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BRINE SHRIMP CYTOTOXICITY ACTIVITY FOR DIFFERENT PARTS OF *HYMENOCALLIS LITTORALIS*

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ABSTRACT

Spider lily or scientifically known as *Hymenocallis littoralis* is an ornamental plant which widely used as folk medicine among Malaysians. This study was conducted to achieve the correlation between brine shrimp lethality test (BSL test) and the anticancer activity in various parts of the *Hymenocallis littoralis* methanol extracts (HLM extract). HLM extract of root, stem, leaves, bulb, anther, and flower was used in this BSL test. Various concentrations of the extract and different time point were used as testing parameters in BSL test. The HLM leaf extract has the highest cytotoxic effect to the nauplii followed by bulb, root, anther, stem, and flower. HLM flower has least LC50 value compared to other parts and concluded it has fewer cytotoxicity effects on *Artemia salina*. It indicates that the extracts are toxic at low concentrations. Further investigation is needed to study on *in vivo* toxicity and cytotoxicity assay in mammalian cell lines of the extracts for its safe application to humans.

Keywords: *Hymenocallis Littoralis*, Various plant parts extracts, Brine shrimp assay

INTRODUCTION

Brine shrimp (*Artemia salina*, fairy shrimp or sea monkeys) lethality assay is commonly used to check the cytotoxic effect of bioactive chemicals [1]. It is a preliminary toxicity screening of plant extracts, fungal toxins, heavy metals, cyanobacteria toxins, pesticides, cytotoxicity testing of dental material and nanostructures [1]. Among other benchtop tests such as crown gall tumors inhibition on discs of potato tubes, frond proliferation inhibition in duckweed, yellow fever mosquito larvae lethality assay, BSL test is low cost, effective and the simplest test [1].

Artemia is a salt-water anostracan crustacean which is commercially available in dormant eggs (cysts) [2]. The larvae are about 22 mm long, are large enough to observe without high magnification and easy to hatch in small space in enormous amount. The eggs are readily available in pet shops and remain viable for years in dry condition. The eggs hatched in artificial seawater in 48 hours to provide nauplii (larvae) for experimental use [3]. The *Artemia* needs suitable salinity level (38 g/L) and warmness for the hatching process [4]. All lifecycle stages of *Artemia* have been exposed for bioassays [5].Laboratory animals such as rat, mice, and rabbit are widely used in toxicological evaluations. However,the high cost and animal's suffering caused by this test had led to the alternative toxicity studies using brine shrimp [6]. Additionally, several studies demonstrated that there is a good correlation between the results for the lethal concentration that kills 50% of the exposed population (LC_{50}) obtained with the Brine Shrimp Lethality Assay using *A. salina* and the results of the Acute Oral Toxicity Assay in mice [6,7]. Henceforth, currently, the brine shrimp lethality test is widely being used in the evaluation of the toxicity level of plant-based samples.

In addition, this test also used to correlate with cytotoxic and antitumor properties for bioactive compounds [8]. It has been observed that LD_{50} values for general cytotoxicity are about one-tenth LD_{50} values in the brine shrimp test [9]. In addition, National Cancer Institute (NCI, USA) has found a significant correlation between the brine shrimp assay and in vitro growth inhibition of human solid tumor cells [10]. It's a preliminary toxicity screen for further experiments on mammalian animal models. The plant is considered to have bioactive substances when the LC_{50} value is lower than 1000 µg/mL [4].

Hymenocallis littoralis is one of the well-known plant species for its medicinal properties. *H. littoralis* is grouped under *Amaryllidaceae* family.

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The various parts of the plants displayed potent biological activity such as antiviral, anticancer, antiparasitic, antibacterial and anti-fungal properties. This plant species has a high amount of alkaloidal content, volatile constituents, phenols, flavonoids and flavonols [11]. Numbers of alkaloids such as lycorine, narciclasine, lycoricidine (bulb) Pancratistatin (bulb and roots in small extent) trisphaeridine and others were isolated from this plant. Thus the preliminary cytotoxicity level of this plant extracts from six main parts was subjected to the brine shrimp lethality assay at three different time zone.

MATERIALS AND METHODS

Plant materials:

H. littoralis plants were bought from a nursery in Jalan Masjid Negeri, Pulau Pinang and the authenticity work was carried out by Mr. Shanmugam (Botanist) from School of Biological Sciences, Universiti Sains Malaysia. Each plant parts were carefully cut and washed with running tap water until removing the dirt prior to the drying process. Each of the plant parts (leaves, stem, root, bulbs, flowers, and another) were cut into small pieces and dried at 40 °C for a week to remove the moisture content. The samples were powdered using a blender (Panasonic, 380V). The powdered plant materials were extracted using methanol solvent by sonication technique to obtain the crude extracts. Ground plant materials were transferred into boiling test tubes and added 5 mL of methanol. The boiling test tubes were arranged in a test tube rack and kept in the sonicator. The sonication was run for 5 min and the extract was filtered through filter paper (Whatman No.1). The technique is repeated for three times using the residues. The filtrate was collected and concentrated in a rotary evaporator (RII0 Buchi, Switzerland) at 40 °C. The concentrated extract was dried in an oven at 40°C for three days to obtain consistent weight and freeze-dried for 2 days. The extracts were stored under refrigeration (-20°C) condition for further analysis [4].

Samples preparation:

H. littoralis plant parts' methanolic extracts (HLM extract) such as root, stem, leaves, bulb, anther, and flower were prepared according to fixed concentrations for brine shrimp lethality test [4]. The stock solution with the concentration of 10mg/mL was prepared freshly. Then working solution with concentrations of 15.63, 31.25, 62.50, 125, 250, 500, 1000 and 2000 µg/mL was prepared freshly. The samples were dissolved in artificial seawater at room temperature and pH 7.

Brine shrimp lethality assay:

Brine shrimp eggs were hatched in artificial seawater prepared from commercial sea salt (38 g/L) [4]. A lamp was placed above the open side of the tank to attract the hatched shrimps near to the tank wall. After 48 h, the shrimps matured as nauplii (*A. salina*) and were used for the assay. The brine shrimp lethality bioassay was carried out on the root, leaves, stem, flower, bulb, and anther of HLM extracts using standard procedure [3,12]. A two-fold serial dilution was carried out in salt water to obtain the working solution. Each concentration was tested in triplicate. A test tube containing salt water was used as a control. Ten milligrams of potassium dichromate (as a positive control) was dissolved in distilled water and serially diluted to obtain test solutions with concentrations of 19.53, 39.06, 78.13, 156.25, 312.50, 625, 1250, 2500and 5000 µg/mL. Each test tube contained 10 shrimp nauplii. The test-tubes were maintained under illumination. Survivors were counted after 12, 24 and 36 hours and the death percentage was determined. The median lethal concentration (LC_{50}) was determined via best-fit line method and the linear equation.

RESULTS

HLM extracts except flower have cytotoxicity effects on *A. salina* nauplii at tested concentrations for observed time point. HLM leaf extract has a high cytotoxicity level compared to other parts at recorded time points. At 12 hour of incubation, the extract kills all the nauplii at the lowest concentration of 15µg/mL. Consequently, the LC_{50} value for HLM leaf extract couldn't determine. This demonstrated the HLM leaf extract has the highest toxic level for *A. salina* at 12 hours (Figure 1d). At 12 hours, HLM bulbs extract with the concentration of 31.25µg/mL completely killed the *A. salina* (Table 1) and the LC_{50} value of 8.50µg/mL. The HLM roots extract killed the nauplii at the concentration of 500 µg/mL at 12 hours, at 24 hours they killed by 250 µg/mL of extract and at 36 hours by 62.50 µg/mL. The LC_{50} value of HLM root extract was 11.73 µg/mL. Moreover, the number of the death rate of *A. salina* increase in direct

proportion to the HLM bulbs extract at the recorded time point. The similar pattern is observed for anther and stem extracts (Table 1). Both extracts have the LC_{50} value of 15.95µg/mL and 16.92 µg/mL respectively. Figure 1a, 1b, 1c and 1d respectively present the 100 % mortality rate of *A. salina* at 12 hours, 24 hours, 36 hours and also the LC_{50} values at 36 hours.

Table-1: The concentrations responsible to kill responsible to kill *Artemia salina* at 100 % mortality rate

Parts	Concentrations (µg/mL)					
	Leaf	bulb	root	anther	stem	flower
12	15.63	31.25	500	1000	500	1000
24	15.63	31.25	250	250	125	1000
36	15.63	31.25	62.5	125	125	250
LC_{50} µg/mL	NA	8.50	11.73	15.95	16.92	64.58

Note: NA: Not applicable

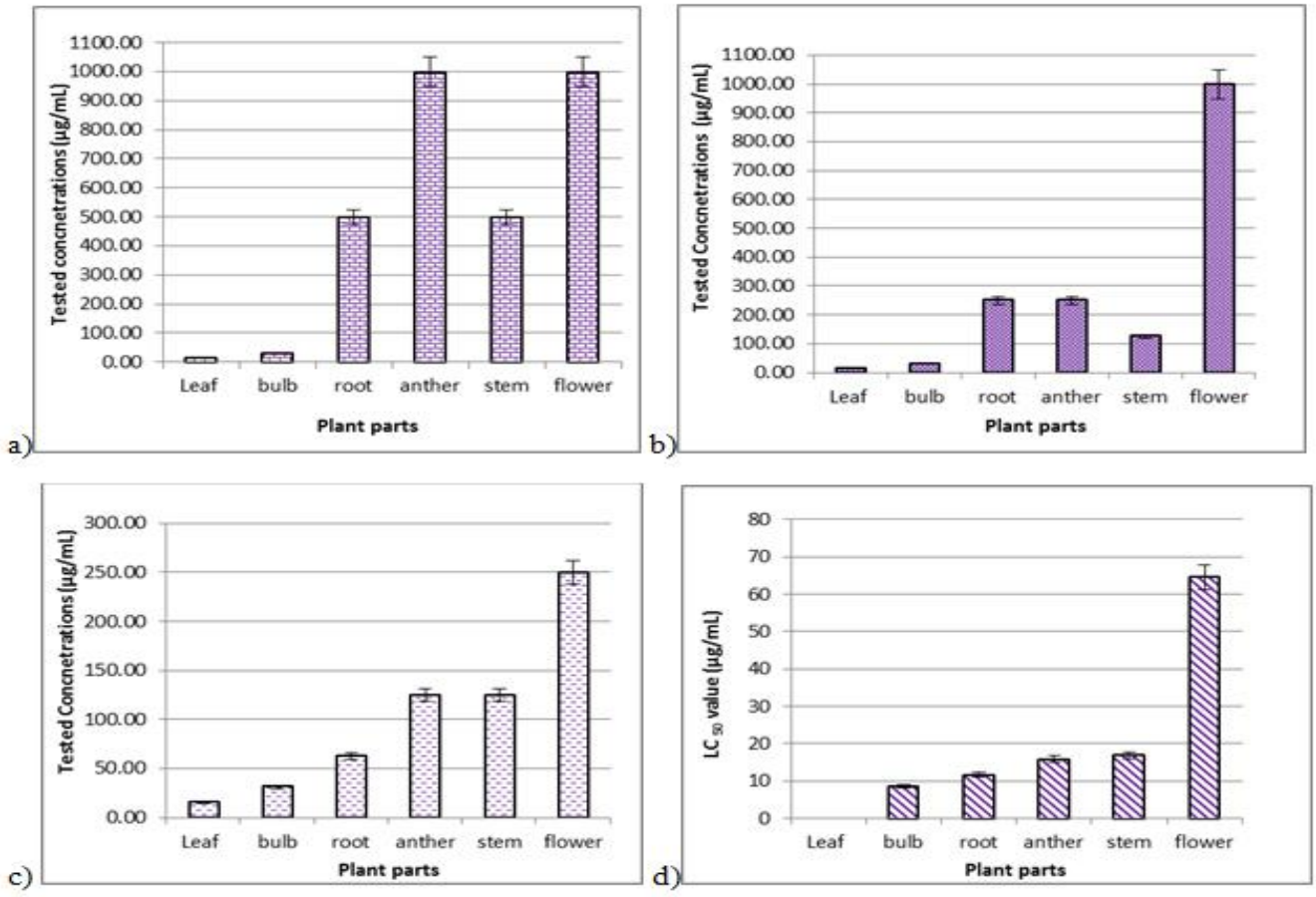

Figure-1: (a) Presents the mortality rate of *A. salina* at 12 hours for all the HLM extracts; (b) presents the mortality rate of *A. salina* at 24 hours all the HLM extracts; (c) presents the mortality rate of *A. salina* at 36 hours for all the HLM extracts and (d) presents the IC_{50} value of HLM extracts at 36 hours.

DISCUSSION

Flower, anther, bulb, leaves, stem and roots of *Hymenocallis littoralis* methanol extracts were used in brine shrimp lethality test. As to date there is no literature report on brine shrimp lethality assay for this plant species. The mortality rate (%) of *A. salina* was determined at three time point which is 12, 24 and 36 hours.

Figure 1a-c shows the mortality rate (%) of *Artemia salina* in all plant parts namely leaf, bulb, root, anther, stem and flower. The leaves extract possesses higher cytotoxicity activity compared to other plant parts' extracts. The literature search suggests that there is less compound identification work that has been carried out so far on the leaves of this plant. HLM leave extract shows prominent wound healing activity at 1 µg/mL (Data to be published). This explained that, there are responsible compound(s) for healing activity in mammalian cells. Therefore, further studies on the phytochemistry and pharmacology of the HLM extract need to be carried out.

At 31.25 µg/mL, all the nauplii were dead for methanolic bulb extract at 12, 24 and 36 hours. This indicates that the bulb possesses high cytotoxicity substances as leaves. As per literature search, there were numbers of compounds with pharmacological activity isolated from the bulb of *Hymenocallis littoralis*. Alkaloid compounds such as lycorine, pancrastatin and haemanthamine with anticancer properties were isolated in a larger amount from the bulb [13]. This alkaloids groups could influence the cytotoxicity activity of this bulb extracts.

The HML root extract shows 100 % mortality with concentration of 500 µg/mL at 12 hours, 250 µg/mL at 24 hours and 62.50 µg/mL at 36 hours. This demonstrated the mortality rate of *A. salina* is directly decline upon the exposure time period. Therefore, the longer the exposure of the sample at even the lower concentration could kill the nauplii. The HLM anthers extract too effect on nauplii's mortality. The extract exhibited a similar pattern as the root extract. The HLM root and anther has wound healing activity at 48 hours in wounded human foreskin mammalian cell line at 1 µg/mL (Data to be published). Thus, these both extracts might have significant activity in cell mobility and mortality.

The HML stem extract has mild cytotoxicity effects at 500 µg/mL of extract concentration and the concentration level decreases to 125 µg/mL for 24 and 36 hours. The sample has cytotoxicity effects against the brine shrimp at the higher concentration for longer exposure time compared to other extracts. The stems extract exhibited slower killing effects compared to bulb, anther, root and leave extracts. It takes 36 hours to reach 100 % mortality rate with 125 µg/mL of extract. The HLM stem extract also possesses wound healing at 1 µg/mL as HLM anther and root extracts.

The HLM flower extract shows lower lethality effects for *A. salina* nauplii. At 12 hours exposure time point, 100 % mortality was observed at only 2000 µg/mL of concentration, meanwhile, for 24-hour time point, 100 % mortality rate was obtained at 1000 µg/mL. The 100 % rate of mortality for 36 hours is at 250 µg/mL of concentration. Flower extract showed low cytotoxicity activity for the brine shrimp and this could be due to the lack of cytotoxicity compounds in flower extract. There is no phytochemistry information or any isolated compound pertaining to the flower of *Hymenocallis littoralis*. Most of the cytotoxicity compounds isolated from this plant were obtained from its bulbs and roots [13,14].

The LC_{50} value for remaining plant portions in descending order is bulb (8.50 µg/mL) > root (11.73 µg/mL) > anther (15.95 µg/mL) > stem (16.92 µg/mL) > flower (64.58 µg/mL). Hence, the bulb of *Hymenocallis littoralis* has highest bioactive substances and displayed good anticancer activity. McLaughin et al. (1998) reported that there is a correlation between brine shrimp lethality assay and anticancer activity because the medium LC50 values for general cytotoxicity are about one-tenth of the LC_{50} values in the brine shrimp test [3].

Meyer et al. (1982) expressed that when the LC_{50} value in brine shrimp lethality assay is lower than 1000 µg/mL, the plant material is considered to have bioactive substances and can be a source for biological activity [6,12]. For example, selected 13 species of the genus *Solanum* possessing molluscicidal activity against *Biomphalaria* 426 a shows activity in brine shrimp lethality assay in the concentration range between 400 to 800 µg/mL [10]. Adoum, (2008) reported that 15 different plant

families used in Hausa and Kanuri folk medicine to cure malaria and cancer shows high LC_{50} value (< 60 µg/mL) in brine shrimp lethality assay [15].

Therefore, *Hymenocallis littoralis* plant parts' methanolic extracts may show good biological activity because all the extracts have the LC_{50} value lower than 1000 µg/mL. The *in vitro* brine shrimp lethality assay shows a good relationship with in vivo toxicological screening [6]. As per Syahmi et al., 2010, *Elaeis guineensis* (LC_{50}: 1 mg/mL; LD_{50}: >5000 mg/kg) methanol extract is proven to be nontoxic in acute oral toxicity and BSL test [16]. Besides Naidu et al., 2014 and Kalala et al., 2015, demonstrated the similar tests for *Mentha spicata* and *Commiphora swynertonii* and the results shows that LC_{50}: 1701 µg/mL; LD_{50}: >5000 mg/kg and LC_{50}: 15.30 µg/mL; LD_{50}: 3400 mg/kg respectively [17,18]. *Datura stramonium* L. (LC_{50}: 12.86 µg/mL; LD_{50}: 821 mg/mL), *Ocimum basilicum* L. (LC_{50}: 9.92 µg/mL; LD_{50}: 965 mg/mL), *Justica pectoralis* Jacq. (LC_{50}: 60.14 µg/mL; LD_{50}: 3531.11 mg/mL) and *Orthosiphon aristatus* (Blume) Miq. (LC_{50}: 16.72 µg/mL; LD_{50}: 5026.31 mg/mL) and *Swietenia* mahogany seed methanol extract (LC_{50}: 680 µg/mL; LD_{50}: >5000 mg/mL) are the other few examples of plant species focused in the brine shrimp and acute oral toxicity correlation studies done on mice by Parra et al. 2001 and Sahgal et al., 2010 [6,4]. These plant extracts with low LC_{50} values were classified under category 4 in the Organization for Economic Co-operation and Development (OECD) guideline 423 as the safer group while the LD_{50} cut off values for animal group is more than 300 mg/mL but less than 2000 mg/mL [19]. According Meyer's versus Gosselin, Smith and Hodge's toxicity scales and Sahgal et al., 2010, correlation studies emphasizes there is a positive correlation between LC_{50} results obtained by the BSL test and LD_{50} results for *in vivo* animal models [7]. Therefore, based on the present results, *Hymenocallis littoralis* plant extract may contain lower toxic effect in acute oral toxicity in mice. This will aid in the investigation of this ornamental plant for therapeutic remedies.

CONCLUSION: *Hymenocallis littoralis's* leaves, bulb, root, flower, stem and anther extracts display cytotoxicity activity against brine shrimp lethality assay. This assay is a primary step for the determination of the presence of bioactive substances for biological activity, anticancer and pesticides activity in a plant. The outcome of the present study demonstrates that the cytotoxicity activity of *Hymenocallis littoralis* is in a descending order; leaves > bulb > root > anther > stem > flower. Hence, this ornamental plant can be further studied as a potent herb for the identification of bioactive in the pharmaceutical industry. Plant products are more safe and economical compared to the modern synthetic medicines. Consequently, this can aid in the development of natural pharmaceutical products.

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