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Brine Shrimp lethality assay (BSLA) of mixed micro algae extracts from Tilapia fish ponds

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Abstract

There are lots of natural products could assist as the starting point in the invention of modern medications due to their biological and pharmacological activities. Microalgae is getting broad attention recently as one of the renewable sources of biomass. However, some natural products are identified to carry toxicological properties as well. To achieve a safe treatment with natural products, studies focusing on both pharmacology and toxicity of medicinal natural sources have been conducted recently. In this study, the methanolic extracts of mixed microalgae were studied for their toxicity with brine shrimp (*Artemia* sp.) lethality assays. Brine shrimp have been extensively applied in bioassay for a variety of toxic substances and simplifying the isolation of bioactive compounds. The LC_{50} from six samples calculated were ranged from 403.98 ppm to 595.79 ppm. The extracts exhibited cytotoxic activity against the brine shrimp and considered as containing active or potent components because their LC_{50} values were less than 1000 ppm. The toxicity test also revealed that mixed microalgae can be safely used as feed enhancement for animals.

Keywords: *Artemia* sp., bioactive compounds, mixed microalgae, toxicity

Introduction

For decades, human has been using extracts from plant materials for the treatments of many diseases. Plants always being a good source of biologically active compounds. The demand of remedial plants and their formulated products is growing greatly in many countries. Therapeutic plants are an essential element of ethnomedicine for both human and animal uses all over the world. They have been in use for centuries to treat disease and improve health. (Gesler, 1992). Among all the natural products, microalgae are one of the main interesting sources. Microalgae are known to produce a range of valuable compounds as a result of their adaptation to different environmental conditions. Malaysia is a tropical country that has an extensive coastline that can be exploited as an ideal habitat for microalgae cultivation on a large-scale production. Research on microalgae in Malaysia is not new. This is evidenced by several publications on microalgae conducted in Malaysia focusing on identification of microalgae strains, utilization of microalgae for wastewater treatments and bioindicators. Studies about natural chemical compositions and chemical defence of microalgae for few years back has come out in the isolation of more than 15000 novel compounds that have been proved to have bioactive characteristics (Metting and Pyne, 1986; Cardozo *et al.,* 2007; Rodríguez-Meizoso *et al.,* 2010). Microalgae are able of producing a diversity of bioactive compounds because they possess a multitude of physiological, biochemical and molecular strategies to survive with stress.

The metabolites produced from microalgae show an extensive range of biological activities such as antitumor, antiviral and antioxidant effects. Toxic metabolites from microalgae also known as phycotoxins. The compounds usually synthesize by dinoflagellates and cyanobacteria, especially those causing harmful algae blooms in aquatic environments. Extreme growth of dinoflagellates may cause discolouration to the sea while growth of cyanobacteria due to excessive nutrition such as nitrogen and phosphorus may cause eutrophication (Chu, 2012). The most common toxins from freshwater microalgae are produced by cyanobacteria such as *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc* species (Chu, 2012). The toxins produced by some microalgae demonstrate the highly effective bioactivity of the compounds they may contain. The cytotoxic activity of these compounds has been applied in anticancer treatments (Sirenko and Kirpenko, 1999).

In order to achieve a safe treatment with any plant products, numerous researches have been focused on the pharmacology and toxicity of medicinal plants. Toxicity is attributed to certain active principles found in plants; these chemical substances interact with living systems and affect normal processes. All chemicals can cause harm to organisms at some level of exposure. Toxicology testing, also known as toxicity testing or safety assessment is

conducted to determine the degree to which a substance can damage organisms. It is frequently done by researchers using standard test procedures to fulfil with governing rules, for example for medicines and pesticides.

Toxicity test are crucial to determine the lethality of materials and to investigate the harmless concentration of drugs to be consumed as medicine (Hood, 2009). One of the methods which commonly used to test the toxicity of plant materials is brine shrimp (*Artemia* sp.) lethality assays (BSLA). The shrimp lethality assay was proposed by Michael *et al*., (1956), and later developed by Vanhaecke *et al*., (1981), and Sleet and Brendel (1983). The benefit of using the brine shrimp in toxicity testing is that the shrimps has a lot of homogeneity in eggs and in newly hatched nauplii which are very sensitive to chemicals. This method also requires a small amount of test material (Pisutthanan *et al*., 2004). Furthermore, the positive correlation between Meyer's toxicity scale for *Artemia salina* with Gosselin, Smith and Hodge's toxicity scale for higher animal models confirmed that the brine shrimp lethality assay is an excellent predictive tool for the toxic potential of natural products extracts in humans.

Materials and Methods

Samples collections
Six samples of mixed microalgae were sampled from different ponds of tilapia fish pond located in Tapak Ternakan Ikan, Universiti Putra Malaysia (UPM). Mixed microalgae were collected using flocculation method and 10% ferric chloride was used as the flocculant. The collected samples were washed for three times and kept in -80ºC for further analysis.

Preparation of extracts from mixed microalgae
After sampling, the mixed microalgae were freeze-dried and turned to powder form. Ten grams of mixed microalgae samples were soaked into 250 mL of 95% methanol. Then, the mixtures were shaken every 30 minutes for six hours to make sure it is well-mixed. After that, the mixtures were kept for 48 hours in room temperature before next procedure. Later, the mixtures were filtered using Whatman filter paper. The filtrate was discarded and the solutions remaining were evaporated using rotary evaporator with the temperature of 40 ºC.

Preparation of test solutions with samples of mixed microalgae
The dilution procedure was done by following McLaughlin *et al.,* (1991). Forty miligrams of each of the test samples were taken and dissolved in distilled water (dH_2O) and finally the volume was made to 20 mL with sea water. Therefore, the concentration of the mixed microalgae extract was 2000 µg/ml. From that concentration a series of dilution (1000, 500, 100, 10 and 1 µg/mL) were prepared with sea water. Control groups consisting of only sea water used in cytotoxicity study to validate the test method and ensure that the results obtained were only due to the activity of the test samples and not due to the starvation of the nauplii. In control groups, 20 nauplii were put into test containing only 20 mL of seawater. If the brine shrimps in these test tubes show a rapid mortality rate, then the test is considered as invalid because the nauplii might die due to some other reason and not because of the cytotoxicity of the compounds.

Hatching of Artemia cysts
In this study, the brine shrimp cysts (Ocean Star International AOSI0116) were obtained from the Laboratory of Ecotoxicology, Department of Biology, UPM. About one gram of the brine shrimp cysts were put into the sea water for hatching. The container used for brine shrimp hatching, consists of dark and illuminated area. This will enable the hatched *Artemia* sp. nauplii to migrate to the illuminated compartment (Sharma *et al*., 2013; Pisutthanan *et al*., 2004). The container was filled with artificial sea water. For every one litre of water, 38 grams of artificial salt were used. The system must be supplied with an aeration system. A regular air flow with average pressure and proper lighting supply is vital for the hatching process. For a successful hatching, the artificial seawater must be at least 90% saturated with oxygen (Vanhaecke *et al.,* 1981). The optimal pH range for the seawater that is essential for the hatching of the eggs is 8.0 ± 0.5 (Sarah *et al.,* 2017). The pH value is very crucial factor for the successful hatching of Artemia eggs. If necessary, the pH should be adjusted using sodium hydroxide or sodium carbonate. Small amount of yeasts was added to the tank as feed to ensure the mortality of the nauplii is not due to starvation. The set up was left for 24 to 36 hours after most of the eggs were already hatched. The already hatched nauplii will migrate to the illuminated area.

LC_{50} determination
The extracts were prepared at five concentration levels namely, 1000, 500, 100, 10 and 1 µg/mL. Then, twenty newly hatched brine shrimp nauplii were put in each test tube, in which they were then exposed to the various concentrations of extracts. The survived nauplii will be counted under a dissecting microscope after 24 hours of

exposure to the tested sample. The percentage of lethality of the brine shrimp was calculated for each concentration. The concentration- mortality data were analysed. The relationship was expressed as a median lethal concentration (LC_{50}) value. The LC_{50} value represents the concentration of the chemicals that killed half of the subjects after a certain exposure period. For the data analysis in this study Microsoft Excel 2010 with additional features named XLSTAT was used to determine the LC_{50} values. The median lethal concentration (LC_{50}) of the test samples was obtained by plotting the percentage of the shrimps killed against the logarithm of the sample concentration (Meyer *et al*., 1982; Ahmed *et al.,* 2010) after 24 hours of exposure.

Results and discussion

Toxicity refer to adverse effects of drugs, metabolites or metabolites of any compounds. Although brine shrimp lethality assay (BSLA) is inadequate in determining the mechanism of action of the bioactive substances in the plant, it is very useful as it provides a preliminary screening that can be ascertained by further specific bioassay once the active compound has been isolated. As already mentioned in the introduction, *Artemia* sp. is one of the most convenient organisms for toxicity testing using BSLA. The principal of a plant or natural resources can be exploited as an anticancer drug is when the organisms contain compounds that are cytotoxic.

Table 1: **The number of brine shrimp nauplii that survived after treated with mixed microalgae extracts, the percentage of mortality and LC_{50} values.**

Samples	Concentrations (ppm)	Numbers of survived nauplii			Average	Mortality (%)	LC_{50} values
		Replicate 1	Replicate 2	Replicate 3			
1	1	19	20	20	19.67	1.67	595.79
	10	18	17	17	17.33	13.33	
	100	16	18	16	16.67	16.67	
	500	13	12	14	13.00	35.00	
	1000	4	9	3	5.33	73.33	
2	1	20	20	20	20.00	0.00	460.83
	10	19	20	18	19.00	5.00	
	100	17	17	16	16.67	16.67	
	500	10	13	11	11.33	43.33	
	1000	5	4	6	5.00	75.00	
3	1	20	20	20	20.00	0.00	475.34
	10	19	18	18	18.33	8.33	
	100	17	17	17	17.00	15.00	
	500	11	13	13	12.33	38.33	
	1000	3	6	4	4.33	78.33	
4	1	20	20	19	19.67	1.67	505.973
	10	19	19	19	19.00	5.00	
	100	18	16	17	17.00	15.00	
	500	14	13	13	13.33	33.33	
	1000	5	3	3	3.67	81.67	
5	1	20	19	20	19.67	1.67	403.980
	10	19	19	18	18.67	6.67	
	100	16	18	18	17.33	13.33	
	500	10	11	12	11.00	45.00	
	1000	3	3	5	3.67	81.67	
6	1	20	19	18	19.00	5.00	528.18
	10	19	18	18	18.33	8.33	
	100	16	17	15	16.00	20.00	
	500	12	14	10	12.00	40.00	
	1000	4	7	5	5.33	73.33	

The results in this study (Table 1) showed that all the methanolic extracts of the mixed microalgae were potent or active against brine shrimp. The degree of lethality was found to be proportional to the concentrations of the extracts tested. In present study, the lethality concentration (LC_{50}) of six samples of mixed microalgae extracts obtained were ranged from 403.98 to 595.79 ppm. The average value for all samples were 495.02 ppm. The variations of LC_{50} values in each sample might be due to the differences in the microalgae species in each sample. This because the mixed microalgae were sampled in different times. In different weather conditions, the species composition will be different. It is acknowledged that the species composition of mixed microalgae varies in each weather conditions due to variation in light intensity, photoperiods, temperature and nutrients concentration but the cultural conditions in this study does not trigger the bloom of toxic species. Using the criteria by Clarkson (2004) toxicity index, the toxicity of mixed microalgae obtained from this study is between low toxicity and medium toxicity. According to Meyer *et al.,* (1982), the crude plant extracts were considered toxic (active) if the LC_{50} value is less than 1000 µg/mL, while non-toxic (inactive) if it is greater than 1000 µg/mL.

However, the phenomenon of toxic microalgal bloom is not observed in this study. This is because the mixed microalgae were originated from tilapia fish ponds. The toxicity level or LC_{50} exhibited in this study is within the range of others microalgae such as *Chlorella* sp., and *Spirulina* sp. Rani *et al*., (2013) have conducted a study for toxicity on one of the most studied microalgae, *Spirulina* platensis. The study found that the LC_{50} of *S. platensis* extracted with methanol was 446.68 ppm (µg/mL). This value almost near to the LC_{50} values of mixed microalgae extracts found in this study. Meanwhile, the LC_{50} value of *Spirulina* sp. obtained by Yudiati *et al*., (2011) was 113.20 ppm.

A study regarding the toxicity of *Chlorella* sp. also conducted by Zahro' *et al*., (2014). *Chlorella* sp. have been used as supplements for many years and were produced in large-scale production. In the study, it was observed that the LC_{50} of *Chlorella* sp. ranged from 267.00 ppm to 415.00 ppm. It showed that the monoculture of *Chlorella* sp. may contained higher bioactive compound compared to present study. The ranges of LC_{50} values obtained from present study also in agreement with the study by Agustin and Kusmiati (2015) that studied on a rhodophyceae microalgae species, *Porphrydium cruentum*. *Porphrydium cruentum* was known as polysaccharides producers and have potential as antioxidant. The BSLA conducted by Agustin and Kusmiati (2015) showed that the LC_{50} values of 513.18 mg/L for exopolysaccharide and 521.82 mg/L for endopolysaccharides of *P. cruentum.*

From this study, we can suggest that mixed microalgae have potential to be used as useful therapeutic medicine and they are considered safe to be consumed as they exhibit almost similar LC_{50} values as *Spirulina* sp. (Rani *et al*., 2013) which has been used as supplements commercially. Besides for human supplements, microalgae also being used in aquaculture industry as fish feed. Many studies have demonstrated that the addition of small quantities (<10% of the diet) of algae in fish feed resulted in positive effects on growth performance and effectiveness of feed utilization (Valente *et al*., 2006; Mustafa and Nakagawa, 1995). Nasir *et al*., (2017) also found that the supplementation of *Spirulina* sp. improves the physical activeness level of the catfish. The concern about mixed microalgae toxicity does not arise because the amount incorporated in animal feed is normally below 5% of the feed composition. It acts as adaptogen which enhance animal health, survival, vitality and resistant to disease.

The results obtained from this study can be used a guide for the isolation of cytotoxic compounds from the methanolic extract of mixed microalgae. However, further researches are compulsory to identify the active materials available in the microalgae extracts. Before an ingredient can be produced to the market as food component for human consumption, market approval is obligatory from regulatory authorities. The potential products must be inspected to determine the potential for toxicity, the possibility for naturally occurring toxins from the cells of the organism itself, heavy metals as well as potential by-products, caused by the degradation of certain pathways or introduced during the production processing. For human consumption as potential superfood or supplements, the amount of dosage will be studied as each plant or any extracts have their own levels of dosage.

As the conclusions, the brine shrimp lethality assay (BSLA) provides initial screening data that can be followed up by further precise bioassays and the extracts of mixed microalgae in this study exhibit cytotoxic activity against the brine shrimp, considered as containing active or potent materials. This report may serve as a track to use mixed microalgae as new source of supplementation or medication which would be very useful.

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