) from *Cinchona* species, Scopolamine (sedative) from *Datura metel* L., and many others which remained in use until present day (Farnsworth and Soejarto, 2009).

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Although many plants have valuable properties, some of them are known to carry toxicological properties as well. Recent studies indicate that although numerous plants are used as food sources, some of them may have mutagenic or genotoxic potential (Tülay and Özlem, 2007). Numerous research studies have recently focused on both pharmacology and toxicity of medicinal plants used by humans. This is of high importance in order to achieve a safe treatment with plant products (Parra et al., 2001).

The toxicity of the plants may originate from different contaminants or from plant chemical compounds that are part of the plant. Various assays are used for the research of potential toxicity of herbal extracts based on different biological models, such as *in vivo* assays on laboratory animals. However, recent studies employed efforts for alternative biological assays that include species of *Artemia salina, Artemia franciscana, Artemia urmiana* and *Thamnocephalus platyurus*. These toxicity tests are considered a useful tool for preliminary assessment of toxicity (Carballo et al., 2002; Veni and Pushpanathan, 2014; Mayorga et al., 2010).

During the past 30 years, the Brine Shrimp Assay has been widely used to test the toxicity of a great variety of plant products. Brine shrimp (*A. salina*) is most extensively studied of the *Artemia* species, estimated to represent over 90% of the studies in which *Artemia* is used as an experimental test organism (Campbell et al., 1994). The Brine Shrimp Toxicity Assay was proposed and developed by Michael et al. (1956) and later adapted by Vanhaecke et al. (1981), Meyer et al., (1982), and Sleet and Brendel (1983).

Brine Shrimp Lethality Assay (BSLA) has been applied as an alternative bioassay technique 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), as well as screening of marine natural products (Carballo et al., 2002).

The technique is economic and utilizes small amount of test material (Pisutthanan et al., 2004). Since its introduction, this in vivo test has been successively employed for bioassay-guide fractionation of active cytotoxic and antitumor agents (Ahmed et al., 2010; Ramachandran et al., 2011). Additionally, several studies demonstrated that there is a good correlation between the results for the lethal concentration that kills 50% of the exposed population (LC_{50}) obtained with the Brine Shrimp Lethality Assay using *A. salina* and the results of the Acute Oral Toxicity Assay in Mice (Parra et al., 2001; Arlsanyolu and Erdemgil, 2006).

Plant collection, storage and preparation of herbal extracts

Collected plant materials (root, bark, branches, stipules, leaves, flowers, fruits or whole plant) were authenticated by comparing with herbarium specimens. After identification, they were washed and dried before examination (Ogugu et al., 2012; Lalisan et al., 2014), either air-dried or dried in an air stove to higher temperature (~40 °C) (Ahmed et al., 2010; Parra et al., 2001; Olowa and Nuñeza, 2013). All were chopped in a grinder mill and stored in desiccators at room temperature (Parra et al., 2001; Veni and Pushpanathan, 2014). For antimicrobial tests, the material was pulverized in sterile electric blender (Lalisan et al., 2014).

The powdered plant sample was soaked in one solvent or mixture of solvents for a longer period of time, either by using laboratory extraction flasks or Soxhlet extractor (Veni and Pushpanathan, 2014), followed by filtration and evaporation, concentrated to dryness under reduced pressure using rotary evaporator (Mayorga et al., 2010). Several other extraction techniques are repercolation with multiple extractions, maceration of the plant material or decoction of a fresh plant material over a period of time (Parra et al., 2001). Depending on the nature of the plant material, some steps of the extraction process were optional (for example, the Soxhlet extraction of plant samples has been employed in order to extract substances of low and medium volatility as well as thermally stable constituents based on polarity gradient of the solvent) (Veni and Pushpanathan, 2014).

Choosing the type of solvent for the extraction process is vital for the toxicity testing, because different solvents show different extracting potential. If possible, extraction should be carried out under mild conditions utilizing solvents of low reactivity (Ghisalberti, 1993). Methanol, 96% ethanol, n-hexane, ethyl acetate, petroleum ether, carbon tetrachloride, dichloromethane and acetone are the most commonly used extracting solvents for this purpose (Ahmed et al., 2010; Ogugu et al., 2012).

In case of air or freeze-dried samples, a polar solvent such as ethyl acetate or methanol is preferable. Alcoholic solvents rupture cell membranes and extract greater amount of endocellular materials, and in this case the separation of pure compounds is left for a later chromatographic separation (Arlsanyolu and Erdemgil, 2006; Ghisalberti, 1993).

If a mixture of solvents is a more suitable option for extraction of some compounds, then a good system with high extraction potential is a 1:1 mixture of two solvents, but the ratio highly depends on the preferred polarity of the mixture (Pimentel Montanher et al., 2002). In case of fresh plant materials, dichloromethane-methanol solvent mixture is ideal for extracting purposes. This is usually followed by re-extraction using less polar solvents in order to separate the lipophilic fraction from the water-soluble fraction (Ghisalberti, 1993).

Studies have shown that there is a significant difference in the obtained LC_{50} results for different solvents' extracts, mainly because some solvents are a poor medium for obtaining specific bioactive components (responsible for the toxicity) from the plant sample, than others (Lalisan et al., 2014).

Dimethylsulfoxide is widely used solvent for the reconstitution of evaporated plant extracts, because brine shrimp nauplii show no significant sensitivity to this solvent up to 11% concentration (Ahmed et al., 2013; Musa, 2012; Kamba and Hassan, 2010). Preparation of stock solution and several dilutions (suggested by McLaughlin, 1991) provide different dose levels, which gives opportunity to determine the linear increase of the toxicity by increasing the concentration of the toxic compound(s) present in the plant extract.

Advantages vs disadvantages of the Brine Shrimp Assay

Brine Shrimp Lethality Assay is a convenient system for monitoring biological activities of various plant species. Although this method does not provide any adequate information regarding the mechanism of toxic action, it is a very useful method for the assessment of the toxic potential of various plant extracts (Gadir, 2012; Naidu et al., 2014). This method provides preliminary screening data that can be backed up by more specific bioassays once the active compounds have been isolated (Pisutthanan et al., 2004).

This test has several advantages which can be summarized as follows:

- Rapidness (the monitoring could be extended to maximum 60 hours, but in most cases, relevant data for the LC_{50} calculations are obtain after 24 hours of exposure),
- Simplicity (no special training needed for the equipment and the assay),
- Low requirements (no aseptic techniques, no special equipment, relatively small amount of test sample is needed and a large number of test organisms of exactly the same age and physiological condition can be easily obtained to start the test),
- Robustness (the cysts are commercially and readily available so that the tests can be carried out worldwide with the same original material and without any problem of provisioning),
- Inexpensiveness (the quantity of cysts required per test is very small so that the price of the biological material is negligible),
- High degrees of repeatability – according to ARC – test (Artemia Reference Centre) (Vanhaecke et al., 1981; Meyer et al., 1982; McLaughlin and Rogers, 1998b; Vanhaecke and Persoone, 1984).

Apart from the great number of advantages this method has, several criteria need to be completed in order to achieve a broad applicability of the Brine Shrimp Assay. This includes the following:

- Standardized experimental conditions in the means of temperature, salinity, aeration, light and pH,
- Same geographical region of the cysts,
- Same age of *Artemia* nauplii at the start of every test,
- Positive and negative controls are essential part of the assay, in order to check the sensitivity of the larvae and the conformity with the standard procedure (Vanhaecke et al., 1981).

Hatching of *Artemia* cysts

The container used for brine shrimp hatching, consists of two unequal chambers with several holes on the divider in between (Fig.1). This will enable the hatched *Artemia* nauplii (Fig.2) to migrate from the hatching compartment into the illuminated compartment (Sharma et al., 2013; Pisutthanan et al., 2004). Both chambers of the container are filled with artificial sea water (3.8%) (Gadir, 2012; Ogugu et al., 2012). In some Brine Shrimp Assays, the seawater might contain other salts such as $MgCl_2 \cdot 6H_2O$, Na_2SO_4, $CaCl_2 \cdot 2H_2O$ or $CaCl_2 \cdot 6H_2O$ (Parra et al., 2001). After filtration through a 1 μm filter under vacuum, the artificial sea water is ready for hatching of the *Artemia* cysts (Vanhaecke et al., 1981).

The container needs to be spacious enough to contain an air pump. Supplying a regular air flow with average pressure and proper light is essential for the hatching process (Veni and Pushpanathan, 2014; Mirzaei and Mirzaei, 2013). This is important because the artificial seawater should be at least 90% saturated with oxygen for a successful hatching (Vanhaecke et al., 1981). In addition, equal distribution of the oxygen in the seawater is achieved by a constant aeration and pressure in the solution for 48 hours in order to reach *Artemia* nauplii mature state.

Small quantities of dry cysts of *Artemia salina* were sprinkled into the dark, larger chamber. Yeast solution 0.06% was added to the hatching chamber for every liter of salt water to feed the larvae after 24 hours, otherwise the *Artemia* larvae would die during the III or IV instar stage (Parra et al., 2001; Sorgeloos et al., 1978). The molting into the further larval stages is dependent on temperature (37 °C) as well as quality and quantity of food (Lalisan et al., 2014). After 36 hours of incubation at room temperature (22-29 °C), the active nauplii free from egg shells were attracted by the light source into the smaller chamber (Sharma et al., 2013; Pisutthanan et al., 2004). It is important that nauplii from the same generation are applied for each concentration of the tested compound (Ogugu et al., 2012).

Fig. 1   Container used for hatching *Artemia* nauplii (Pellosi et al., 2013).

Fig. 2 *Artemia salina* nauplius (by Juan Camilo Jaramillo).

Influence of the experimental conditions over the sensitivity of *Artemia* nauplii

The pH value of the seawater is a very important factor for the hatching of the *Artemia* eggs. Optimal pH range is 8.0 ± 0.5. If necessary, the pH should be adjusted using NaOH or Na_2CO_3, to avoid lethality of the *Artemia* larvae caused by decrease of pH during incubation (Vanhaecke et al., 1981; Parra et al., 2001).

Temperature variations affect the hatching process of *Artemia* cysts. Warmer medium temperature enables faster hatching of the cysts (Table 1).

It has been shown that 50% of the cysts hatch within 30 hours at 20 °C, while at 24 °C this percentage is already obtained after 21 hours. With regard to the molting rate,

it appears that at 24 °C more than 60% of the larvae molt into the II instar stage 16 hours after hatching, whereas at 20 °C it takes 35 hours to arrive at the same stage (Sorgeloos et al., 1978).

The light stimulus influences the hatching of *Artemia* cysts significantly. Sorgeloos explained how the embryological development of hydrated embryos that are not stimulated by light, can be delayed until the light trigger is applied. Apparently, the cysts started hatching after being exposed to light, which gave statistically significant differences between the dark and the light series (Sorgeloos, 1973).

Additionally, Vanhaecke et al. (1981) indicated that the most sensitive age for the majority of the tested compounds were the 48 hour-old nauplii at the stage of instar II-III.

Table 1. Percentage of hatched and molted cysts depending on the temperature of the medium*

Temperature of the medium	Percentage of hatched cysts	Hatching time	Developmental stage reached
20 °C	50%	30 hours	I (first) stage
24 °C	50%	21 hours	I (first) stage

Temperature of the medium	Percentage of molted larvae	Hatching time + molting time	Developmental stage reached
20 °C	~60%	30 hours+35 hours	II (second) stage
24 °C	>60%	21 hours+16 hours	II (second) stage

*Based on the results in the research paper of Sorgeloos et al. (1978)

Furthermore, the larvae of I instar stage only consume their yolk as a food source and they are more resistant to positive control (chromic acid) due to their poorly developed epithelium of the digestive tract that disables the normal absorption of nutrients and toxic compounds from the external medium (Sorgeloos et al., 1978; Vanhacke and Persoone, 1984; Sleet and Brendel, 1985; Kokkali et al., 2011).

Therefore, even under given constant experimental temperature, salinity, light, pressure and pH conditions, the sensitivity of *Artemia* nauplii in different instar developmental stages differs markedly among different strains from different geographical regions.

Preparation of the Brine Shrimp Assay

The Brine Shrimp Lethality Assay has been developed for toxicity testing of various concentrations of pure compounds and crude plant extracts. Firstly, pure compounds and crude plant extracts were dissolved in a solvent suitable for the tested herbal sample. However, a mixture of several solvents may be a more suitable solvent system for some plant samples.

By dissolving the plant extract in a convenient solvent, a stock solution is made, which can be used for serial dilutions to prepare different concentrations. This is useful in the means of toxicity testing, because it is of high importance to determine the concentration range in which there is a linear correlation between the concentration and the lethality of the brine shrimps. Most experiments that involve the Brine Shrimp Lethality Assay for toxicity assessment of herbal extracts include a concentration range of 10, 100 and 1000 µg/ml (Parra et al., 2001).

The results for the toxicity of tested herbal preparations gained by using crude plant extracts were more accurate than by testing pure compounds isolated from the same plant. This difference in the toxicity results is probably due to the chemical complexity of the crude or partially purified extract, which seemed to be essential for the bioavailability of the active constituents of the examined plant. On the other hand, isolated pure compounds seemed to lose this specific bioactivity (Mayorga et al., 2010).

Prepared crude plant extracts in various concentrations can be tested by applying certain volume of the extract in vials containing brine solution or by applying the tested volume of extract on filter paper discs which are placed in vials afterwards (Meyer et al., 1982). Each vial contains the tested crude plant extract, artificial sea water and 10-15 brine shrimp nauplii. Ten nauplii were selected and transferred into each sample vial, and the final volume in each vial is adjusted with artificial seawater (Gadir, 2012). If there is no possibility to apply exact number of brine shrimps, all remaining alive nauplii are immobilized and the calculations could be made by counting the total number of dead shrimps in each vial (Pisutthanan et al., 2004; Ghisalberti, 1993).

Adding dry yeast suspension as food for the brine shrimps is optional (Gadir, 2012), because feeding the brine shrimps during the assay is insignificant when it comes to determining toxicity.

The negative control enables elimination of other factors that contribute to the total number of dead nauplii. The solvent used to dissolve the crude plant extracts is a relevant negative control for this purpose.

The toxicity of tested plant samples was determined by comparing their LC_{50} values with highly toxic substances suitable to be used as positive controls for this test, such as: vincristine sulphate (Ahmed et al., 2010), potassium dichromate (Naidu et al., 2014), thymol (Sharma et al., 2013; Mirzaei and Mirzaei, 2013), cyclophosphamide (Moshi et al., 2010), pure DMSO (Arlsanyolu and Erdemgil, 2006), caffeine (Gadir, 2012) etc.

Data analysis

Depending on the conditions of the experiment, survivors have been counted by magnifying glass or a microscope after certain period of time (6, 12, 24, 36, 48, 54 and/

or 60 hours), although most toxicity studies which use the Brine Shrimp Assay determined the toxicity by counting the survived nauplii after 24 hours of exposure to the tested sample. Usually, no deaths were observed to occur in the negative control after 24 hours.

After 24 hours of exposure, the median lethal concentration (LC_{50}) of the test samples has been obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration (Meyer et al., 1982; Ahmed et al., 2010; Moshi et al., 2010). LC_{50} values were estimated using a probit regression analysis (Finney, 1971). Finney's statistical method (Table 2) was incorporated in several software packages such as Stata, MatLab, R and IBM SPSS, which enable computerized calculations of LC_{50} with confidence intervals (Fig.3). These calculations may not give the exact lethal concentration of the examined compound or extract that kills 50% of the population, but without doubt it represents a significant prelimi-

nary data for further toxicity testing assays. In most studies, the experiment has been conducted in triplicate, so that gained results were statistically reproducible (Sharma et al., 2013; Pisutthanan et al., 2004; Parra et al., 2001).

Toxicity testing criteria

The toxicity of herbal extracts expressed as LC_{50} values is commonly valorized either by comparison to Meyer's or to Clarkson's toxicity index.

According to Meyer's toxicity index, extracts with $LC_{50} < 1000$ µg/ml are considered as toxic, while extracts with $LC_{50} > 1000$ µg/ml are considered as non-toxic (Meyer et al., 1982).

Clarkson's toxicity criterion for the toxicity assessment of plant extracts classifies extracts in the following order: extracts with LC_{50} above 1000 µg/ml are non-toxic, LC_{50} of

Table 2. Finney's table for transformation of percentage of mortality to probit values (Finney, 1952).

| %	0	1	2	3	4	5	6	7	8	9
0	__	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.25	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
__	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09
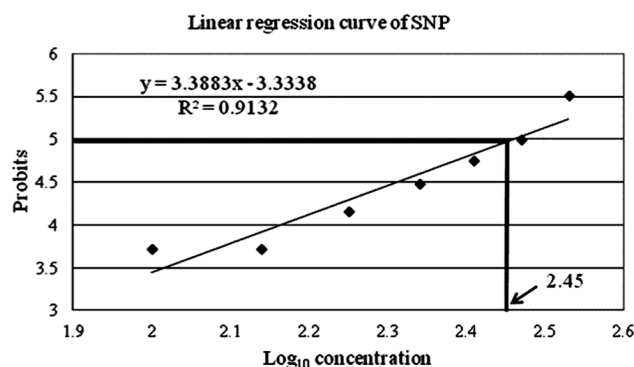
Fig. 3   Obtaining the logarithmic value of the concentration by interpolation from the linear correlation between probits and log(c) (Saha et al., 2014).

500 - 1000 µg/ml are low toxic, extracts with LC_{50} of 100 - 500 µg/ml are medium toxic, while extracts with LC_{50} of 0 - 100 µg/ml are highly toxic (Clarkson et al., 2004).

Correlation between Brine Shrimp Assay and other animal models

Due to the ethical issues in toxicological tests, substituting animals with alternative models is very important. The effectiveness of the *Artemia salina* bioassay for predicting the toxicity of plant extracts was evaluated by comparing the LC_{50} results for the brine shrimps with the LD_{50} results for acute toxicity in rats and mice (Sharma et al., 2013; Naidu et al., 2014; Parra et al., 2001).

Syahmi et al. have shown that LC_{50} values obtained by Brine Shrimp Lethality Assay are in good correlation with LD_{50} values obtained by examination of the acute toxicity of *E. guineensis* methanolic extracts using laboratory Swiss albino mice (Syahmi *et al*, 2010). Furthermore, using Sprague Dawley rats showed that LD_{50} values for the acute toxicity examined by oral route of *Mentha spicata* methanolic extract are significantly correlated with the LC_{50} results obtained by the Brine Shrimp Lethality Assay (Naidu et al., 2014). The LD_{50} values for the mice and rats models were above 5000 mg/kg, and the LC_{50} values for the brine shrimps were above 1000 µg/ml, indicating that these extracts are non-toxic.

It has been shown that a good correlation exists between the LC_{50} results obtained by force feeding female Sprague Dawley rats with a feeding needle of aqueous extracts of various plant species and the LC_{50} results obtained by 24 hour exposure of brine shrimps to those aqueous extracts. The LC_{50} results for the brine shrimps were above 5 mg/ml,

Table 3. LC_{50} versus LD_{50} values in Brine Shrimp Lethality Assay and *in vivo* study - Swiss albino mice; toxicity classification according to Meyer's and Gosselin, Smith and Hodge's criteria

Plant extract	LC_{50} (Mg/ml) obtained by Brine Shrimp Lethality Assay	LD_{50} (mg/kg) obtained by using Swiss albino mice as experimental models	Toxicity class
Aloe vera (L.) Burm.	3.59	120.65	Toxic/Very toxic
Artemisia absinthium L.	15.74	2499.10	Toxic/Moderately toxic
Citrus aurantium L.	3.99	476.94	Toxic/Very toxic
Cymbopogon citratus (DC. Ex Nees) Stapf	9.83	460.00	Toxic/Very toxic
Datura stramonium L.	12.86	821.93	Toxic/Moderately toxic
Justicia pectoralis Jacq.	60.14	3531.11	Toxic/Moderately toxic
Musa x paradisiaca L.	15.10	383.97	Toxic/Very toxic
Ocimum basilicum L.	9.92	956.50	Toxic/Moderately toxic
Ocimum gratissimum L.	18.76	2081.00	Toxic/Moderately toxic
Ocimum tenuiflorum L.	18.75	1540.00	Toxic/Moderately toxic
Orthosiphon aristatus (Blume) Miq.	16.72	5026.31	Toxic/Slightly toxic
Pimenta dioica (L.) Merr.	32.78	2560.00	Toxic/Moderately toxic
Piper auritum Kunth	26.67	1802.00	Toxic/Moderately toxic
Plantago major L.	4.74	182.54	Toxic/Very toxic
Plectranthus amboinicus (Lour.) Spreng.	52.29	4902.92	Toxic/Moderately toxic
Plectranthus amboinicus (Lour.) Spreng. Aqueous extract	82.27	8193.00	Toxic/Slightly toxic
Ruta graveolens L.	5.39	219.45	Toxic/Very toxic
Senna alata (L.) Roxb.	7.74	1459.32	Toxic/Moderately toxic
Stachytarpheta jamaicensis (L.) Valh	14.51	2035.12	Toxic/Moderately toxic
Thuja occidentalis L.	11.94	440.00	Toxic/Very toxic

*according to Meyer's toxicity index for BSLA (Meyer et al., 1982)
**according to Gosselin, Smith and Hodge scale (Gosselin et al., 1984)

and the results obtained for the acute oral toxicity in laboratory rats also indicated no evidence of toxicity. The results have shown that in both the brine shrimp and the laboratory animals there were no signs of acute toxicity (Shafii et al., 2011).

The cytotoxic effect of the bioactive compound Quassin present in different fractions of *Quassia amara* has been studied using Brine Shrimp Lethality Assay and experimental rats. From the examined fractions of this plant, the methanol fraction has shown the least toxic activity in brine shrimps, as well as in experimental rats which all survived after dosage with single limit test dose of 2000 mg/kg and 5000 mg/kg (Obembe et al., 2014).

A cytotoxicity study using Brine Shrimp Lethality Assay and Acute Oral Toxicity in Mice for the toxicity testing of *Swietenia mahagoni* (Linn.) seed methanol extract has shown that the plant is nontoxic. The LC_{50} results obtained for this plant extract using the Brine Shrimp Lethality Assay correspond to LD_{50} values between 2500 and 8000 mg/kg indicating that according to the Brine shrimp Lethality Assay it is nontoxic. The correlation between the LC_{50} results obtained by the BSLA and the LD_{50} results obtained in mice has been confirmed with the obtained LD_{50} values for mice above 5000 mg/kg, meaning the plant is nontoxic for mice as well (Sahgal et al., 2010).

Parra et al. 2001 examined the toxicity effects of 20 plant extracts from different species using the Brine Shrimp Lethality Assay (BSLA) and *in vivo* studies with rats (Table 3).

The positive correlation between LC_{50} results obtained by the BSLA and LD_{50} results for *in vivo* animal models is confirmed by comparing the toxicity class of each plant extract according to Meyer's versus Gosselin, Smith and Hodge's toxicity scales (Table 3). The predicted oral LD_{50} dose for humans in Gosselin, Smith and Hodge's toxicity scale is based on the oral LD_{50} doses for rats as follows: less than 5 mg/kg is considered super toxic, 5-50 mg/kg is extremely toxic, 50-500 mg/kg is very toxic, 500-5000 mg/kg is moderately toxic, 5000-15000 mg/kg is slightly toxic and above 15000 mg/kg is considered practically non-toxic (Gosselin et al., 1984).

This comparison confirmed that the Brine Shrimp Lethality Assay is an adequate method for preliminary toxicity testing in humans by using the brine shrimps as alternative model for the mice and rats *in vivo* models.

Conclusion

Many plant species possess pharmacologically active constituents which contribute to their wide use in folk medicine and in the design of drugs. On the other hand, many plants which have been described as being curative can also be associated with harmful effects. The concentration of a substance is the most important determinant of the outcome: if it reaches a sufficiently high concentration in the susceptible biological system, it could lead to toxic effects.

In order to gain relevant data which can be extrapolated to human population, it was of high need that *in vivo* testing using animal models was involved. For years, researchers have been using rats and other animal models that show a high correlation with the human population. Recently, this type of testing has been limited because of ethical and economic aspects. Therefore, alternative toxicity assays are widely used for the testing of the toxicity potential of plant products. Brine Shrimp Lethality Assay seemed like an appropriate solution, especially because it could still classify as *in vivo* testing. *Artemia salina* nauplii is one of the alternatives for the biological toxicity assays of herbal extracts, and this test turned out to be significantly correlated with several other animal models. The preliminary toxicity data obtained by conducting the Brine Shrimp Lethality Assay gives LC_{50} values which are a convenient platform for further toxicity studies.

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Резиме

Токсиколошка евалуација на растителни производи со примена на модел со солени ракчиња (*Artemia salina* L.)

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Клучни зборови: тест за леталност на солени ракчиња, тестирање на токсичност, растителни екстракти, пробит анализа, LC_{50}

Природните производи наоѓаат голема примена во развојот на нови лекови, поради бројните биолошки и фармаколошки својства кои ги поседуваат. Од друга страна, тие може да поседуваат и токсиколошки својства. Затоа, во терапијата со овие производи, податоци за безбедност се обезбедуваат преку бројни студии чија што главна цел на истражување се однесува на испитување на фармаколошките и на токсиколошките карактеристики. Овие студии истовремено се насочени и кон пронаоѓање на алтернативни биолошки тестови, од кои Тестот за леталност со солени ракчиња (Brine Shrimp Lethality Assay) се покажал како најсоодветен за оваа цел.

Овој метод дава можност за прелиминарна проценка на токсичноста на растителните екстракти, така што има многубројни предности, од кои што брзината, едноставноста и минималните услови за изведување на тестот се само дел од нив. Сепак, за да биде соодветна неговата примена во токсиколошки цели, потребна е стандардизација на експерименталните услови (температура, pH и концентрација на сол на медиумот, аерација и светлина).

Одредувањето на токсичноста на хербални екстракти со примена на овој метод е во концентрациски опсег од 10, 100 и 1000 µg/ml. Смртноста на ракчињата се определува најчесто после 24 часа експозиција, преку пресметување на средната летална концентрација (LC_{50}). Средната летална концентрација (LC_{50}) на испитуваните примероци се пресметува преку графички приказ на зависноста меѓу процентот на мртви ракчиња наспроти логаритамските вредности од соодветните концентрации на испитуваниот примерок и примена на пробит регресиона анализа. Потоа овие вредности се споредуваат со Мејер-овиот или Кларксон-овиот критериум за токсичност. Позитивната корелација помеѓу Мејеровата скала за токсичност на *A. salina* и Госелин, Смит, Хоџовата скала за токсичност за виши животински модели потврдува дека Тестот за леталност со солени ракчиња претставува одлична алатка за предвидување на токсичниот потенцијал на растителните екстракти кај луѓето.