IN-VIVO TOXICITY EFFECT OF BIOCHEMICAL CONSTITUENT OF *CLINACANTHUS NUTAN* 80% METHANOL LEAF EXTRACT ON ZEBRAFISH EMBRYO

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ABSTRACT
Medicinal plants are used for therapeutic purposes against several diseases. Most of these plants contain high concentration of secondary metabolites that are of benefit to animals. When use in high concentration, plant extract can cause several side effects including malformation of tissue organs and mortality. Therefore, the aim of this research was to study the toxicity effect of *Clinacanthus nutan* 80% methanol leaf extract on zebrafish embryo. Leaf was extracted with 80% methanol, fertilized Zebrafish embryos were exposed to the different concentration of the crude extract for 0-7 days. Results showed high yield of 30.12 %, there is significant difference ($P<0.001$) between the control and the treated groups. There was low toxicity with significant difference between the groups that were exposed to different concentrations and those that were maintained in embryo media. It was concluded that the low toxicity observed for this plant makes it beneficial as a medicinal supplement that can be used in the prevention of diseases.

Keywords: *Clinacanthus nutan*, embryos, medicinal plants, toxicity, zebrafish

INTRODUCTION
Recently, *Clinacanthus nutans* or Sabah Snake Grass belonging to the family Acanthaceae was shown to contain phytochemicals that prevent cancer via tea concoction (Kamarudin *et al*., 2017). It is a small shrub and has been used traditionally in tropical Asia for the treatment of inflammatory diseases (Kamarudin *et al*., 2017). This herb is commonly known as "Belalai Gajah" in Malaysia and many other names from different countries. It is also used externally for treat skin rashes, insect and snake bites, diabetes mellitus, fever, diuretics, herpes simplex virus (HSV), and varicella-zoster virus (VZV) (Alam *et al*., 2016). Besides, it is also reported as having antihepatitis, antiherpes, antiinflammatory properties (Hashim *et al*., 2017)) and even used to prevent and cure cancer (Zulkipli *etal*., 2017). Previous study has shown that the aerial part of the plant such as stem and leaf contains high amount of bioactive constituents when it is extracted using specific organic solvents (Yeo *et al*., 2018). *Clinacanthus nutans* leaf can also be consumed as uncooked raw material, mixed juice, tea, and fresh drink (Kumar *et al*., 2015). Fresh leaf of this herb are highly demanded for patients with cancer, diabetes, and general ailments (Ghazali *et al*., 2016)]. The major principle phytochemical constituents of *Clinacanthus nutans* are stigmasterol, β-sitosterol, lupeol, betulin, six known Cglycosyl flavones, vitexin, isovitexin, schaftoside, isomollupentin, 7-O-β-gluco pyranoside, orientin, isoorientin, two glycoglycerolipids, a mixture of nine cerebrosides and a monoacylmonogalactosylglycerol, and five sulfur-containing glucosides (Alim *et al*., 2017). Zebrafish (*Danio rerio*) has emerged as one of the simple and ideal *in vivo* models of research due to its small size and easy handling. There is increased popularity of this organism among scientists because of its rapid use in developmental, genetic and biomedical research (Fonseka *et al*., 2016). Hence, the objectives of this research are to determine it toxicity effect on zebrafish embryo.

MATERIALS AND METHODS
Collection and identification of plant samples
Clinacanthus nutans (leaf) was collected at University Agriculture Park, Universiti Putra Malaysia. A plant sample was identified at Institute of Bioscience (IBS) University Putra Malaysia by botanists and voucher number of SK2893/15 was allocated to it. Cleaning, separation of the leaf from the stem as well as drying was carried out for two weeks at the Biotechnology laboratory Universiti Putra Malaysia. Leaf was grounded to semi-powder form and extracted with 80% methanol.

Extraction of leaf was carried out at biotechnology laboratory as reported by Renshaw*et al*.,2015. A total of 100g of semi powdered leaf was soaked in 1000 mL of 80% methanol in 2.5 liters flat bottom flask and allowed to stand with ceaselessly daily shaken for 3 days at 25°C. Extracts were filtered with What-man filter paper and concentrated with a rotary evaporator at 42°C to semi-solid form. Crude extract was then weighed, transferred to sample bottles and stored at 4°C (Ojinnaka*et al*., 2016).

Doses were prepared by dissolving (100 mg) of crude extract in 1 mL of 100% dimethyl sulfoxide (DMSO) to obtain a stock solution (100 mg/mL). DMSO was used to solubilize the crude extract, further dilution (sub-stocks solution) was carried out by adding distilled water using this formula $M_1V_1=M_2V_2$ in microliter (µg/mL). A two-fold serial dilution was used to prepare the concentration of interest (7.81-1000 µg/mL) in 96-well microplate (Sigma Aldrich, USA).

Zebrafish embryos were supplied by Danio Assay Laboratories Serdang (Sdn), Malaysia. The newly fertilized zebrafish embryos were transferred into 96 well plates (one embryo/well). Plant crude extracts were prepared at different concentration (7.81-1000 µg/mL) in 200 µL of embryo media in 96-well plates. The embyos/larvae were then exposed to different concentration of the crude extract by transfering the prepared crude extrade at different concentration to the 96 well plate containing embryos/larvae. The well plate were gently handle and incubated at room temperature (25-28°C).

Statistical analysis

The experiments were repeated three times independently. Data were expressed as mean ± standard error of mean (mean ± S.EM). Toxicity effect of crude extract was calculated and analyzed using one-way ANOVA followed by Dunnett's post hoc test, where $P<0.05$ was considered statistically significant between different concentrations of the crude extract and the control (fish maintained in embryo media) The LC_{50} was calculated and analyzed using Graph Pad Prism version 5.0 software.

RESULTS AND DISCUSSION

Result of percentage yields the plant extract

The result of percentage yields the plant extract is 30.12 %.

Result of Acute toxicity effect of the crude extract

The result of acute toxicity test of crude extract on zebrafish embryo at day 2 shows calculated that all embryos exposed to 200 µg/mL and those that were maintained in embryo media survived at day 2. The result also revealed that 80% of the fishes exposed to 400 µg/mL survived at day2 but less than 50% and 30% of the fishes exposed to 800 µg/mL and 1600 µg/mL survived at day 2. (Figure 1)**.**

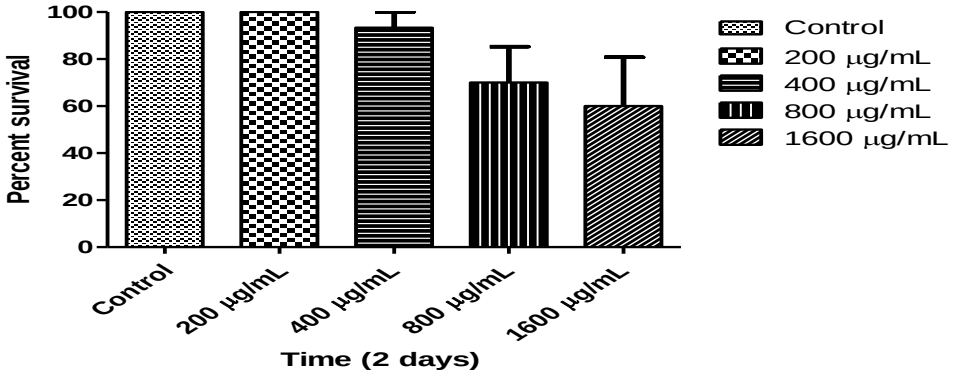

Figure 1: Acute toxicity effect of the crude extract on the survival rate of zebrafish embryo treated with different concentration (200-1600 µg/mL). The percentage of survival (n=3) is shown versus number of days post exposure. The values represent mean ± SEM from three independent experiments.

Chronic toxicity effect of the crude extract

The result of chronic toxicity test of crude extract on zebrafish embryo at day 7 shows calculated that all embryos exposed to 7.81 µg/mL and control group survived at day 7. High mortality was recorded at groups exposed to 125, 250 and 500 µg/mL (Figure 2)**.**

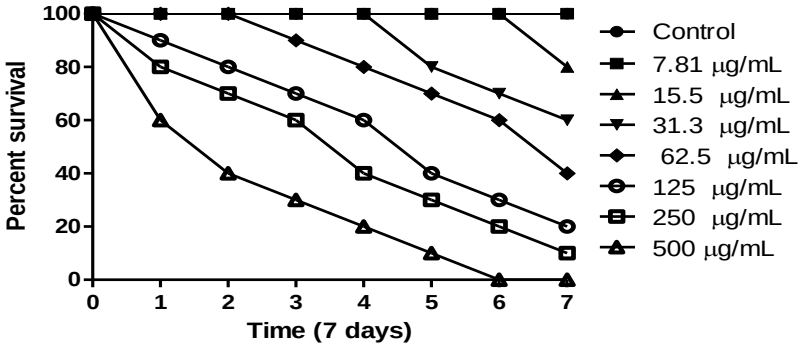

Figure 2 Chronic toxicity effect of the crude extract on the survival rate of zebrafish embryo treated with different concentration (7.81-500 µg/mL). The percentage of survival (n=3) is shown versus number of days post exposure. The values represent mean ± SEM from three independent experiments. ***P<0.05 represented significantly different values of the percentage survival.

The effect of crude extracts on survival shows that higher concentration of this plant extract significantly affect survivability of the embryos. This finding is similar to the result reported on *Clarias gariepinus* which show increased death rate of juveniles fish with increased concentration of C. papaya seeds extract (Nwabueze, 2014). Effects *Oreochromis niloticus* fingerlings and adult following treatment with different concentrations also followed a concentration gradient (Toh *et al*., 2014). Even though, plant bioactivities compound such as saponin, flavonoid and terpenoid may be responsible for most of the antioxidant as well as therapeutic potential of crude extracts, it is also implicated as the major compounds that are responsible for the death and teratogenic defect of embryo and larvae after exposure to different concentrations of the extracts (Bajalan*et al*., 2016).

CONCLUSION **AND RECOMMENDATION**

It was concluded that the low toxicity observed for this plant makes it beneficial as a medicinal supplement used in prevention of diseases. Phytochemical studies to identify and isolate bioactive constituents of the plant are highly recommended.

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