

## Artigo

## Influence of Solubility of Ethanol Extracts in *Artemia salina* Tests

dos Santos, H. D.; de Oliveira, F. F.; de Oliveira, R. A.\*

Rev. Virtual Quim., 2017, 9 (4), 1535-1545. Data de publicação na Web: 20 de julho de 2017

<http://rvq.s bq.org.br>

### Influência da Solubilidade de Extratos Etanólicos em Testes com *Artemia salina*

**Resumo:** O teste com o microcrustáceo *Artemia salina* é amplamente utilizado por pesquisadores de produtos naturais como um método fácil e rápido de triagem biológica de produtos naturais, incluindo extratos brutos. Valores de Concentração letal mediana ( $CL_{50}$ ), obtidos a partir da correlação entre concentração de extrato e quantidades de *A. salina* mortas,  $\leq 1.000 \mu\text{g mL}^{-1}$  é considerada como indicativo de possível atividade biológica. Nesse método, extratos vegetais devem ser dissolvidos em água salina. No entanto, a completa dissolução da amostra a ser testada dificilmente é alcançada. Assim, o fator solubilidade representa um forte parâmetro de interferência na determinação do valor de  $CL_{50}$ , afetando a avaliação do real potencial biológico do produto natural. Neste contexto, quando a completa solubilização do extrato não é observada, nesse trabalho é sugerido uma adaptação metodológica baseada no uso do tensoativo DMSO ou Tween 80, seguido de filtração, antes dos ensaios *in vitro*. Adicionalmente, o modelo Probit é recomendado para se obter valores de  $CL_{50}$  estatisticamente significativos. Assim, a adaptação metodológica proposta aumenta a precisão dos resultados de  $CL_{50}$ .

**Palavras-chave:** Extratos de plantas; Concentração letal mediana; Solução salina.

### Abstract

Tests with the microcrustacean *Artemia salina* are widely used by researchers of natural products as a quick and easy method applied to biological activity screening of natural products, including crude extracts. Median lethal concentration ( $LC_{50}$ ) values, obtained by means of the correlation among extract concentration and the quantity of *A. salina* dead,  $\leq 1.000 \mu\text{g mL}^{-1}$ , were considered as an indicative of biological activity. In this method, plant extracts should be dissolved in saltwater. However, the complete dissolution of samples to be tested is difficult to be reached. Thus, the solubility factor represents a strong interference parameter in determining the  $LC_{50}$  value, affecting the real biological potential evaluation of the nature product. In this context, when complete solubilization of extract is not observed, is herein suggested a methodological adaptation based on the use of DMSO or Tween 80, followed by filtration of the solution, before the *in vitro* assays. Additionally, the Probit model is recommended to obtain statistically significant values of  $LC_{50}$ . Thus, the proposed methodological adaptation enhances the precision of the  $LC_{50}$  results.

**Keywords:** Plant extract; Median lethal concentration; Saline solution.

\* Universidade Estadual de Santa Cruz, Departamento de Ciências Exatas e Tecnológicas, Campus Soane Nazaré, CEP 45662-900, Ilhéus- BA, Brazil.

✉ [rosilene@uesc.br](mailto:rosilene@uesc.br)

DOI: [10.21577/1984-6835.20170089](https://doi.org/10.21577/1984-6835.20170089)

## Influence of Solubility of Ethanol Extracts in *Artemia salina* Tests

Hemerson D. dos Santos, Fernando F. de Oliveira, Rosilene A. de Oliveira\*

Universidade Estadual de Santa Cruz, Departamento de Ciências Exatas e Tecnológicas,  
Campus Soane Nazaré, CEP 45662-900, Ilhéus- BA, Brazil.

\* [rosilene@uesc.br](mailto:rosilene@uesc.br)

*Recebido em 29 de agosto de 2016. Aceito para publicação em 8 de julho de 2017*

1. Introduction
2. Materials and Methods
  - 2.1. Plant Matter
  - 2.2. Preparation of the solutions
  - 2.3. Lethality test conducted on *Artemia salina*
  - 2.4. Statistical analysis
3. Results and Discussion
4. Conclusions

### 1. Introduction

Within the broad field of research conducted on natural products, many works have been developed sought to obtain new bioactive compounds.<sup>1-5</sup> Thus, tests involving the screening of plant extracts represent an important tool in the search for molecules with therapeutic properties.

Biological assays using extracts, aiming to investigate properties such as cytotoxicity, genotoxicity, antifungal, anti-inflammatory and others, in general are laborious, demand long period and are of elevated cost. Nevertheless, it's possible to adopt screening methods, such as the *Artemia salina* (brine shrimp) lethality test (TAS) which is simple

and easy to execute, is cost-effective, and present good correlation with some biological activities.<sup>6-9</sup>

The lethality test conducted with *A. salina* (microcrustacean) is a biological trial widely used to evaluate the potential cytotoxicity of ethanolic extracts from plant species. Basically, the microcrustacean is treated with the extract solution, in concentration ranging from of 1,000 to 10  $\mu\text{g mL}^{-1}$  are submitted to a 24 hour period After counting the number of dead *A. salina*, is determined the median lethal concentration ( $\text{LC}_{50}$ ) of extract. Then, based on the knowledge of the value of the  $\text{LC}_{50}$ , it is possible to infer the biological potential of the studied plant extract.

This microcrustacean is a primitive aquatic arthropod of the Artemiidae family.<sup>10</sup> It

contains eleven pairs of appendices that are responsible for its ability to swim, its cuticle breathing, and their feeding, which is popularly used to feed fish in aquariums. *A. salina* has an egg-laying reproduction process; their cysts are approximately 0.2 to 0.3 mm in size and hatch from the egg within 24 to 36 hours in the form of swimming larvae called *nauplii*. They reach a size of 0.45 mm on average and reach an adult phase within three weeks, achieving a maximum size of 12 mm. The hatching can occur in seawater filtered for 48 hours, reaching an ideal *nauplius* stage for the execution of *A. salina* tests.<sup>11</sup>

The *A. salina* is used in biological tests, mainly in natural products, due to its correlation with various biological activities, such as the anti-tumor activity reported by McLaughlin et al.<sup>9</sup> It is well-known that LC<sub>50</sub> values of below 1,000 µg mL<sup>-1</sup> refer to the plant matter capable of presenting biological activity.<sup>6</sup> Low lethality can be considered if an LC<sub>50</sub> value is above 500 µg mL<sup>-1</sup>, moderated for LC<sub>50</sub> between 100 and 500 µg mL<sup>-1</sup> and strongly lethal when the LC<sub>50</sub> value is less than 100 µg mL<sup>-1</sup>.<sup>9</sup>

Due to the importance and practicality of the *A. salina* test, many researchers use it in the screening for ethanol extracts from plant species that have biological potential. This fact is measured by the great number of published researches in which was used this method. Examples of LC<sub>50</sub> determined using *A. salina*, reporting studies of some plant extracts include: *Montrichardia linifera* (60.4 µg mL<sup>-1</sup>),<sup>12</sup> *Plectranthus neochilus* Schltr (210.3 µg mL<sup>-1</sup>),<sup>13</sup> *Lychnophora trichocarpha* (672.4 µg mL<sup>-1</sup>),<sup>14</sup> *Bromelia antiacantha* Bertol (362.1 µg mL<sup>-1</sup>),<sup>15</sup> *Sonchus oleraceus* (5,120.0 µg mL<sup>-1</sup>),<sup>16</sup> *Pithecellobium cochliocarpum* (257.5 µg mL<sup>-1</sup>)<sup>17</sup> and *Mimosa caesalpinifolia* Benth (1,765.0 µg mL<sup>-1</sup>).<sup>18</sup>

To evaluate the lethality conducted on *A. salina* it is necessary to correlate the concentration of the species extract in a saltwater medium with the quantity of micrustacean *nauplii* dead. Different methodologies cite the use of DMSO

surfactant to aid in achieving better solubility of ethanol extracts.<sup>12,14,16,19-21</sup>

In our experience with lethality tests conducted on *A. salina* with essential oils, it was found that the use of Tween 80 as a surfactant was appropriate for the species of *Piper*: *P. cernuum* Vell., *P. glabratum* Kunth., *P. hispidum* Sw., and *P. aff. Madeiranum*.<sup>22</sup> Nevertheless, upon applying the *A. salina* tests in ethanol extracts, our research group, in many cases, observed the formation of background body in the samples during the trials. This fact that made it difficult to make precise associations between the real concentration of the substances that were soluble in saltwater and the quantity of dead *nauplii*. It is our belief that the solid particles present in the saltwater medium can interfere in the death of *A. salina*, thus leading to an incorrect outcome, in which the expected result would be that the death of the *nauplii* would be caused by intake or contact with soluble compounds.

Taking into account the aspects observed as regards the solubility of ethanol extracts in saltwater mediums, the present study suggests an adaptation in methodology of TAS to establish a more precise LC<sub>50</sub> value of the soluble compounds, in an attempt to better correlate the concentration of the soluble constituents of ethanol extract, with the quantity of dead *nauplii*, and in this manner obtain better results related to the triage of the biological potential of plant species.

## 2. Materials and Methods

### 2.1. Plant matter

The leaves of the species were collected in the southern regions of the State of Bahia, Brazil, and their respective exsiccates were deposited in the Herbarium at the State University of Santa Cruz. The plants subjected to the studies are listed in Table 1.

**Table 1.** Species of plants subjected do the *Artemia salina* lethality test (TAS)

Specie of plant	Family	Collection municipality	Exsiccate number <sup>a</sup>
<i>Piper dilatatum</i>	Piperaceae	Una	14104
<i>Lantana macrophylla</i>	Verbenaceae	Ilhéus	15250
<i>Campomanesia dichotoma</i>	Mirtaceae	Ilhéus	16381
<i>Kielmeyera itacarensis</i>	Clusiaceae	Itacaré	21426
<i>Sphagneticola trilobata</i>	Asteraceae	Ilhéus	20326

<sup>a</sup> Herbarium of the Universidade Estadual de Santa Cruz, Bahia, Brazil

The leaves were dried at 50 °C, in a stove with forced ventilation, until reaching a constant weight. Each dried and grounded plant material (10.0 g) were subjected to exhaustive extraction with 100 mL of ethanol, in four replicates. After concentration, the ethanol extracts was maintained cooled until the moment of the biological assays (TAS).

## 2.2. Preparation of the solutions

The saltwater (salinity ~ 3.5%) used in the *A. salina* tests and the preparation of the solutions was collected beforehand in Ilhéus, Bahia, Brazil, and then filtered.

Three different methodologies were adopted in the preparation of the solutions, as described below:

### Method 1 – DMSO

The solution (1,000 µg mL<sup>-1</sup>) was prepared from 0.1000g of ethanol extract in saltwater containing 1% DMSO (Merck). Next, dilutions of 75, 50, 25, 10, and 1% of the stock solution were performed, obtaining a final volume of 5.0 mL in test tubes, in five replicates.

### Method 2 – Tween

The solution (1,000 µg mL<sup>-1</sup>) was prepared from 0.1000g of saltwater extract with the aid of 1% of non-ionic, polysorbate 80 surfactant – Tween 80 (Goldlab). This mixture was vacuum filtered with an analytical filter paper. After removing the filtrate, the solid

was washed six times with distilled water and dried in an oven at 105 °C. In this manner, the remaining solid mass was subtracted from the initial quantity to quantify the real concentration of the soluble plant extract, followed by dilutions at 75, 50, 25, 10, and 1% in five replicates.

### Method 3 – DMSO-f

The ethanol extracts were treated in a manner similar to that used in method 1. However, after the dilutions had been obtained, the mixture was vacuum filtered with analytical paper. After removing the filtrate, the solid was washed six times with distilled water and dried in an oven at 105 °C. In this manner, the mass of the remaining solid was subtracted from the initial quantity to quantify the real concentration of the soluble plant matter, followed by dilutions at 75, 50, 25, 10, and 1% in five replicates.

## 2.3. Lethality test conducted on *Artemia salina*

The methodology for the *A. salina* test was based on the method described by Meyer. et al,<sup>6</sup> with adaptations. The *A. salina* cysts were acquired commercially and were submitted to hatching in a 48 hour period, under lighting provided by a 40W incandescent lamp in a container with one part protected from the light, containing filtered saltwater.

Next, in the tubes containing the solutions, with the aid of a Pasteur pipette and a magnifying glass, ten *nauplii* were added to each test tube. After 24 hours of contact with the extract, the number of dead (when no movement was presented) and live (those that still moved in the medium) *nauplii* were recorded. Control groups were prepared for filtered seawater and seawater with 1% of surfactant, in triplicate, with ten *nauplii* in 5 mL of filtered seawater.

#### 2.4. Statistical analysis

Two methods were used to obtain LC<sub>50</sub>: the graph method of interpolation, by means of the number of live and dead *A. salina* within the concentration,<sup>16</sup> and the Probit model, which correlates the various concentrations of extract and number of occurrences (deaths of *A. salina*).

In the Probit model, the response of each sample is a random variable with binomial distribution. This model allows one to obtain a LC<sub>50</sub> value with a confidence interval of 95%. The calculations were performed using the Statistic 7 software.

A test of multivariate analysis was also performed using the BioEstat 5.0 software, to discriminate the results of the solubilities with each referent methodology, as well as the lethality and non-lethality of the ethanol extracts obtained in the *A. salina* test.

### 3. Results and Discussion

The behavior of the solubility of the various extract and percentage values of solubility with the use of Tween and DMSO-f, referent to methodologies 1, 2, and 3, can be seen in Table 2.

**Table 2.** Characteristics of the various stock solutions of the ethanol extracts in the different methodologies used in the preparation of the solutions.

Species	Methods		
	DMSO	Tween* (%)	DMSO-f * (%)
<i>P.dil</i>	Coalescing	96	49
<i>L.mac</i>	Viscous	71	67
<i>K.ita</i>	Diffuse in the medium	39	55
<i>C.dic</i>	Coalescing	54	46
<i>S.tri</i>	Diffuse in the medium	100	89

\*Soluble percentage in the saltwater medium; *P.dil* – *P. dilatatum*; *L.mac* - *L. macrophylla*; *K.ita* - *K. itacarensis*; *C.dic* – *C. dichotoma*; *S.tri* – *S. trilobata*; DMSO – Methodology with DMSO, without filtration; Tween – Methodology with filtered Tween 80; DMSO-f - Methodology with filtered DMSO.

It was observed that, among the studied species, the Tween surfactant enhanced the solubility of ethanol extract, as compared to the DMSO-f, with the exception to the *K. itacarensis* species. This variation of solubility is expected, concerning the diversity of the different substances present in the studied species. The solubility of extracts is an

important factor for the lethality test. Thus, greater solubility is indicative that a large part of the chemical components present in the plant extract are available in saline solutions to result the mortality of *A. salina*. The presence of solid materials in the saline medium certainly influences the outcome of the test. This is because the *A. salina* has a

cutaneous respiration system, that is, the surface of its body presents cuticles that facilitate the diffusion of oxygen through cells that contains copper ions that are associated to blood pigmentation. Consequently, this microcrustacean must be in constant movement for it to be able to breathe and feed itself.<sup>23</sup> The aggregation of these particles in the body of *A. salina* can compromise its breathing. Thus, the presence of particles in its environment certainly interferes in its movement and survival.

The low solubility of the extract can interfere in the LC<sub>50</sub> value, which is defined through the extract's concentration values in a saline medium, that is, of a soluble mass in a known volume, taking into consideration the erroneous conclusion drawn when faced with the outcome of the *A. salina* test and leading to an incorrect classification for the species under study. Therefore, it is important to guarantee that the maximum of the sample of the plant extract be soluble in a saline solution medium.

The low solubility of the extract interfere in the LC<sub>50</sub> value, which is defined by means of the extract's concentration values in a saline medium, that is, the soluble mass in a known volume. It's necessary to consider that the erroneous conclusion drawn from the outcome of the *A. salina* test induces an incorrect classification of the species under study. Therefore, it is very important to guarantee that the maximum of the sample of the plant extract be soluble in the saline solution used in the bioassays with *A. salina*.

Extracts of plant species, in concentrations ranging from 1,000 to 10 µg mL<sup>-1</sup>, were submitted to the *A. salina* tests in five replicates of each concentration. After 24 hours of contact, the percentage of dead *nauplii*, correlated with each concentration, were counted. The interpolation method (number of live and dead *nauplii* within the concentration) and the Probit method, which is a statistical method used to estimate critical doses in dose response trials, as discussed by Finney.<sup>24</sup> The LC<sub>50</sub> values obtained can be seen in Table 3.

**Table 3.** Assessment of the lethality of ethanol extracts in the *A. salina* test, using different methodologies

	LC <sub>50</sub> (µg mL <sup>-1</sup> )			LC <sub>50</sub> (µg mL <sup>-1</sup> )			IC-95%		
	Interpolation			Probit					
	DMSO	Tween	DMSO-f	DMSO	Tween	DMSO-f	DMSO	Tween	DMSO-f
<i>P.dil</i>	882*	462*	601*	919*	531*	911*	833-1,005	489-575	831-991
<i>L.mac</i>	385*	1,077	1,255	527*	1,252	1,556	512-544	992-1,512	1,070-2,043
<i>K.ita</i>	882*	1,020	1,000	861*	1,641	5,825	778-946	1,323-1,959	4,206-7,445
<i>C.dic</i>	833*	258*	1,165	826*	504*	1,863	695-958	476-532	1,456-2,267
<i>S.tri</i>	172*	342*	520*	508*	435*	555*	487-529	426-445	518-592

\*LC<sub>50</sub> lower than 1000 µg mL<sup>-1</sup>; DMSO - methodology with DMSO without filtration; Tween – methodology using Tween 80; DMSO-f – methodology with filtrated DMSO; *P.dil* – *P. dilatatum*; *L.mac* – *L. macrophylla*; *K.ita* – *K. itacarensis*; *C.dic* – *C. dichotoma*; *S.tri* – *S. trilobata*. No mortality was found in the control groups of seawater and seawater with surfactants

Though the estimate of the LC<sub>50</sub> per interpolation is a simple form of correlating the data of the extract's concentration with the number of dead *A. salina*, this method

has proven to be effective only for the qualitative treatment of the data. As it is reliable in the inference of the level of lethality or non-lethality of the species, the Probit model uses probabilistic calculations to estimate the  $LC_{50}$  value.

This model counts each response (death of *nauplii*) within applied extract concentrations conducted upon *A. salina* as a random variable with a binomial distribution. In this manner, this model allows for a better estimate of the median lethal concentration value.

Upon examining Table 3 data, it was observed that all the studied species, using the DMSO, without filtration, were classified as lethal within the *A. salina* bioassay. Different results were obtained using the other methods. These results corroborates that the presence of solids in the aqueous environment of *A. salina* bioassays also interferes in the mortality of the *nauplii*.

That is, *L. macrophylla* and *K. Itacarensis*, the  $LC_{50}$  values were 1252.0 and 1641.0  $\mu\text{g mL}^{-1}$ , respectively, when using the method in which was added the surfactant Tween-80, indicating the non-lethality of the ethanol extracts from these species. The  $LC_{50}$  values of 1556.4 and 5825.3  $\mu\text{g mL}^{-1}$  were obtained in the respective order for these same extracts, using the DMSO-f method. Through these results, it's possible to infer that soluble constituents of the ethanol extracts, when using the DMSO method without filtration, and with the presence of a insoluble particles, indicates a lethal  $LC_{50}$  value. This proves that insoluble material in the aqueous medium significantly influenced the mortality rate of the microcrustaceans.

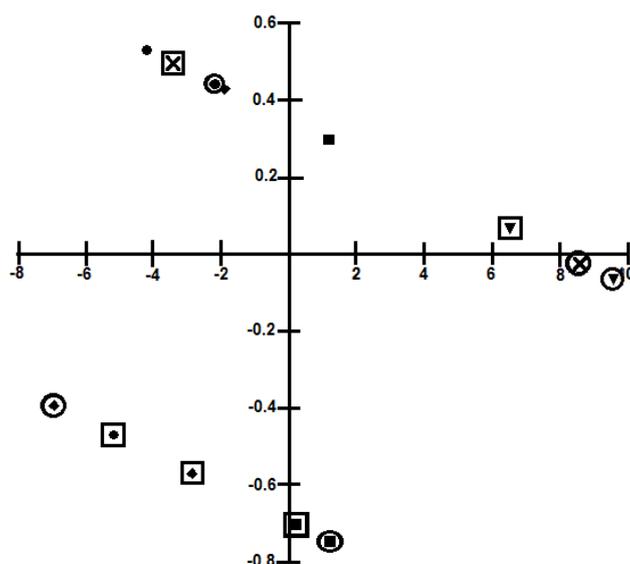
Through the *A. salina* bioassay using the DMSO method without filtration, the extract from *C. dichotoma* showed  $LC_{50}$  value of

826.6  $\mu\text{g mL}^{-1}$ , which indicates biological activity. Nevertheless, by means of the DMSO-f method was indicated a non-lethality of this extract. We suggest that the low solubility of this extract contributed to the inefficiency of the availability of constituents with biological activity in the saltwater environment.

There are wide range of behaviors of the variable involved in the *A. salina* test, a discriminant analysis was performed to associate the behavior of the different solubilities with the methodologies and classification of the lethality of the extracts.

Through the diagram of the discriminant analysis (Figure 1) obtained using the software BioEstat 5.0, was observed that extracts of the same species were classified as lethal (upper vertical axis) and non-lethal (lower vertical axis) in the *A. salina* test, by means of the application of the three methods. The points on each diagonal (Figure 1) are distanced between each other via their solubility in saltwater provided by surfactants, in which, as the points decrease, more soluble extracts can be found. Solubility was considered to be similar for the same extracts of the two methodologies in which the DMSO was applied, such as surfactants. In this sense, the points of the extracts that obtained the same classification in the test were overlapped.

It was observed that the *C. dichotoma* extract presented lethality, though it had solubilized less than the *L. macrophylla* extracts, in which lethality was not present, that is, in this case, the chemical nature of the extracts represented a larger determining factor in the  $LC_{50}$  values as compared to the solubility of the samples. In other words, for extract with a greater power of biological activity, small quantities are already sufficient to present positive results of lethality.



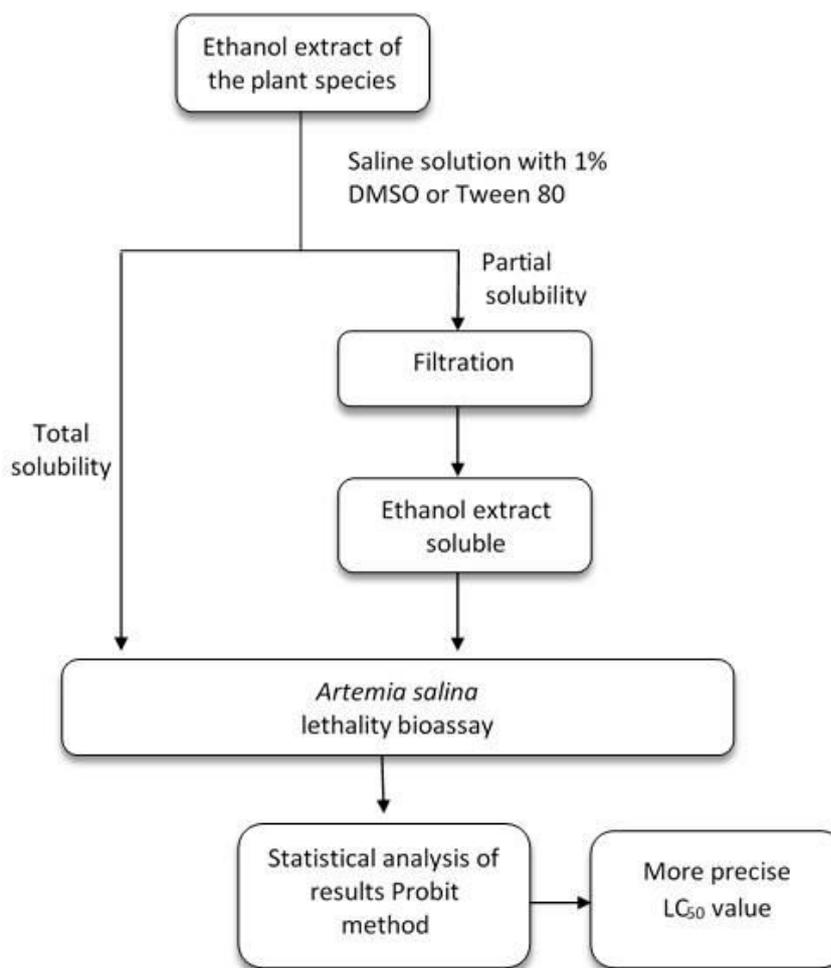
Ethanol extracts using the DMSO methodology (without edges), Tween (rounded edges) and DMSO-f (square edges). Of the species of (X) – *P. dilatatum*. (■) – *L. macrophylla*. (◆) – *K. itacarensis*. (●) – *C. dichotoma*. (▼) – *S. trilobata*. On the lower vertical axis, non-lethal species can be observed, while on the upper vertical axis, lethal species can be observed. The species are more soluble from the left to the right on the horizontal axis.

**Figure 1.** Discriminant analysis as regards the lethality of the ethanol extracts using the DMSO, Tween and DMSO-f

The proposed adaptation for the *A. salina* test method applied for ethanol extract of plant species is illustrated in Figure 2. It can be observed that the values of median lethal concentration are influenced by two interfering variables: the solubility of the extracts, which themselves depend on the affinity of the many chemical components of the plant species with the chemical properties of the surfactant, and non-soluble parts of the extract, which result in the movement and breathing of the *nauplii*. The extracts with more apolar characteristics are not solubilized in saltwater mediums, maintaining a background image. As each plant species has its own specific chemical composition, the solubility of the extracts becomes a particular characteristic of each plant species and should be tested to choose the best methodology (Figure 2).

Although an extract may not be highly soluble in saltwater, it is possible to increase its solubility by adding surfactants, the most common of which are DMSO and Tween 80. However, the presence of the insoluble part of the extract present in the saltwater medium appears as an interfering variable in the  $LC_{50}$  values, as proposed in Figure 2.

Therefore, in this work is suggested the use of surfactants to increase the solubility of the ethanol extracts in saltwater, followed by filtration to determine the concentration of soluble constituents, should the formation of a background image appear in the sample. By means of these procedures, it's possible to reach greater reliability in the *A. salina* test, which is an important screening process used by researchers of Natural Products.



**Figure 2.** Diagram of the proposed methodological model to determine LC<sub>50</sub> values in the biological triage test of ethanol extracts when submitted to the *A. salina* bioassay

#### 4. Conclusions

The methodology proposed in this study led to more reliable results as regards the LC<sub>50</sub> values of the ethanol extracts. The LC<sub>50</sub> values for the plant species were reliable when obtained using the methods that involves filtration of the background image of aqueous solution of the ethanol extracts. In this light, the removal of the background present in the solutions of the samples that will be submitted to the biological triage test with *A. salina* is recommended.

#### Acknowledgments

The authors thanks to Universidade Estadual de Santa Cruz (UESC) for financial support, including scientific initiation grant to HDS.

#### References

- Baratto, L.; Lang, K. L.; Vanz, D. C. Investigação das atividades alelopática e antimicrobiana de Mikania laevigata (Asteraceae) obtida de cultivos hidropônico e tradicional. *Revista Brasileira de Farmacognosia* **2008**, *4*, 577. [CrossRef]

- <sup>2</sup> Xue, Y.; Zhang, Y.; Cheng, D.; Daddy, S.; He, Q. Genetically engineering *Synechocystis* sp. Pasteur Culture Collection 6803 for the sustainable production of the plant secondary metabolite p-coumaric acid. *Proceedings of the National Academy of Sciences, India - Section A* **2014**, *26*, 9449. [CrossRef] [PubMed]
- <sup>3</sup> Imai, Y.; Sato, S.; Tanaka, Y.; Ochi, K.; Hosaka, T. Lincomycin at Subinhibitory Concentrations Potentiates Secondary Metabolite Production by *Streptomyces* spp. *Applied and Environmental Microbiology* **2015**, *11*, 3869. [CrossRef]
- <sup>4</sup> Holnik, R.; Hussein, A. A.; Souza, B. M. C. Comparação do teor de taninos entre duas espécies de espinheira-santa (*Maytenus aquifolium* Mart. e *Maytenus ilicifolia* Mart. ex Reissek) cultivadas no Horto Medicinal do Refúgio Biológico Bela Vista - RBBV da Itaipu Binacional - Foz do Iguaçu, PR - Brasil. *Revista Brasileira de Plantas Mediciniais* **2015**, *3*, 385. [CrossRef]
- <sup>5</sup> Speed, M. P.; Fenton, A.; Jones, M. G.; Ruxton, G. D.; Brockhurst, M. A. Coevolution can explain defensive secondary metabolite diversity in plants. *New Phytologist* **2015**, *4*, 1251. [CrossRef] [PubMed]
- <sup>6</sup> Meyer, B.; Ferrigni, N.; Putnam, J. Brine Shrimp: A Convenient General Bioassay for Active Plant Constituents. *Planta Medica* **1982**, *5*, 31. [CrossRef] [PubMed]
- <sup>7</sup> Alves, T. M. A.; Silva, A. F.; Brandão, M. Biological screening of Brazilian medicinal plants. *Memórias do Instituto Oswaldo Cruz* **2000**, *3*, 367. [CrossRef]
- <sup>8</sup> Magalhães, A. F.; Tozzi, A. M. G. De A.; Santos, C. C. Saponins from *Swartzia langsdorffii*: biological activities. *Memórias do Instituto Oswaldo Cruz* **2003**, *5*, 713. [CrossRef]
- <sup>9</sup> McLaughlin, J. L.; Chang, C.; Smith, D. L. Simple Bench-Top Bioassays (Brine Shrimp and Potato Discs) for the Discovery of Plant Antitumor Compounds. *Abstracts of Papers of the American Chemical Society* **2009**, *9*, 112. [CrossRef]
- <sup>10</sup> Kondo, M. Lipovitellin do crustáceo, *Artemia salina*. A análise bioquímica do complexo lipovitellin dos grânulos de vitelo. *Journal of Biological Chemistry* **1980**, *14*, 6727. [PubMed]
- <sup>11</sup> Mahdhi, A.; Messina, C.; Kamoun, F.; Santulli, A.; Bakhrouf, A. Determination of biological characteristics of Tunisian *Artemia salina* populations. *Biologia* **2012**, *1*, 143. [CrossRef]
- <sup>12</sup> Amarante, C. B.; Müller, A. H.; Póvoa, M. M.; Dolabela, M. F. Estudo fitoquímico biomonitorado pelos ensaios de toxicidade frente à *Artemia salina* e de atividade antiplasmódica do caule de aninga (*Montrichardia linifera*). *Acta Amazonica* **2011**, *3*, 431. [CrossRef]
- <sup>13</sup> Arcanjo, D.; Albuquerque, A.; Melo-Neto, B. Bioactivity evaluation against *Artemia salina* Leach of medicinal plants used in Brazilian Northeastern folk medicine. *Brazilian Journal of Biology* **2012**, *3*, 505. [CrossRef] [PubMed]
- <sup>14</sup> Ferraz Filha, Z.; Lombardi, J. A.; Guzzo, L. S.; Saúde-Guimarães, D. A. Brine shrimp (*Artemia salina* Leach) bioassay of extracts from *Lychnophoriopsis candelabrum* and different *Lychnophora* species. *Revista Brasileira de Plantas Mediciniais* **2012**, *2*, 358. [CrossRef]
- <sup>15</sup> Junior, L. Avaliação das atividades antimicrobiana, citotóxica, moluscicida e antioxidante de *Bromelia antiacantha* Bertol (Bromeliaceae). *Revista Brasileira de Plantas Mediciniais* **2010**, *4*, 406.
- <sup>16</sup> Lima, J. M.; Silva, C. A.; Rosa, M. B. Prospecção Fitoquímica de *Sonchus Oleraceus* e sua Toxicidade Sobre o Microcrustáceo *Artemia salina*. *Planta Daninha* **2009**, *1*, 7. [CrossRef]
- <sup>17</sup> Lima, C. M. P.; Soares, R. F.; Bastos, I. V. G. A. Avaliação da toxicidade aguda do extrato das cascas de *Pithecellobium cochliocarpum* (Gomez) Macbr. *Revista Brasileira de Plantas Mediciniais* **2014**, *4*, 832. [Link]
- <sup>18</sup> Lopes Citó, A. M. G.; Araújo, B.; Monção, B. Chemical constituents and toxicological

studies of leaves from *Mimosa caesalpinifolia* Benth., a Brazilian honey plant. *Pharmacognosy Magazine* **2014**, *39*, 456. [[CrossRef](#)] [[PubMed](#)]

<sup>19</sup> Carlos, R. M.; Filho, S.; Souza, A. G. Avaliação da bioatividade dos extratos de cúrcuma (*Curcuma longa* L. Zingiberaceae) em *Artemia salina* e *Biomphalaria glabrata*. *Revista Brasileira de Farmacognosia* **2009**, *4*, 919. [[CrossRef](#)]

<sup>20</sup> Mayorga, P.; Pérez, K. R.; Cruz, S. M.; Cáceres, A. Comparison of bioassays using the anostracan crustaceans *Artemia salina* and *Thamnocephalus platyurus* for plant extract toxicity screening. *Revista Brasileira de Farmacognosia* **2010**, *6*, 897. [[CrossRef](#)]

<sup>21</sup> Carvalho, A. F.; Silva, D. M.; Silva, T. R.; Scarcelli, E.; Manhani, M. R. Avaliação da atividade antibacteriana de extratos etanólico e de ciclohexano a partir das flores de camomila (*Matricaria chamomilla* L.). *Revista Brasileira de Plantas Mediciniais* **2014**, *3*, 521. [[CrossRef](#)]

<sup>22</sup> Assis, A.; Brito, V.; Bittencourt, M. Oliveira, F.; Oliveira, R. Essential oils composition of four Piper species from Brazil. *Journal of Essential Oil Research* **2013**, *3*, 203. [[CrossRef](#)]

<sup>23</sup> Chang, E. S.; Thiel, M.; *Physiology*, 4a. ed. Oxford University Press: New York, 2015.

<sup>24</sup> Finney, D. *Probit Analysis*, Cambridge University Press: United Kingdom, 2009.