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Screening natural products against *Artemia salina*

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Abstract

This protocol outlines a bioassay method using *Artemia salina* (brine shrimp) for the analyses of natural product extracts and fractions. The brine shrimp bioassay serves as a simple and cost-effective tool for toxicity screening and/or for preliminary cytotoxicity testing.

Materials

Solvents and solutions

- *Artemia salina* cysts
- Dimethyl sulfoxide (DMSO)
- Deionized water
- Iodized NaCl salt
- Potassium dichromate ($K_2Cr_2O_7$)

Equipment

- 96-well plate
- Air pump
- Incubator
- Light source
- Pipette (1-10 and 1-100 µL)

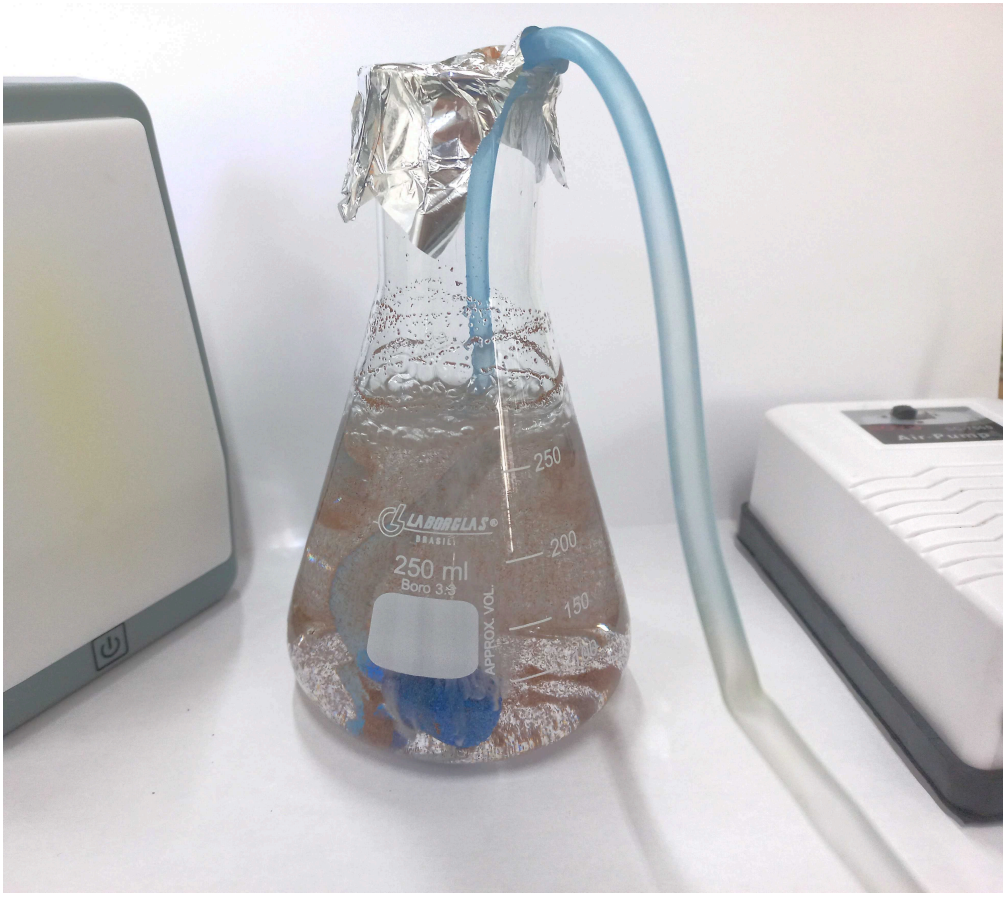
Background

1 *Artemia salina* is a microcrustacean adapted to high salinities that, under appropriate conditions, can hatch in 24 to 36 hours, becoming a nauplius without complete formation of the digestive tract. Due to its easy of handeling, rapid development, and low cost, it is adopted as a model for natural product screening and ecotoxicological tests. It is utilized for detecting toxic substances (Campos *et al.*, 2024; Médice *et al.*, 2024) and for preliminary cytotoxicity testing (Albuquerque *et al.*, 2014; Solis *et al.*, 1993). Toxicity is one of the main factors leading to the abandonment of drug candidates. Therefore, the use of preliminary methods for its assessment is essential in bioprospecting, helping to prevent the discontinuation of costly studies. From a different perspective, preliminary cytotoxicity results may justify additional, more complex studies in anticancer drug discovery programs. Limitations of this assay include the requirement for a saline environment, which can lead to issues related to sample solubility, and low sensivity (Göethel *et al.*, 2022). Nevertheless, when conducted under optimal conditions, it is a highly reproducible model and an effective test for preliminary toxicity results (Salay *et al.*, 2024).

Day 01

2 **Preparing brine shrimp for hatching**

- In a 250 mL Erlenmeyer flask, add 250 mL of deionized water, 4.8 g of iodized NaCl salt, and 0.25 g of dehydrated *Artemia* cysts.
- Incubate for 24 hours at 25 °C with constant agitation by aeration and lighting.



3 **Sample preparation and controls**

- Samples: solubilize samples in DMSO, according to final test concentration. Note: Our results suggest that *A. salina* nauplii are resistant up to 1% DMSO in final test concentration.

  Suggested test concentration (final concentration in well) for preliminary, single point concentrations tests:

  - Natural product extracts: 1000 µg/mL;
  - Natural product fractions: 100 µg/mL.
- Positive control: dissolve $K_2Cr_2O_7$ in deionized water to obtain a concentration of 20 mg/mL (final concentration in well: 200 µg/mL).
- Negative control: DMSO 100% (final concentration in well: 1%).

Day 02

4 **Exposing *Artemia salina* nauplii to test samples and controls**

- Sterilize a clean 96-well plate under UV light.
- Transfer 99 µL of solution containing 15-20 nauplii, without cysts, in each well of the 96-well plate.
- Transfer 1 µL of sample solution (or controls) to each well.

  Every sample is tested in triplicate and controls in quintuplicate

  Negative control: DMSO 100%

  Positive control: $K_2Cr_2O_7$
- Count and register dead *Artemia salina* that are eventually found in wells prior to incubation.
- Incubate in the absence of light at 25 ºC for 24 hours.

Day 03

5 **Assessing mortality**

- Count the number of dead *A. salina* nauplii in each well. A nauplius is considered dead if it remains completely motionless for at least 10 seconds. Use an optical microscope or magnifier lense to assist in observation.
- After recordingthe number of dead individuals, add a few drops of ethanol to kill the remaining live nauplii. This step ensures an accurate count of total number of organisms.
- Finally, count and record the total number of nauplii in each well.
- Be sure to exclude from the final calculations any nauplii that were found dead on Day 2, prior to the start of incubation.
- Calculate mortality using the formula below:

$$Mortality = \left(\frac{Dead\ nauplii\ in\ one\ well}{Total\ nauplii\ on\ the\ well}\right) x\ 100$$

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