Brine Shrimp Lethality Assay of Methanolic Extract of Orthosiphon Stamineus Benth. Leaves

Nur Farah Amalina Mughni¹, Fatin Fathiah Mohammad Yusop¹

¹Kolej Kemahiran Tinggi MARA Lenggong, Kg. Padang Luas, Mukim Durian Pipit, 33400 Lenggong, Perak, Malaysia
*Email: amalina.mughni@mara.gov.my
Phone: +6057510007; Fax: +6057510006

ABSTRACT

The in vitro lethality of brine shrimp (BST), which is a simple tool for preliminary assessment of toxicity where one of the simplest biological responses to observe is lethality, since there is only one condition which are either dead or alive. The lethality of the methanol extract of Orthosiphon stamineus leaf from 10 different locations were subjected to brine shrimp after 24 h of exposure to the test solutions according to protocol reported by Meyer et al. (1982). The extracts were found to show reaction and dose dependent to toxicity on brine shrimp nauplii and the LC50 value of KBPP, TPPM, HLSM, SUMM, PPKM and DSJM were found to be 223.95 µg/ml, 252.04 µg/ml, 305.46 µg/ml, 323.47 µg/ml, 291.00 µg/ml and 281.90 µg/ml respectively. The other four locations which are PTNS, SNNS, SKTM and CJPM were found to be 163.90 µg/ml, 130.06 µg/ml, 106.09 µg/ml and 123.26 µg/ml respectively. This result revealed that these four locations gave positive results because about 50% of species presented positive results when LC50 <200µg/ml. A methanolic extract of this plant demonstrates moderate or low toxic activity against brine shrimp nauplii.

Keywords: Brine shimp lethality, Orthosiphon stamineus

INTRODUCTION

Based on the statistics carried out by the World Health Organization (WHO), 80% of the world population prefers to choose herbs as medicine to treat ailments. Herbal medicine in particular is the main ingredients in traditional medical methods and common element in homeopathy, Ayurveda, Chinese medicine and some other alternative healing methods. WHO also states that about 119 medical substances are extracted from various plants and 74% of them are still used in exactly the same way these herbs were used in the past. Up to date, the extracted substances from herbs have become the basis of the manufactured medication for diseases including those with cardiovascular problems, asthmas and hypertensions (WHO, 1998).

Orthosiphon stamineus Benth which is also known as “Misai Kucing” is a genus in the family of Lamiaceae. “Misai Kucing” plant is an herbal species originated from South-East Asia regions. In traditional medicine, folks believe that Orthosiphon stamineus possesses diuretic properties, antiallergic, anti-inflammatory, antihypertensive and antitumor. It is also used to treat diabetes, rheumatism and gout (Burkhill, 1966).

In this study, the leaves of Orthosiphon stamineus Benth plant from different locations were studied. This plant is locally known as “Misai kucing” or literally cat’s whiskers
because of its unique flower which resembles cat’s whiskers. All parts of this plant possess therapeutic properties such as the leaves of the plant are believed to contain antioxidant property (Akowuah et al., 2005; Khamsah et al., 2006; Yam et al., 2007), anti-inflammatory property (Yam et al., 2008) and diuretic property (Arafat et al., 2008).

Brine shrimp is an aquatic crustacean of genus, class of branchiopoda and it is order of anostraca. Brine shrimp can be found worldwide in water ranging from brackish to the ultrasaline. The eggs of brine shrimp almost 0.2 mm in diameter and can be stored for longer periods as long as it remains dehydration. When eggs of the brine shrimp were added to saline solutions, eggs will adsorb the water and embryogenesis process occurs. This process occurs between 16 to 36 hours. Then, the embryo will develop antennae and mandibles, breaks away from the hatching membrane and become a lively and free-swimming nauplius (Asem et al., 2010).

Meyer et al., 1982 has developed the in vitro lethality of brine shrimp (BST), which is a simple tool for preliminary assessment of toxicity where one of the simplest biological responses to observe is lethality, since there is only one condition which are either dead or alive (Montanher et al., 2002). This bioassay also provides the forefront screen than can be backed up with a more detailed assay once the active compound has been isolated. BST is competent, fast and low-cost assessments that need just a quite small amount samples. The method is easily understood and uses small amounts of analyzing the content (Pisutthanan et al., 2013). It appears that BSLT is predictive of cytotoxicity and pesticidal activity (Ghisalberti, 1993).

**MATERIALS AND METHODS**

**Plant materials**

*Orthosiphon stamineus* leaves from KBPM; Kepala Batas (Pulau Pinang), CJPM; Changkat Jering (Perak), DSJM; Desaru (Johor), and SUMM; Sungai Udang (Melaka) were obtained from herbal suppliers. Leaves were also collected from nursery, hillside and garden in SKTM; Sungai Kok (Terengganu), HLSM; Hulu Langat (Selangor), PPKM; Paser Putih (Kelantan), TPPM; Taiping (Perak), PNSM; Pantai (Negeri Sembilan) and SNSM; Sendayan (Negeri Sembilan). A voucher specimen (no. 11469) was deposited at the herbarium of School of Biological Sciences, University Sains Malaysia.

**Extraction**

Methanol extract was used for this study. The *Orthosiphon stamineus* leaves were washed with water, sliced and dried in the oven for 3 days at 55oC. The dried samples were ground and extracted with methanol for 5 days. The pooled methanol extracts were evaporated using a rotary evaporator.

Fifty grams of leaf powder from each place of the plant was extracted by 500 millilitres of methanol at 40oC using maceration technique. The extracts were then filtered and dried using a rotary evaporator.
Hatching the Brine Shrimp

Brine shrimp lethality bioassay is commonly used in the bioassay for the bioactive compounds (Meyer et al., 1982). Artemia salina was applied as a practical observation for screening. 1.0 g of the brine shrimp’s eggs were obtained and hatched in filter seawater for 48 h to mature shrimp called nauplii.

Bioassay

The toxicity analysis was conducted on brine shrimp nauplii using Meyer method. Exactly 10 mg of crude extract was dissolved in 10 ml of methanol and the mixture was sonicated to ensure homogeneity of the extract in the solvent.

Next, 5000 µl, 2500 µl, 1250 µl, 625 µl, 312.5 µl and 156.25 µl of this solution were transferred into separate vials. The solvent in the vials was evaporated to dryness in a fume hood and 5.0 ml of seawater was added to each of the vial to give final concentrations of 1000 μg/ml, 500 μg/ml, 250 μg/ml, 125 μg/ml, 62.5 μg/ml and 31.25μg/ml of the sample, respectively. For the bioassay, 10 brine shrimp larvae were introduced into the test tubes. After 24 hours, the numbers of surviving shrimps at each concentration of the extracts were examined to determine the LC$_{50}$ values of the samples. A control was prepared at the same time using seawater without addition of extract.

Positive control in a toxicity study is a commonly approved toxicity agent and caused by the test agent is in contrast to the outcome for the positive control. In the present study potassium dichromate was used as positive control. Potassium dichromate was analysed at very low concentration (40, 10, 5, 1.25, 0.625, 0.313, 0.156, 0.078 μg/ml) (Mclaughlin et al., 1993; Meyer et al., 2007).

After 24 hours, the tubes were inspected and the surviving nauplii in each tube were counted. The lethal concentrations of the sample resulting in 50% mortality of the brine shrimp (LC$_{50}$).

Calculation

The mortality endpoint of this bioassay is defined as the absence of controlled forward motion during 30 second of observation. The percent of lethality of the nauplii for each concentration and control was calculated. For each tube, count the number of dead and number of live nauplii, and determine the % death.

Statistical Analysis

The percentage lethality was calculated from the mean survival larvae of extracts treated tubes and control. LC$_{50}$ values were obtained by best-fit line method.

RESULTS AND DISCUSSION

The brine shrimp lethality bioassay has been used regularly in preliminary screening to detect toxicity of the test sample extracts towards brine shrimp nauplii, which also offer an indicator of possible toxicity properties of the analyze materials. The lethality of the methanol extract of Orthosiphon stamineus leaf from 10 different locations were
subjected to brine shrimp after 24 hours of exposure to the test solutions according to protocol reported by Meyer et al. (1982).

The methanol extract of ten different locations showed lethality against brine shrimp. The result of the toxicity screening of *O. stamineus* (% mortality at different concentrations and LC$_{50}$ values) were tabulated in the Table 1. The value of LC$_{50}$ higher indicates the lower of the toxicity in the test sample.

Brine shrimp lethality bioassay, the test sample extract from six locations showed LC$_{50}$$>$200µg/ml, which revealed that the extract is pharmacologically inactive (Anderson et al., 1991) and suggest that these samples are practically non-toxic. The extracts were found to show reaction and dose dependent to toxicity on brine shrimp nauplii and the LC$_{50}$ (table 1) value of KBPP, TPPM, HLSM, SUMM, PPKM and DSJM were found to be 223.95 µg/ml, 252.04 µg/ml, 305.46 µg/ml, 323.47 µg/ml, 291.00 µg/ml and 281.90 µg/ml respectively. The other four locations which are PTNS, SNNS, SKTM and CJPM were found to be 163.90 µg/ml, 130.06 µg/ml, 106.09 µg/ml and 123.26 µg/ml respectively. This result revealed that these four locations gave positive results because about 50% of species presented positive results when LC$_{50} <200$µg/ml. However, Serano et al. (1996) considered this value as low.

**Table 1**: Brine Shrimp lethality data assay. All data are presented as Mean +SD, n=2

<table>
<thead>
<tr>
<th>Plant</th>
<th>Regression line</th>
<th>LC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KBPP:KepalaBatas,Penang;TPPM:Taiping,Perak; PNSM:Pantai,Negeri Sembilan; SNSM: Sendayan Negeri Sembilan; HLSM: Hulu Langat, Selangor;SKTM: Sungai Kok, Terengganu;SUMM: Sungai Udang,Melaka; PPKM: Pasir Puteh,Kelantan;CJPK:ChangkatJering,Perak;DSJM: Desaru,Johor</td>
<td>$y= 76.778x – 130.44$</td>
<td>223.95 ± 5.30</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.8613$</td>
<td></td>
</tr>
<tr>
<td>TPPM</td>
<td>$y= 73.026x - 125.37$</td>
<td>252.04 ± 3.22</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.9134$</td>
<td></td>
</tr>
<tr>
<td>PTNS</td>
<td>$y= 61.559x – 86.327$</td>
<td>163.90 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.959$</td>
<td></td>
</tr>
<tr>
<td>SDNS</td>
<td>$y= 65.353x – 88.166$</td>
<td>130.06 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.8957$</td>
<td></td>
</tr>
<tr>
<td>HLSM</td>
<td>$y= 61.663x – 103.23$</td>
<td>305.46 ± 1.13</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.8597$</td>
<td></td>
</tr>
<tr>
<td>SKTM</td>
<td>$y= 54.012x – 59.41$</td>
<td>106.09 ± 1.45</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.9905$</td>
<td></td>
</tr>
<tr>
<td>SUMM</td>
<td>$y= 55.94x – 90.4$</td>
<td>323.47 ± 4.43</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.9301$</td>
<td></td>
</tr>
<tr>
<td>PPKM</td>
<td>$y= 63.592x – 104.22$</td>
<td>291.00 ± 4.39</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.9342$</td>
<td></td>
</tr>
<tr>
<td>CJPM</td>
<td>$y= 66.286x – 88.591$</td>
<td>123.25 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.8520$</td>
<td></td>
</tr>
<tr>
<td>DSJM</td>
<td>$y= 55.982x – 87.161$</td>
<td>281.90 ± 4.60</td>
</tr>
<tr>
<td></td>
<td>$R^2= 0.8768$</td>
<td></td>
</tr>
</tbody>
</table>
A methanolic extract of this plant demonstrates low or nontoxic activity against brine shrimp nauplii which may be due to the fact that chemical compounds present in the methanol extract of *Orthosiphon stamineus* leaf is less or not toxic. In general, polyphenols are the most important compound present in the *Orthosiphon stamineus* Benth leaf such as polymethoxylated flavonoids and caffeic acid derivatives. Yanez et al. (2004) proved that the cytotoxicity of the polyphenols assayed was moderate or null on Melan-a. This study is supported by other study by Kuntz et al. (1999) which studied the effect of 36 flavonoids on 2 colon cancer cell lines (HT-29 and Caco-2), concluding that almost all flavonoids which have been studied had an antiproliferative effect and showed no cytotoxicity. In another study, the low cytotoxicity of 4-methoxylated flavones was demonstrated and compound had an antiproliferative effect on 2 animal tumor cells (LLC-MK2 and C6).

Han (2007) evaluated the possible toxic effect after following fourteen days oral administration of methanol extract of *Orthosiphon stamineus* in female Sprague Dawley (SD) rats. This study revealed that LD<sub>50</sub> was shown to be higher than 5g/kg due as no lethality occurred in the younger female SD rats, which is considered as practically nontoxic. This study concludes that the methanol extract of *Orthosiphon stamineus* seems to be deficient toxic effect which can compromise the medicinal uses of this plant in herbal medicine.

Brine shrimp lethality bioassay can be done on other pharmacological activities beside cytotoxicity such as antimicrobial, pesticide and antitumor activities of the compounds (Meyer *et al*., 1982). A number of novel antitumor and pesticidal natural products have been isolated using brine shrimp lethality bioassay (Meyer *et al*., 1982).

**CONCLUSION**

The extracts were found to show reaction and dose dependent to toxicity on brine shrimp nauplii and the LC50 value of KBPP, TPPM, HLSM, SUMM,PPKM and DSJM were found to be 223.95 µg/ml, 252.04 µg/ml, 305.46 µg/ml, 323.47µg/ml, 291.00 µg/ml and 281.90 µg/ml respectively. The other four locations which are PTNS, SNNS, SKTM and CJPM were found to be 163.90 µg/ml, 130.06 µg/ml, 106.09µg/ml and 123.26 µg/ml respectively. This result revealed that these four locations gave positive results because about 50% of species presented positive results when LC50 <200µg/ml. A methanolic extract of *Orthosiphon stamineus* leaves from different locations demonstrated moderate or low toxicity activity against brine shrimp nauplii which may due to the fact that the major chemical compounds present in the methanol extract of this plant are less or not toxic.

**REFERENCES**


