Bangladesh J Pharmacol. 2017; 12: 186-189

VISUAL EXPERIMENT

Brine shrimp lethality assay

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Published: 5 June, 2017

DOI: 10.3329/bjp.v12i2.32796

ABSTRACT

Brine shrimp lethality assay is an important tool for the preliminary cytotoxicity assay of plant extract and others based on the ability to kill a laboratory cultured larvae (nauplii). The nauplii were exposed to different concentrations of plant extract for 24 hours. The number of motile nauplii was calculated for the effectiveness of the extract. It is a simple, cost effective and requires small amount of test material.

INTRODUCTION

Now-a-days brine shrimp (*Artemia salina,* fairy shrimp or sea monkeys) lethality assay is commonly used to check the cytotoxic effect of bioactive chemicals. It is a preliminary toxicity screening of plant extracts (Ghosh et al., 2015; Kibiti and Afolayan, 2016; Oberlies et al., 1998; Sufian and Haque, 2015; Syahmi et al., 2010), fungal toxins (Harwing and Scott, 1971), heavy metals (Saliba and Krzyz, 1976), cyanobacteria toxins (Hisem et al., 2011), pesticides (Michael et al., 1956), cytotoxicity testing of dental material (Pelka et al., 2000) and nanostructures (Maurer-Jones et al., 2013). Subsequently animal model is recommended for its establishment. Other bench top assays are crown gall tumors inhibition on discs of potato tubers (Galsky et al., 1980), frond proliferation inhibition in duckweed (Mclaughlin et al., 1998) and yellow fever mosquito larvae lethality assay (Spielman and Williams, 1966). Among them, brine shrimp lethality test is the simplest one, low cost and effective.

The larvae (nauplii; singular nauplius), about 22 mm long, are large enough to observe without high magnification and small enough for hatching in enormous amount without extensive workspace in a laboratory.

This assay was first proposed by Michael et al. in 1956. Subsequently, it was further developed by others. This lethality assay has been successively employed as a bioassay guide for active cytotoxic and anti-tumor agents in 1982 (Meyer et al., 1982).

For the bioactive compound of either natural or synthetic origin, this is a rapid and comprehensive test. It is also an inexpensive and simple test as no aseptic techniques are required. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and relatively small amount of sample (2-20 mg or less) is necessary.

MATERIALS AND EQUIPMENTS

1. Rectangular glass jar
2. Measuring cylinder (1000 mL)
3. Table salt (27 g)
4. Spatula
5. Brine shrimp eggs (a few gram)
6. Air pump

7. Analytical balance
8. Pasteur pipette
9. Light source
10. Microtip pipette
11. Test tubes (12 × 100 mm)
12. Magnifying glass
13. Test sample of plant extract

PREPARATION OF REAGENTS

Serial dilution of extract: Clean test tubes were taken and labeled. Plant extract of 10 mg was weighed by an analytical balance. Then stock solution was prepared by dissolving 10 mg of plant extract (soluble in water) in 1 mL of water. Concentrations of 1 mg/ mL, 100 µg/ mL, 10 µg/ mL and 1 µg/ mL were prepared by serial dilution from the stock solution. Five test tubes were labeled as 1-5. Then 1 mL of prepared solution was taken into the respect test tubes containing 10 nauplii and 1 mL of seawater. The number of dead nauplii was counted after 24 hours.

VIDEO CLIP

Brine shrimp lethality assay: 4 min 9 sec

PROTOCOL

Hatching brine shrimp

1. Measure 3 liters of water using measuring cylinder and pour into the rectangular jar
2. Weigh about 27 g of table salt by an balance and add it into the jar containing water
3. Mix the water with a spatula
4. Place the tip of an airline from a air pump into the bottom of the jar maintaining proper aeration
5. Add about 15 g of brine shrimp eggs at the top water level of the jar and mix with the water
6. Switch on a light (60-100 Watt bulb) placed a few inches away from the jar
7. After 20-24 hours, the nauplii will hatch
8. Observe the eggs and nauplii
9. Collect the nauplii after the next 24 hours
10. Hatched nauplii must be separated from the empty egg. It can be done by turn off the air and switch off the lamp. The empty egg will float while the brine shrimp will concentrate in the water column.
11. Transfer 10 nauplii to a test tube using a Pasteur pipette

Toxicity testing

12. Expose the nauplii to different concentrations of the plant extract
13. Count the number of survivors and calculate the percentage of death after 24 hours

CALCULATION

The mortality endpoint of this bioassay is defined as the absence of controlled forward motion during 30 sec 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,

$$\%\text{Death} = \frac{\text{Number of dead nauplii}}{\text{Number of dead nauplii + Number of live nauplii}} \times 100$$

Concentration	Number of live nauplii	Number of live nauplii
	Day 1 (12 PM)	Day 2 (12 PM)
0	10	10
1 mg/mL	10	3
100 µg/mL	10	3
10 µg/mL	10	3
1 µg/mL	10	10

DISCUSSION

The present study shows that hatched nauplii is unique for the preliminary cytotoxicity study. Most researchers prefer this method to use. Although, another method is the inhibition of hatching of the eggs (Carballo et al., 2002) which is similar to brine shrimp lethality assay for marine natural products, but seems to be less sensitive to detect toxicity of macroalgae extracts.

Several factors are to be considered before starting this assay. Each egg looks like a small grain of fine sand. A teaspoonful contains thousands of eggs. The eggs will remain viable for several years if kept cool and dry. The egg will hatch into nauplii in presence of salt solution. The percentage of salt solution varies (2-4%). Best result is obtained in presence of sea salt instead of iodinized salt or reagent grade salt.

The use of seawater is recommended. If it is not available, distilled water can be used. Tap water should not be used if it contains chlorine. In that case, the tap water must be unused overnight in order to remove chlorine.

The pH adjustment of the water is important for hatching the eggs. The optimal pH range is 8.0 ± 0.5. The pH should be adjusted using sodium hydroxide or sodium carbonate, to avoid lethality of the nauplii caused by decrease of pH during incubation.

The eggs will float on the surface. Penetration of the salt solution into the eggs initiates the development into the nauplii. Oxygen is required. This is obtained directly from the air by the floating eggs. At room temperature, nauplii will emerge about 20-30 hours after wetting. However, at 30°C, the emergence time is about 20 hours.

Presence of light does not have greater hatching viability than the nauplii exposed to natural light or no light. Completely dark situation has hatching viability of 90%.

After 36–48 hours incubation at room temperature (28–30°C) under conditions of strong aeration and continuous illuminations, the nauplii hatched within 24-30 hours. The nauplii move freely in the columns of liquid. It usually takes a position at the top air-liquid interface.

Air is bubbled through the plastic tube extending to the bottom of the jar to keep all the eggs in continuous motion.

During the study period, the nauplii do not receive food. The death of the nauplii may be due to the effect of the plant extract or starvation. To ensure the mortality effect of plant extract, control sample containing only nauplii is also used. In any case, hatched nauplii can survive for up to 48 hours without food because they still feed on their yolk-sac.

Ordinary yeast may be added as food source of the nauplii. It is added in every second day.

Michael et al (1956) used low concentration of sodium chloride in the culture solution for nauplii. In addition, calcium, potassium and magnesium were used. The pH of the solution was adjusted to 10.0 using sodium hydroxide. Subsequently, the composition of the culture solution was modified. The optimum temperature for culture is 30ºC. This method was originally used to detect the effectiveness of pesticides.

Nauplii show the body movement in response to light, either toward the source of light (positive phototaxis). Positive phototaxis was greatest at the low intensity of 2.8^{12} quanta·m^{-2}·s^{-1}.

Bioactivity of crude extracts can be detected by this brine shrimp lethality assay. It is a guide for active cytotoxic and antitumor agents. This simple and effective procedure permits convenient standardization.

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PRECAUTION

False positive result may be given due to the solvent used. DMSO is the preferred solvent used. Other solvent may be used cautiously.

CONFLICT OF INTEREST

The manuscript was reviewed even one of the authors is directly involved in the editorial team of this journal.

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