

**A MODIFIED MICROPLATE CYTOTOXICITY ASSAY  
WITH BRINE SHRIMP LARVAE (*Artemia salina*)**

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**Summary**

*Artemia salina* nauplii are widely used in toxicity assays of synthetic and natural products. Microassays are particularly useful because they allow testing of several products, serially diluted, in a sole microplate. Despite this, following the original method, we have observed systematically about 30% mortality in nontreated controls. Thus, we introduced several modifications that improved the viability percentage and motility of nauplii. To standardize the present method, we used a dilution series (100.00–0.19 µg/ml) of potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) and calculated the concentration that killed 50% of the nauplii (LC<sub>50</sub>) by PROBIT analysis. The above modifications allowed us to obtain nauplii cultures that have systematically 100% viability and good motility until 44 h after hatching. The LC<sub>50</sub> of K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> (12.60 µg/ml) was similar to that previously reported (12.50 µg/ml). We propose this modified microassay to determine the toxicity of synthetic and natural products, as an alternative that allows the biological material to remain in better condition throughout the entire assay, and good reproducibility of results.

**Key words:** microassay, brine shrimp, *Artemia salina*, toxicity, natural products, synthetic products.

*Artemia salina* L (Artemiidae), commonly known as the brine shrimp, is a small crustacean used to determine the toxicity of a wide variety of products. This assay is considered as one of the most useful tools for a preliminary assessment of toxicity (1). It has been used for detection of fungal toxins (2), and to determine the toxicity of plant extracts (3), heavy metals (4), cyanobacteria (5), algae (6), and dental materials (7). Furthermore, a microplate assay has been described (8). This micromethod facilitates the testing of diverse products serially diluted in 96-well microplates and volumes of 200 µl; in the former method, individual 5 ml vials are used.

The microplate assay allows determination of the medium lethal concentration (LC<sub>50</sub>) of practically any soluble product in natural or artificial seawater, in a simple, cheap, and rapid manner. Nevertheless, following the original microplate method (8), we have observed systematically about 30% mortality in nontreated nauplii cultures. In addition, larvae have low motility and cannibalism is frequently observed. In this article, we introduce a set of modifications to improve the biological quality of nauplii throughout the entire assay.

### **Methods**

The procedure described below is a modification of two previously published methods (8, 9).

#### **Hatching.**

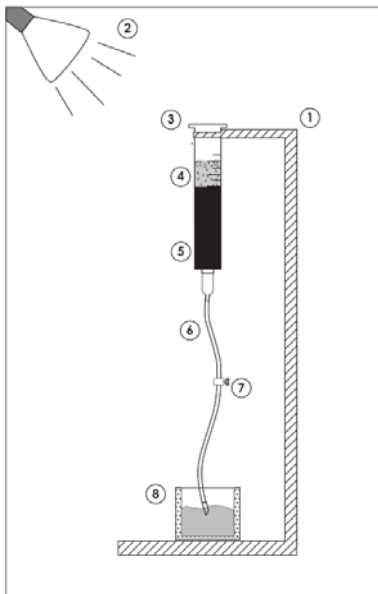
A total of 100 mg of dry brine shrimp eggs (Great Lake, Salt Lake City, UT, USA) were spread into a Hatch n' Feeder the Brine Shrimp Corral (ToM Oscar Enterprises, Inc., CA, USA), which was submerged into a glass tank, designed for thin layer chromatography, with the following inside dimensions: 27.5 cm × 27.5 cm × 7.5 cm (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The tank contained 3 L artificial seawater (40 g salt [Instant Ocean from Aquarium Systems, Mentor, OH, USA]/L), supplemented with 6 mg dried yeast (BD Bioxon, Becton Dickinson de México, SA de CV, Cuautitlán Izcalli, Edomex, México)/L. The pH was adjusted to 8.5 with 0.1 M Na<sub>2</sub>CO<sub>3</sub> (Sigma-Aldrich). Air was then bubbled through the liquid. The pressurized air was taken from the laboratory facilities, and the air pressure into the corral was regulated with a valve. The eggs were incubated in darkness for 36 h at 25 °C.

#### **Larvae Collection and Concentration.**

Nauplii were separated from empty shells and nonhatched eggs using the aforementioned corral. Phototropic nauplii were attracted with a light source to the top of the tank and transferred with a Pasteur pipette into a sterile polystyrene Petri dish (60 × 15 mm diameter; BD Biosciences, San Jose, CA, USA) containing 5 ml fresh artificial supplemented seawater; from there they were transferred into a nauplii concentrator that we designed and constructed. This item consisted of a 10 ml plastic disposable syringe without a piston (BD Biosciences) that was wrapped with carbon paper, leaving uncovered the upper quarter of the syringe length, to hold phototrophic nauplii. The syringe was held to a vertical support with a universal clamp and its distal tip connected to a plastic flexible pipe (about 20 cm long) that has a valve in the middle of its length to drain fluid and concentrate those nauplii caught in the Petri dish. The tip of the pipe was introduced into a 25 ml beaker that contained the drained supplemented artificial seawater. A light source was put in the side of the syringe to attract nauplii to the uncovered portion of the syringe (Figure 1).

**Bioassay**

The microassay was performed in sterile, flat-bottom 96-well polystyrene microplates covered with a low evaporation lid (Costar, Corning, NY, USA). All wells were filled with supplemented artificial seawater. The wells located in the outer perimeter of the microplate were filled with 200  $\mu$ l, and the remaining wells with 100  $\mu$ l. The latter were distributed as follows: to three rows, with 10 wells each,  $10 \pm 1$  nauplii suspended in 100  $\mu$ l supplemented seawater were added. These organisms were taken from the previously described concentrator. These cultures corresponded to 100% viability controls. In the first well of the remaining three rows, 100  $\mu$ l of a  $K_2Cr_2O_7$  stock solution (0.4 mg/ml of supplemented seawater) was dispensed, which was used as a standard (1,10). Starting from these, three dilution series (100.00–0.19  $\mu$ g  $K_2Cr_2O_7$ /ml of supplemented seawater) were performed. Then,  $10 \pm 1$  organisms from the concentrator, suspended in 100  $\mu$ l supplemented seawater, were added. The microplates were covered and incubated in a shaker (Clinical Rotator, Eberback Co., Ann Arbor, MI, USA) at 100 rpm/min in a sealed chamber, having 100% humidity with a soft light source for 24 h at 25 °C. Nauplii were observed using a stereoscopic microscope (Mod. 570 American Optical Co., Buffalo, NY, USA) with 12.5 $\times$  magnification. Larvae that remained immobile for more than 10 s were considered dead (11). Then, 50  $\mu$ L of absolute methanol (JT Baker, Mallinckrodt Baker SA de CV, Xalostoc Edomex, México) was added to each well, and after 15 min the total number of shrimp larvae in each well was counted. The assay was performed twice (n = 30).



**Figure 1.** Concentrator of phototropic nauplii. Numbers in the scheme correspond to the following parts of the item: (1) vertical support and universal clamp; (2) light source; (3) syringe without piston; (4) phototropic nauplii; (5) carbon paper cover; (6) drainage pipe; (7) valve; and (8) reservoir of drained seawater.

**Data analysis:**

LC<sub>50</sub> values were determined by applying a PROBIT analysis with the aid of the SPSS 10.0 computation package (SPSS Inc., Chicago, IL, USA).

**Results**

The hatching of nauplii started at approximately 16 h under the described conditions. In the bioassay, the viability of nontreated nauplii was 100% and any alteration in their behavior was observed, especially low motility or cannibalism. Mortality was proportional to the potassium dichromate concentration. The LC<sub>50</sub> of potassium dichromate on *A. salina* was 12.60 µg/ml and the confidence interval (CI) 95% = 10.37- 15.39 µg/ml (Figure 2).

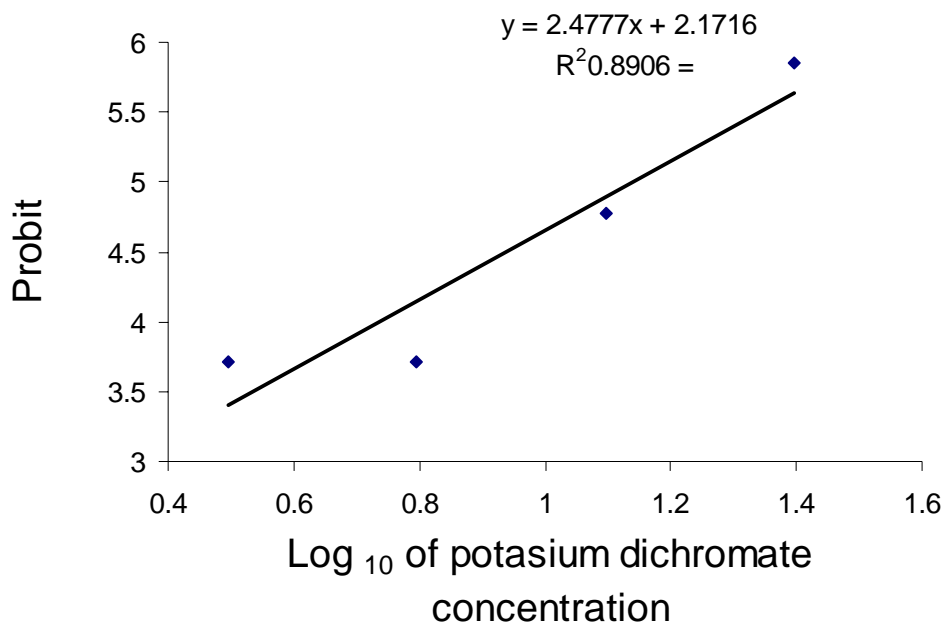


Figure 2. Dose-mortality lineal plot. Obtained with a Probit analysis using various concentrations (µg/ml) of potassium dichromate as lethal agent on *A. salina* nauplii.

### Discussion

For the purpose of this study, the age of the brine shrimp was reported relative to the initial hydration of the eggs. We chose potassium dichromate as a standard because of its well-known toxicity in this kind of assay (1,10). The results allowed us to control the microassay and to analyze the data in an easy and quick manner. We propose this modified microassay as an alternative that allows the maintenance of nauplii in good condition throughout the entire experiment, having a good reproducibility of results. Using potassium dichromate as a standard, we found a LC<sub>50</sub> (12.60 µg/ml) practically equal to that previously reported by González-Pérez [12.50 µg/ml (10)]. Both of these values are included in the range reported by other workers for chromium on freshwater organisms [0.067–59.9 µg/ml (12)].

During the past 25 years, the brine shrimp assay has been widely used to test the toxicity of a great variety of products. It was recently shown that the LC<sub>50</sub> of 20 medicinal plant extracts determined using brine shrimp larvae correlated well with the corresponding oral lethal dose (LD<sub>50</sub>) in mice. Thus, *A. salina* nauplii could provide a simple method to predict the acute toxicity of plant extracts (9), avoiding the use of laboratory animals as demanded by an increasing number of organizations across the world (13). Nonetheless, the possibility of substituting laboratory animals with *A. salina* needs to be further investigated.

### Acknowledgements

The authors wish to gratefully acknowledge to Dr. Alicia Lagarto-Parra and M.S. Silvia González-Hernández for their technical assistance and B.S. Myrna Yadira Said-Martínez for the art work. This work was partially supported by IMSS/FOFOI, grants 2002/216 and 2005/9/I/508.

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