TOXICITY EVALUATION OF *VITEX TRIFOLIA* ETHANOL EXTRACTION USING ZEBRAFISH (*DANIO RERIO*) EMBRYO

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Abstract. *Vitex trifolia (V.trifolia)* commonly known as chaste tree and has been proven to have important medicinal benefits in herbal medicine. The aim of this research was to determine the toxicity effect of *V.trifolia* ethanol extraction on zebrafish embryo (*Danio rerio*) at different concentrations. Toxicology tests were performed on *Danio rerio* embryos exposed to *V.trifolia* extraction at various concentrations from 0.24 to 1000 g/ml for 24, 48, 72, and 96 hours. Observation was done on the survival rate, hatching rate, heartbeat rate, scoliosis rate, and melanin pigmentation formation. Data was analyzed using version 27 of the SPSS. Result shows that the *V.trifolia* has an LC_{50} value of 7.681 g/ml. At higher concentrations with the range between 62.5 to 1000 g/ml, no hatching was observed, whereas hatchability of *Danio rerio* embryos was observed at lower concentrations with the range between 0.244 to 31.25 g/ml. There was no scoliosis observed in any concentration of zebrafish larvae treated with *V.trifolia* extract. For the heartbeat of *Danio rerio* embryos, normal heartbeat was obtained at concentration of 0.24 to 31.25 ug/ml up to 96 post fertilization (hpf). However, at 62.5 ug/ml the embryo heartbeat only presence until 24 hpf, followed by zero heartbeat at 48 hpf onwards. Melanin pigmentation was detected at 48, 72, and 96 hpf. In conclusion, the *V.trifolia* ethanol extraction shows a mild toxicity effect at higher doses when tested on the zebrafish embryo.

Keywords: Toxicity assessment, *vitex trifolia,* zebrafish embryo (*Danio rerio*)

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Introduction

For thousands of years, medicinal plants have been used to cure a variety of human illnesses, and they provide a rich supply of innovative medicines. The practices of medicinal plants depend on the assumptions, principles and perceptions of various cultures and contexts, such as disease prevention, diagnosis, improvement, and rehabilitation [1]. However, persistently high use of medicinal herbs can cause health exposure due to the presence of toxic heavy metals substances [2]. Therefore, the evaluation on the toxicity effect of natural products is very critical.

Vitex trifolia (*V.trifolia*), a member of the Verbenaceae family, is an aromatic shrubby tree that can grow up to 4 m tall and can be found throughout most of India, from the foot of the Himalayas to the south. *V.trifolia* also can be found in tropical and subtropical regions, with some species also found in temperate zones [3]. Bark is pale, grey, smooth, and variable, with simpler trifoliate leaflets that are mostly sessile, elliptical, blue, or purple flowers in terminal, pedunculate, panicle cymes, and globose drupes. The leaves are widely used medicinally. Powdered leaves have insecticidal and antibacterial activity. The fruit is used in amenorrhoea. The leaves also contain concubine, agnuside, casting, orientinluteolin-glucoside. The fruit contains a new alkaloid vitamin (0.01 %) [4]. *V.trifolia* possesses larvicidal, wound healing, anti-HIV, trypanocidal, and anti-cancer properties [5]. It is also an anti-bacterial, anti-inflammatory, and antipyretic agent. In some countries, they are also used as sedatives for rheumatism, headaches, and the common cold. Traditional medicines use *V.trifolia* species to treat wounds, allergies, asthma, and pain in the body [6]. Several extraction methods are used for *V.trifolia* species, including water and ethanol leaf extracts made by decoction, Soxhlet, or maceration [7].

Plant ethanol extraction has been linked to various activities such as genetic improvement, anti-stress, hunger enhancement, immune stimulation, maturation of culture organisms, aphrodisiac and anti-pathogenic properties in shrimp, and fish aquaculture. Thus, since they appear to become more biodegradable than synthetic substances, their use may minimize treatment costs and be more environmentally friendly. However, since the effect of plant products on fish is dose-dependent and there is also a risk for overdosing, it is of great importance to assess the optimal concentration of ethanol extraction [8]. Toxicity monitoring is carried out to scan the chemical properties of a sample to ensure that there are no elements that are life-threatening in it. The value of toxicological research is also to include a dosages graph against impact of toxicity, to examine the safety of sample products, and to check the investigation methods for toxicity [9]. Acute toxicity tests offer time and/or concentration information that induces a noticeable effect or detectable reaction in 50 % of the affected group of research specimens. Testing is known to be ecologically important and morally defensible, easy, and cost efficient. Acute toxicity studies have been provided timely and useful information on whether more toxicity tests had been carried out [10].

Several animal models have been used to study the impact of plant extracts on toxicity. The toxicity of *V.trifolia* leaf ethanolic extracts was studied using zebrafish (*Danio rerio*) embryos in this study. The zebrafish embryo is a new and reliable animal model for assessing the toxicity of various components. The zebrafish is a tropical freshwater fish belonging to the Cyprinidae family. The use of zebrafish embryos in toxicity studies is mostly due to their ease of use and cost-effectiveness in the laboratory [11]. Furthermore, the embryo's developmental process is quick, and its embryonic development is similar to that of higher

types of vertebrates. The current study aims to use this model to examine the toxicity of *V.trifolia* leaf ethanolic extracts by exposing embryos to different treatment dosages.

Materials and Methods

Sample Collection

Fresh leaves of *V.trifolia* were collected from Nutrition Laboratory, Faculty of Medicine and Health Science, UPM Serdang Malaysia. The plant was sent for confirmation and the ID is MFI 0140/19.

Sample Preparation

Fresh *V.trifolia* leaves were oven-dried at 40 °C [12]. The dried *V.trifolia* leaves were then powdered. 500 ml of 95 % ethanol was mixed with 50 g of grind *V.trifolia*. The mixture was then shaken for 24 hours in an automatic shaker at 37 °C and 250 rpm. After a 24-hour period, the extract was filtered three times with Whatman paper. A 275.50 ml *V.trifolia* extract was evaporated in a rotary evaporator at 50 °C until the extract became waxy. The extract was then dried for 2 minutes in a 45 °C oven before being stored at -20 °C for future use.

Zebrafish Breeding Process

In order to separate the eggs from the zebrafish, a fish tank was cleaned and a shield was placed in the tank. Total 1000 ml of tap water and 5 drops of anti-chlorine were added to the fish tank. The fish tank was filled with two female and two male zebrafish. The fish tank was then covered with a box and placed in a dark place for 17 hours to allow hormones to be released. The box was removed after 17 hours, and the male and female zebrafish mated for 1 to 2 hours. After that, 1000 ml of tap water, a half drop of methylene blue, and five drops of anti-chlorine were mixed. The purpose of methylene blue is to stain animal cells or eggs to make their nuclei more observable. Few petri dishes were prepared and filled up with the mixture. Thirty eggs were transferred from the fish tank into each petri dish. The embryos in the petri dishes were then placed at room temperature for 24 hours [13].

Zebrafish Embryo Assay

Prior to zebrafish embryo assay, a serial dilution of *V.trifolia* extract was prepared. Distilled water was used to dilute 0.1 g of *V.trifolia* extract with 1000 L of stock solution,. Serial dilutions of the 100 % solution mixture were made at 500 g/ml, 250 g/ml, 125 g/ml, 62.5 g/ml, 31.25 g/ml, 15.63 g/ml, 7.8 g/ml, 3.91 g/ml, 1.95 g/ml, 0.98 g/ml, 0.49 g/ml, and 0.244 g/ml. Dimethyl sulfoxide (DMSO) at concentration of 100 % was used as a positive control, while distilled water was used as a negative control [13].

One embryo was placed in each of the 96 well plates 24 hours post-fertilization (hpf) using a pasteur pipette. There were 3 replicates per assay (n=3). The treatment was administered by pipetting 200 ul of diluted sample into 96-well plates at various concentrations. The embryo was examined under an inverted microscope after 24, 48, 72, and 96 hours of treatment, with an emphasis on key parameters such as survival rate, melanin pigmentation, hatching rate, heartbeat rate, and scoliosis rate [13]. The heartbeat was

observed and counted using an inverted microscope. The heart rate was measured in bpm (beats per minute). Scoliosis was also observed and recorded under an inverted microscope [14].

Data Analysis

Statistical Package for the Social Sciences (SPSS), Version 27.0. Armonk, New York was used to analyze the data. The data was interpreted as a mean standard deviation. All data were interpreted using $p< 0.05$, which indicates that the result is significant. The probit analysis was used to calculate the LC_{50} by plotting the mortality value against the test extract concentration [13].

Results and Discussion

The Effect of V.trifolia Ethanol Extraction on Danio Rerio Embryo Survival Rate

The ability of a biological being to respond in a specific scenario as evidenced by its optimal state of requirement is referred to as its survival rate. Part of the best condition that can influence the continuity of the unexplored zebrafish organism includes humidity, hydraulic conductivity and consistency and even the metabolic waste component. Greater persistence reveals also that life-creating zebrafish may survive the environment where even the lower courage level implies that the formative year's type cannot step further through the built-in air. Accordingly, the usual criteria for zebrafish growing lifetime survival rates are expected to reduce as a concentration of the standard solution. At higher doses, the sample has a highly secondary metabolites substance [15].

According to the findings, the survival rate of the zebrafish embryo decreased as the concentration of the *V.trifolia* ethanol extraction increased. It also showed a lower overall survival rate after a slightly steady decrease at higher doses (125-1000 g/ml). The survival rate of the zebrafish embryo has been shown to remain stable (0.24-3.9 g/ml) but through default (62.5 g/ml-1000 g/ml) during 48 hours. Furthermore, the survival rate of embryo zebrafish tested with ethanol *V.trifolia* extract over a 72-hour concentration sequence shows a marginally consistent decrease (15.63-31.25 g/ml) ($p< 0.05$). In such a complex 96-hour sequence of concentrations, the survival rate of embryo zebrafish (*Danio rerio*) measured with ethanol *V.trifolia* extract indicates a stabilized survival rate of 72 hours. The survival rate improved at lower concentrations (0.244-62.5 µg/ml), but then at higher concentrations, the survival rate declined (Figure 1).

Based on the survival rate study, result showed that lower concentration of *V.trifolia* (0.244-7.8 µg/ml) had no toxicity ($p< 0.05$) effect towards the zebrafish embryo, however, at higher concentration (125-1000 µg/ml) showed some toxicity effect ($p> 0.05$). The negative control has a 100% survival rate, indicating that the negative control was alive for the zebrafish embryo survival rate. Illustrates the same negative control segment that has no effect on the survival rate of developing zebrafish as seen in all the studies. Finally, the structure has been highlighted as one of the key components to be emphasized because it can influence the psychological research of zebrafish ongoing species, affecting the survival rate. According to the survival rate results, the survival rate increases for the lower concentration but decreases for the higher concentration [16].

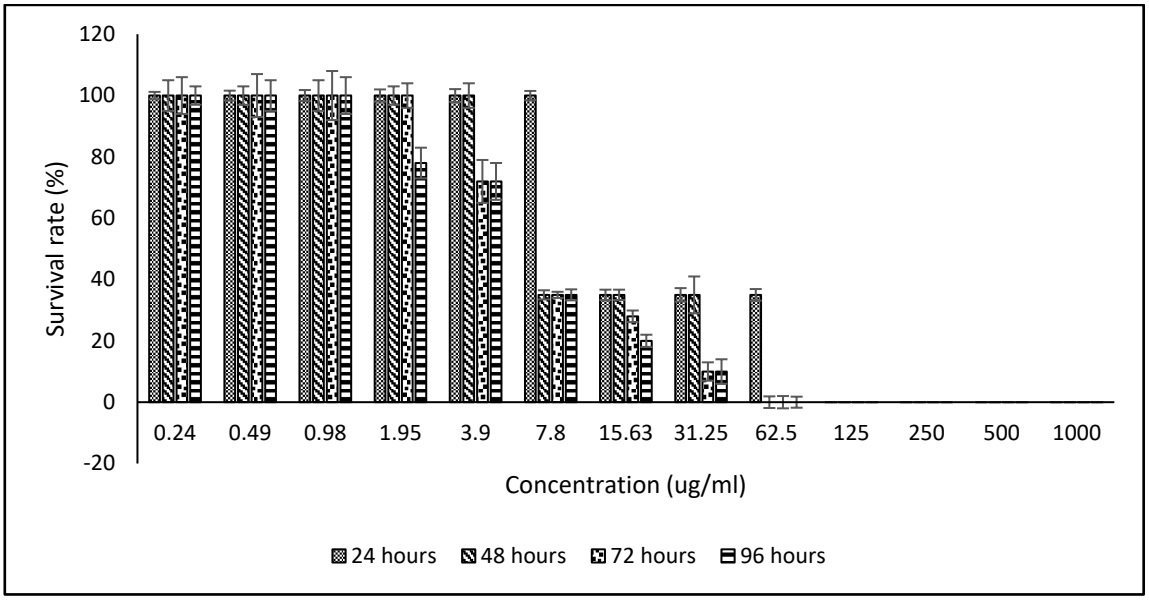

Figure 1: The survival rate 24 hpf, 48 hpf, 72 hpf and 96 hpf of zebrafish embryos after exposed to *V.trifolia* extract. At lower concentrations (0.24-3.9 ug/ml) 100 % of embryos were survived at 24 to 48 hpf. At 7.8 ug/ml and above concentrations, the embryos survival rate decreased below than 50 % from 48 hpf. Data were expressed at mean ± SD.

The Effect of Vitex Trifolia Ethanol Extraction on Heartbeat Rate of Danio Rerio Embryo

At 24, 48, 72, and 96 hpf, the effect of *V.trifolia* ethanol extraction on the heartbeat rate of zebrafish embryos was observed (Figure 2). By all concentration sequences, the ethanol *V.trifolia* extract had an ideal heartbeat, but the heartbeat counting eventually bottomed out at a higher concentration (125-1000 g/ml) ($p < 0.05$). Because the zebrafish was dead, no heartbeat rate was recorded from 62.5 to 1000 g/ml. As a positive control, no zebrafish embryo heartbeat rate was detected after 24, 48, 72, and 96 hpf.

In evaluating cardiac ability, the measurement of heartbeat becomes relevant in given the fact that the deviation of heart rhythm may have been the factor so as the influence of investigated defective cardiovascular disease had enhanced among the most valuable model creations for cardiac analysis. Normal heartbeat in all concentration was evaluated but in higher concentration does not reveal heartbeat rate. The heartbeat of zebrafish embryo after exposed of *V.trifolia* was within normal range which is 120-180 bpm. Throughout contrast, some few interpretations could indeed influence the heartbeat of zebrafish, such as the component variable, inherited precipitated model approach, and various others. It often means the rate of heartbeat to adapt, resulting in tachycardia or bradycardia going to rely on the zebrafish model's production function possession. From a predictor of toxic effect through zebrafish embryos, because as a genetic condition, the approximate neuronal feature including its embryo is security guard [17].

The normal heartbeat rate at the lower concentration was determined evaluate the effects of the heartbeat rate. But the heartbeat rate is not revealed by greater proportion. The negative control demonstrates similar outcomes at a lower concentration, which is the normal rate with heartbeat. Neither heartbeat rate findings were seen for the positive control [18].

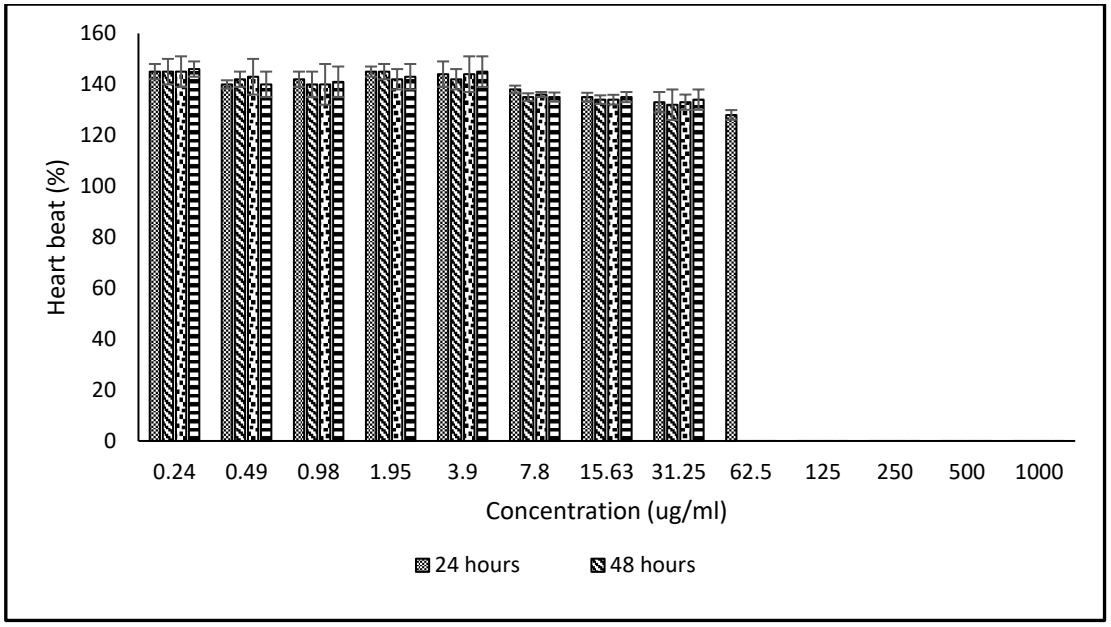

Figure 2: The heartbeat rate of zebrafish embryo after exposed to *Vitex trifolia.* Normal heartbeat was obtained at concentration of 0.24-31.25 ug/ml up to 96 hpf. At 62.5 ug/ml the embryo heartbeat only presence until 24 hpf, followed by zero heartbeat at 48 hpf onwards. Data were expressed at mean ± SD.

The Effect of Vitex Trifolia Ethanol Extraction on Danio Rerio Embryo LC_{50} Value

The LC_{50} of the *V. trifolia* extraction assessed on *Danio rerio* embryo model was plotted using the mortality rate against test extract concentration (Figure 3). The LC_{50} value was 7.681 µg/ml which was considered secure to be used on zebrafish embryo because the *V.trifolia* extract exhibits milder toxicity effects mostly on zebrafish (*Danio rerio*) embryo. For ethanol *V.trifolia* extract, the concentration would be less than 199.53 µg/ml, which had been considered safe to be ingested by the zebrafish embryo.

The LC_{50} value analysis of *V.trifolia* is 7.681 µg/ml, which indicates that *V.trifolia* extract shows milder effects of toxicity on the embryo of zebrafish. The LC_{50} estimates of *V.trifolia* samples and permanent are arranged. In general, higher LC_{50} values suggest that the level of toxicity is low and higher concentrations were expected to result in 50 % of an organism's mortality rate, however lower LC_{50} demonstrates that the toxicity of high bioactive components, such as phytochemicals, is a heavy metal that causes the embryos of zebrafish. In addition, elsewhere in analysis demonstrated that the median lethal concentration of *V.trifolia* was indeed protected to take supplements by human.

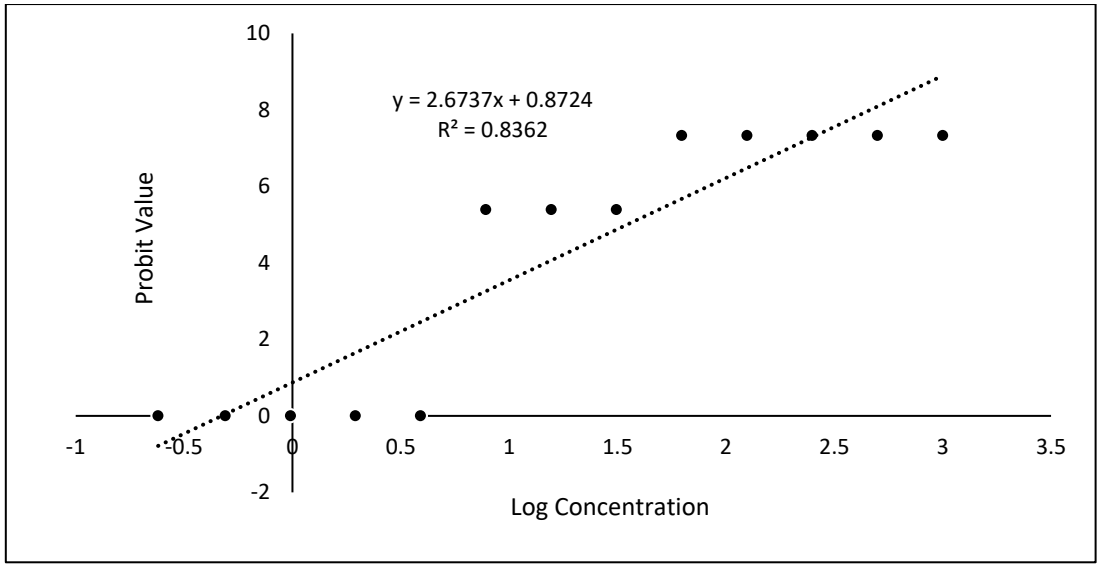

Figure 3: The Lethal Concentration (LC_{50}) value assessed on zebrafish embryos after exposure to *V. trifolia* extract

The Effect of Vitex Trifolia Ethanol Extraction on Danio Rerio Embryo Hatching Rate

From 48 to 72 hpf, the hatching rate of zebrafish embryos exposed to varying concentrations of *V. trifolia* ethanol extraction was determined (Figure 4). The effect of *V. trifolia* extract on the hatchability of *Danio rerio* embryos revealed disrupted hatchability in all *V. trifolia* samples as the concentration of the test extract increased. The rate of hatching of the embryo at a lower concentration (0.24-31.25 g/ml) was 100 %, whereas at the highest concentration (62.5-1000 g/ml), the rate of hatching of the embryo presented or not even on the zebrafish embryo (*Danio rerio*) began from 48 hours to 96 hours.

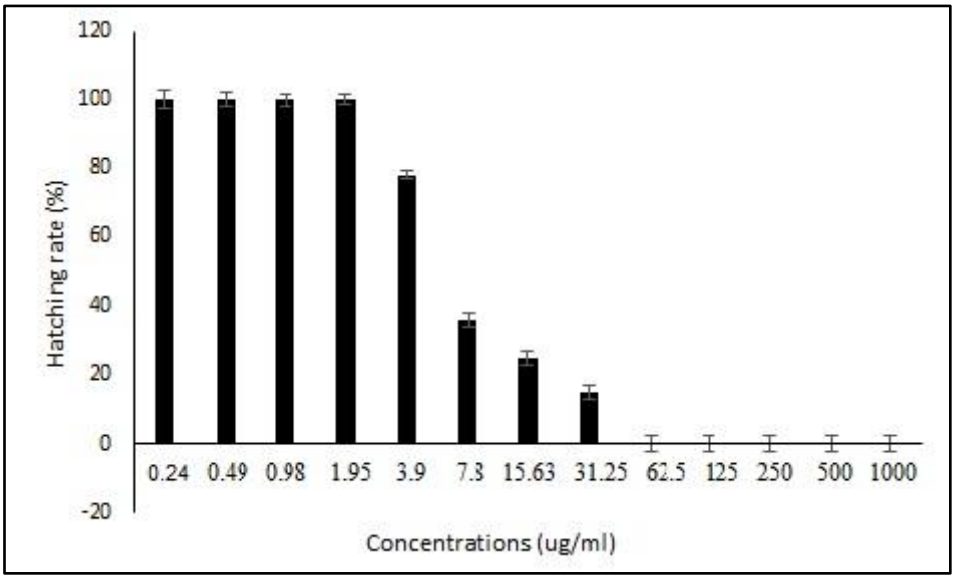

Figure 4: The hatching rate of zebrafish embryo after exposed to *Vitex trifolia*

The study indicates rising the light output led to an increase in an improvement in antioxidants along with flavonoids and phenolic compounds and terpenoids substances. Especially in contrast with roots and stems, bioactive compounds became greater throughout the leaves. With through advanced control, ethanol extract enhanced in specimens across all parts of the plant [19]. A zebrafish was assumed have been hatched at lower concentration (0.24-1.95 µg/ml), but higher concentration (125-1000 µg/ml), No hatchings were observed. Its deficiency of hatchability may indicate a delay in growth or low growth. The embryos of zebrafish (*Danio rerio*) begin to hatch in normal conditions at 48 hpf, but after 72 hpf they become hatchable by a percentage [20].

The Effect of Vitex Trifolia Ethanol Extraction on Danio Rerio Embryo Scoliosis Rate

According to the results from Figure 5, there was no scoliosis in any of the hatched zebrafish embryos at all concentrations. In the negative control, no scoliosis was observed in the hatched embryos.

Figure 5: The hatched embryo's normal appearance in the absence of scoliosis

In a scoliosis analysis, no scoliosis was found in higher and even lower concentrations of zebrafish larvae. This indicates that the control negative samples and the experimental condition do not reveal any harmful effect on the entire test extract in distinct. Observed scoliosis on zebrafish larvae, whether recognize, begins from 48 hours through 96 hours. Unless the spine's irregular curvature enables the backbone would have a "C" or "S" like structure [21]. In addition, melanin pigmentation has shown there were no ordinary abnormalities, as melanin pigment has been prevalent at 48 hpf, 72 hpf and 96 hpf, while it may be missing at 24 hpf but even though melanin originally generates mostly from neural crest throughout embryogenesis. For the establishment of neural crest signal transduction in zebrafish, the eye seems to be essential. Neural crest cells spread distally around the eyes in differentiated cells embryos and are therefore not observed on the affected side during 23-24 hpf [22].

The Effect of Vitex Trifolia Ethanol Extraction on Danio Rerio Embryo Melanin Pigmentation Rate

According to the current study, melanin pigmentation was presented at 48, 72, and 96 hpf but not at 24 hpf (Figure 6) due to melanocytes emerging from the neural crest during

embryonic development. In zebrafish, the eye is critical for organizing neural crest cell proliferation. In wildtype embryos, neural crest cells travel anteriorly around the eyes and are not visible on the eye surface until 23 to 24 hpf [23]. Melanin pigmentation was lacking at 24 hpf and present at 48, 72, and 96 hpf in the negative control, but not in the positive control since all embryos were dead.

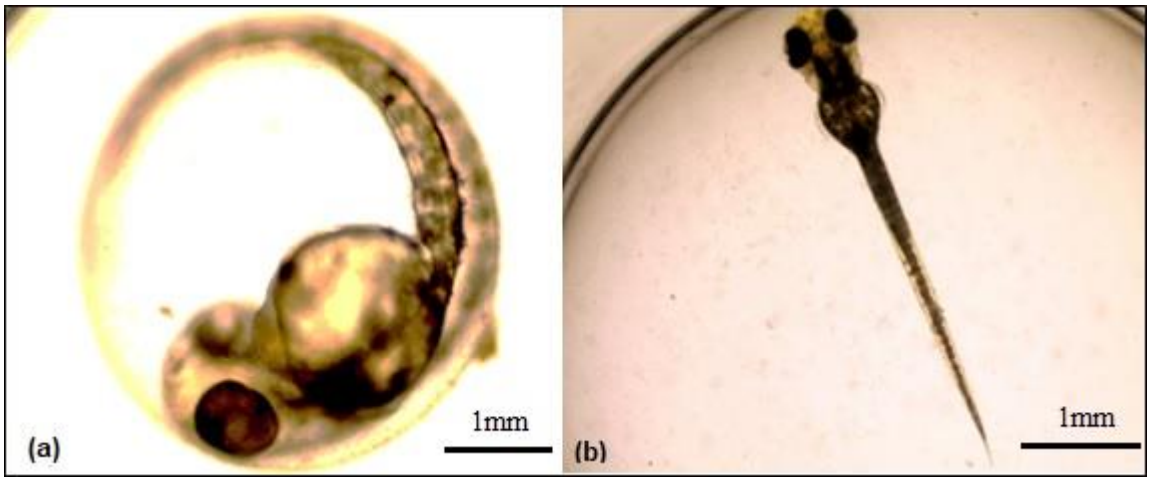

Figure 6: The absence of melanin pigmentation at 24 hpf (a) compared to the presence of melanin pigmentation at 96 hpf (b) on zebrafish embryos.

Conclusions

In conclusions, the effect of toxicity to *V.trifolia* upon this embryo model of *Danio rerio* via the toxic effects analyses assessment was assessed. The results consequently assert that perhaps the extraction of ethanol *V.trifolia* seems to be the absolute best extract because it showed a lower toxic effect in overall variables such as scoliosis, determination of the LC_{50} value, hatching rate, survival rate, hatching rate and embryo heartbeat of zebrafish. Therefore, although due to its potential pharmaceutical companies, this plant extract is safe to consume, somehow it showed a higher concentration toxicity effect as measured throughout the embryo model of zebrafish. It is also recommended that further analytical phytotoxin assessment research should indeed be carried out to assess the various aspects of *V.trifolia* that exhibit potential toxicity. It might assist to identify the toxic substances that could be modified during method and to important experiences safe and therefore more viable substitutes. The toxic assessment evaluated the effect of *V.trifolia* intervention upon this embryo model of zebrafish. This research will have a major impact on the assessment of the toxicity of traditional medical use. Readers might benefit from experiments by using zebrafish (*Danio rerio*) embryos for the toxicity evaluation of ethanol *V.trifolia* extract.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards under approval code: MSU-RMC-02/FR01/05/L3/003.

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