Artemia salina lethality and histopathological studies on Bacopa monnieri leaf extract

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ABSTRACT

Bacopa monnieri leaf aqueous and methanolic extractions at 1, 3, 5 and 24 hours were determined for the highest amount of total phenolic compound and used for evaluating the toxicity test against Artemia salina at varied concentrations as 0, 5, 50, 100, 500, 1000, 2,500 and 5,000 ppm, by determining the median and 90% lethal concentration, LC_{50} and LC_{90}, respectively, within 24 hours. The result revealed that the total phenolic compound measurements in 1, 3, 5 and 24 hours were 70.58±2.74, 70.52±1.63, 67.11±2.51 and 79.01±2.87 mg of gallic acid equivalent per gram of aqueous extractions, and 37.88±1.57, 43.08±2.74, 45.86±2.68 and 55.31±3.60 mg of gallic acid equivalent per gram of methanolic extractions, respectively. Due to the highest amount of total phenolic compound, the 24-hour aqueous extract of B. monnieri leaf expressed the 24-h LC_{50} and LC_{90} values in A. salina and they were 3,577.90 and 6,440.22 ppm, respectively. Under histological analysis, pathological lesions like cellular swelling, and elongation of the epithelial cells and edema were observed. More additional, cells protruding into the lumen and absence of microvilli were also found.

Key words: Artemia salina, Brine shrimp, Bacopa monnieri, Histopathology, Leave, Total phenolic compound.

INTRODUCTION

The current trend in toxicology is the reduction of experiments on higher vertebrates (Doke and Dhawale, 2015). The genus Artemia commonly known as the brine shrimp, is a small crustacean, which is an important model organism in scientific research (Nunes et al., 2006). Artemia is characterized by common features such as small body size, reproduction strategy, short life cycle, adaptability to wide ranges of salinity and temperature (Gajardo and Beardmore, 2012). The brine shrimp toxicity assay was proposed and developed by Michael et al. (1956). The brine shrimp lethality assay is considered one of the most useful tools for the preliminary assessment of general toxicity (Hamidi et al., 2014). It has been established as a safe, practical and economic method to determine the bioactivity of plant constituents. This test has also been used to screen the toxicity of heavy metals; as well as screening of nanoparticles (Rajabi et al., 2015). But no information about the histopathological changes in this crustacean after exposure the test substances. The medicinal plants or herbs are widely studied for their potential uses in Ayurvedic medicine to treat variety of diseases (Tripathi et al., 2012). In recent years, B. monnieri has received much attention worldwide due to its wide spectrum of pharmacological activities. B. monnieri is the small smooth creeping fleshy plant with the numerous branches. It grows to a height of 60-90 cm and its branches are 5-35 cm long (Fig. 1, Charoenphon et al., 2016). Phytochemical analysis has shown that B. monnieri contains bacosides, bacopasides, and bacopasaponins (Agrawal et al., 2006), flavonoids, glycosides, and saponins. B. monnieri has been used in ethnomedicine (Verma, 2014). The traditional uses or phytochemical properties of B. monnieri are antidepressant, antidiabetic, anti-inflammatory, antimicrobial, and antioxidant activities (Subasri and Pillai, 2014). The present research work was conducted to evaluate the Artemia salina lethality and histopathological studies on Bacopa monnieri leaf extract.

MATERIALS AND METHODS

Plant collection and extraction: Fresh, mature, green leaves of B. monnieri were washed with tap water and air dried in shade for 24 hours and dried in a hot air oven at 70°C for 6 hours, and crushed with a blender. The extraction procedure was determined by the method of Kjanijou et al. (2012) and Jiraungkoorskul (2016). Five grams of leaf powder were extracted with 100 ml of distilled water and methanol on a shaker at 180 rpm for 1, 3, 5, and 24 hours at room temperature. The whole mixture was then filtered through a fresh gauge plug, and centrifuged at 4,000 rpm for 10 minutes. Finally supernatant was filtered with a Whatman number 1 filter paper, the clear filtrate used as a stock solution.

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for total phenolic compound measurement and lethality bioassay.

**Total phenolic compound measurement**: Total phenolic compound was determined using Folin–Ciocalteu reagent according to methods of Jiraungkoorskul (2016) and McDonald et al. (2001) with modifications. Briefly, the 50 µl of the aqueous and methanolic extraction in each time (1, 3, 5 and 24 hours) was mixed with 250 µl of 10% Folin-Ciocalteus and 200 µl of 0.7 M sodium carbonate then add distilled water until 5 ml and incubated at room temperature for 2 hours in the dark room. The mixture was measured at 724 nm by using a spectrophotometer. Quantification was based on the standard curve of the gallic acid and expressed as gallic acid equivalent.

**Brine shrimp lethality bioassay**: The brine shrimp lethality assay was assigned to determine the toxic effect of plant extract. It followed the method by Meyer et al. (1982). Due to the highest amount of total phenolic compound, the required concentrations (0, 5, 50, 100, 500, 1000, 2500 and 5000 ppm) were prepared through mixing up of the 24 hour aqueous extraction with variable amounts of 2.5% NaCl. Ten *Artemia salina* were added into five replicates of each concentration of the leaf extract. The bioassay was maintained at 26±1°C throughout the test. The mortality was recorded for a maximum of 24 hours of exposure. They were considered dead or moribund if they stopped moving for a prolonged period even after gentle probing with a small spatula. The LC$_{50}$ was analyzed by the probit method of Finney (1971) using the SPSS 18.0 (Statistical Package of Social Sciences) software. It estimated the lethal concentration and the slope of the regression line with its confidence interval (p<0.05).

**Specimen preparation for light microscopic study**: For histopathological analysis, the brine shrimp was treated in the LC$_{50}$ concentration of the 24 hour aqueous plant extraction. Under the histology technique, only live *A. salina* was examined. The procedures were performed following the methods of Kjanijou et al. (2012) and Jiraungkoorskul (2016). Briefly, *A. salina* was fixed in 10 % buffered formaldehyde for 24 h, dehydrated through a graded series of ethanol, and cleared with xylene solution. It was embedded in a block using melted paraffin at the embedding station. The paraffin blocks were sectioned at 5 µm thickness using a rotary microtome, and stained with Harris’s hematoxylin and eosin (H&E). The glass slides were examined for abnormalities using the Olympus CX31 light microscope and photographed by a Canon EOS 1100D digital camera.

**RESULTS AND DISCUSSION**

The total phenolic compound from leaves of *B. monnieri* in each time extraction at 1, 3, 5 and 24 hours were 70.58±2.74, 70.52±1.63, 67.11±2.51 and 79.01±2.87 mg of gallic acid equivalent per gram of aqueous extractions, and 37.88±1.57, 43.08±2.74, 45.86±2.68 and 55.31±3.60 mg of gallic acid equivalent per gram of methanolic extractions, respectively (Figure 2). The properties of the aqueous leaf extract of *B. monnieri* against *A. salina* were presented in Figure 3. The result of brine shrimp assay was expressed in percentage of mortality. The dose dependent mortality was observed, as the percent of mortality (x) was positively correlated with the concentration (y) of the leaf extract as evident from established regression equations (y=71.558x). The percentage mortality increased as the concentration of aqueous extract of *B. monnieri* increased. The 24-hour aqueous extract of *B. monnieri* leaf expressed the 24-h LC$_{50}$ and LC$_{90}$ values in *A. salina* as 3,577.90 and 6,440.22 ppm, respectively. *B. monnieri* showed a significant effect against brine shrimp. The correlation ($R^2$) between concentration and mortality was 0.9348.
The general morphology of an adult *A. salina* was an elongated body with two eyes in front, a linear intestinal tract, and 11 pairs of legs alongside the body (Fig. 4A). In the control group, the alimentary tract was a tubular structure which is composed of three clearly distinguishable pats, i.e., the foregut, midgut and hindgut that were freely suspended in hemolymph. The epithelium lining of the entire gut consisted of a single cell layer. Enterocytes of the foregut and hindgut were cuboidal and lined by a thin cuticle, whereas midgut enterocytes were cuboidal to columnar and possessed an apical brush border (Fig. 4B). The histopathological lesions after exposure to *B. monnieri* at 24 hours extraction as 3,577.90 ppm were observed primarily in the midgut. Lesions with deformation or elongation of the epithelial cells were observed (Fig. 4C). Additionally, there were vesicles in the cytoplasm of the epithelial cells (Fig. 4D). Moreover, cells protruding into the lumen, blebbing cells and absent microvilli were also observed (Fig. 4E).

Several research workers suggested that medicinal plants extracts have the natural antioxidant activities (Yanishlieva *et al.*, 2006; Surveswaran *et al.*, 2007; Veeru *et al.*, 2009) and also have direct relationship between antioxidant activity and total phenolic content (Conforti, *et al.*, 2009; Piluzza and Bullitta, 2011). The present result revealed that the total phenolic compound measurement of *B. monnieri* in 1, 3, 5 and 24 hours extraction was 70.58±2.74, 70.52±1.63, 67.11±2.51 and 79.01±2.87 mg of gallic acid equivalent per gram of aqueous extractions, and 37.88±1.57, 43.08±2.74, 45.86±2.68 and 55.31±3.60 mg of gallic acid equivalent per gram of methanolic extractions, respectively. These results were in agreement with earlier reports. Alam *et al.* (2012) extracted *B. monnieri* with methanol for 7 days and reported the total phenolic compound measurement was 21.54 mg GAE/g of extract. Tupe *et al.* (2013) extracted 1 g of *B. monnieri* with 100 ml methanol for 3 hours and reported the total phenolic compound measurement.
Fig 4: *Artemia salina* (A) gross morphology; (B) longitudinal section of midgut showing midgut cells in cuboidal shape overlay with brush border (*) and histopathological studies on *Bacopa monnieri* leaf extract showing (C) elongation of the epithelial cells; (D) vesicles in the cytoplasm of the epithelial cells (arrows); and (E) cells protruding into the lumen, blebbing cells with absence of microvilli (H&E 1000X).

The compound measurement was 12.94 mg GAE/g of extract. Moreover, Shah et al. (2012) extracted 70 g of *B. monnieri* with 1000 ml water for 72 hours and reported the total phenolic compound measurement was 58 mg GAE/g of extract. It supported that aqueous extract had total phenolic content more than methanolic extract, that similarly with the present study.

Using brine shrimp lethality bioassay tested the toxic effect of the aqueous extract of leaves of *B. monnieri* and found to show a little toxicity as expressed the 24-h LC50 and LC90 values in *A. salina* as 3,577.90 and 6,440.22 ppm, respectively. The lethal concentration value of this plant suggested that it is a relatively nontoxic plant. The classification of toxicity based on LC50 values stated by the Organization for Economic Co-operation and Development are as follows: very toxic ≤ 5 mg/kg; 5 > toxic ≤ 50 mg/kg; 50 > harmful ≤ 500 mg/kg; and 500 > no label ≤ 2000 mg/kg (OECD, 2002). Therefore, an LC50 of more than 2000 mg/kg of plant extract is an indication that *B. monnieri* leaf extract does not possess any toxic effects. Each of the different concentrations samples showed different mortality rates. When graphed, the concentrations versus mortality percentage showed an approximate linear correlation. These results were not in agreement with earlier reports because most of the studies on *B. monnieri* toxicity have been done using crude extracts. D’Souza et al. (2002) reported that bacoside showed the maximum activity with a LC50 of 38.3 µg/ml. The researchers have reported the LC50 and LC90 of *B. monnieri* in different doses, time and solvent extraction. This activity could be explained by the phenols, flavonoids and bacosides present in the extract. The morphological characteristics of the digestive tract of *A. salina* in the present study was similarly with the earlier reports (Kikuchi, 1971; Hootman and Conte, 1974; Gunasekara et al., 2011). In this study, histopathological alterations were observed in the midgut including edema, swelling, and the deformation or elongation of epithelial cells. Moreover, cells protruding into the lumen and absent microvilli were also found in some areas. Due to rare information about Artemia histopathological study, these histopathological changes were in similar pattern with our previous reports (Kjanijou et al. 2012; Pavananundt et al. 2013), suggested that the substances led to morphological damage in the epithelial cells of the midgut, which is likely where these substances are absorbed. Regardless of the type of substance used, the similarity of the detrimental changes in the organism indicates that these alterations are a common response to cellular toxicity (Kjanijou et al. 2012; Pavananundt et al. 2013). In conclusion, Artemia lethality and histopathological studies can be the alternative tool for evaluation of the natural product or chemical substances.

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