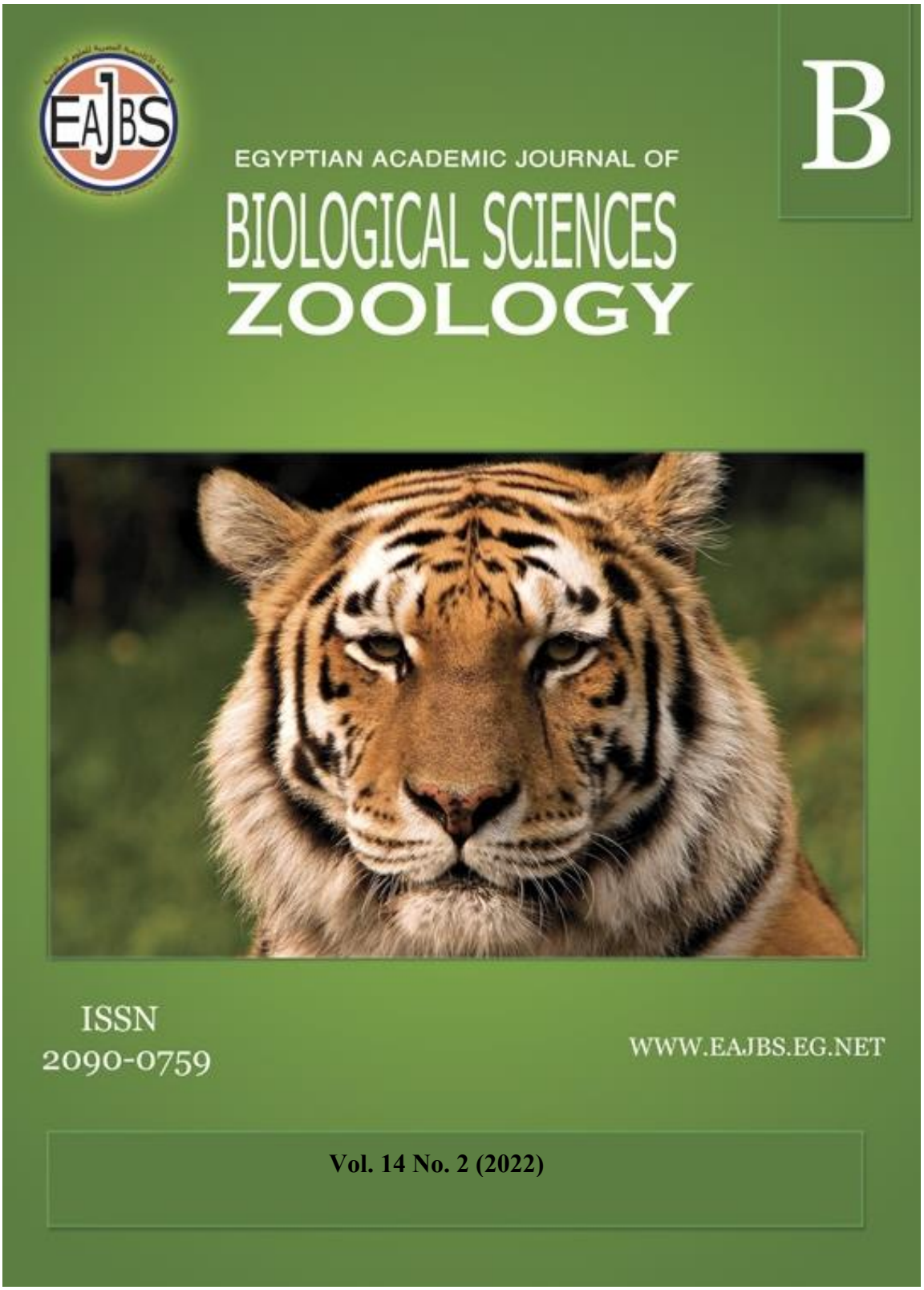

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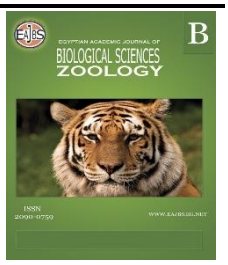

Toxicity and Teratogenicity Effects of Aqueous Leaf Extract of *Phyla nodiflora* in Zebrafish (*Danio rerio*) Embryos

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ABSTRACT

In India, *Phyla nodiflora* plant has often been used as traditional medicine for several years. In the current research, Zebrafish (*Danio rerio*) were used as animal model to examine the toxic and teratogenic effects of the plant on embryonic development. Zebrafish embryos were treated with concentrations of 6.25, 12.5, 25, 50, 100 and 200 µg/ml of the aqueous leaf extract of *P. nodiflora*. The Organization for Economic Co-operation and Development (OECD) guidelines of safety level was met by the extract, which was found to have a median lethal concentration (LC_{50}) value of 147.02 µg/ml. However, the teratogenicity assessment found that embryos exposed to extracts of concentrations 50 µg/ml and above exhibited a variety of developmental abnormalities. The magnitude of the defects was observed to be concentration-dependent. In addition, delayed hatching was seen at concentrations of 100 and 200 µg/ml owing to stunted growth and early death. The surviving embryos at the test concentration of 100 µg/ml indicated a substantial decrease in heart rate. As a result, the treated embryos had bent tail tips, scoliosis, edema in the yolk sac and curved tail. The current investigation has produced preliminary findings on the possible toxicity and teratogenicity of *P. nodiflora* leaf extract on zebrafish embryos.

INTRODUCTION

Plants have been exploited as a useful source of chemical compounds with a wide variety of pharmacological effects, many of which have been developed into medications that are employed in therapeutics (Ghasemzadeh *et al*., 2015). The promising potential of plants, particularly those with a history of ethno medicinal applications in alleviating a range of illnesses and ailments, has also led to the establishment of a broad array of herbal remedies and supplements around the world. However, despite the beneficial effects on human health, herbs and products derived from them have also been associated with cases of adverse side effects resulting from their ingestion.

Thus, the toxicological assessment of herbal products is an essential step within the framework of herbal product development to protect and ensure consumer safety. By convention, various mammalian models such as mice, rats, and rabbits have been widely

used in toxicological studies (Caballero & Candiracci, 2018). Because the whole animal system is typically closely related to human toxicity, the use of animal models is considered a gold standard in toxicological testing (Jayasinghe & Jayawardena, 2019).

However, in recent years, the use of zebrafish (*Danio rerio*) as an alternative to the classical higher vertebrate models has gained increasing attention. The wide usage of zebrafish is mainly attributed to its high genetic similarity to humans; zebrafish possess approximately 70% homology with humans, and about 84% of its genes appear to be related to human disease (Howe *et al*., 2013). Presently, compared to adult zebrafish, embryos are increasingly being used for toxicological evaluations due to their optical transparency, which permits direct visualization of the model's developmental stages without a need for surgical procedures (Jayasinghe & Jayawardena, 2019). In addition, teratogenic effects upon exposure to chemical substances can be easily observed in zebrafish, giving the excellent predictive ability of the bioassay in evaluating developmental toxicity in mammals (Gao *et al*., 2014). Moreover, testing on the zebrafish model can also be completed in a short timeframe, which is extremely valuable, and the embryos exhibit a good dose response to toxicity (Zhang *et al*., 2003).

Phyla nodiflora Linn. (Verbenaceae) known as *Lippia nodiflora* is an ever green, creeping, branched herb distributed in India, Sri Lanka, Ceylon, South, and Central America, and Tropical Africa. Traditionally, *Phyla nodiflora* has been reported to be used in treating colds, bronchitis, coughs, muscle weakness, poor blood circulation, bone fractures, snake bites, and scabies (Teoh *et al*., 2019). *P. nodiflora*, emphasizes the need for more research to properly establish the toxicity profile of the plant and determine the safe levels for its practical usage in healthcare.

Pharmacological properties reported on the plant leaves included anti-proliferative (Hofer *et al*., 2013), cytotoxicity (Abd Latip & Abd Mutalib, 2019; Teoh *et al*., 2019;), antimalarial (Srinivasan, 2013), antidiabetic (Balamurugan and Ignacimuthu, 2011), and antioxidant properties (Abd Latip & Abd Mutalib, 2019). Individual bioactive constituents responsible for these biological properties have yet to be identified. However, in our molecular network-based dereplication of the chemical constituents of the plant, it is shown to be rich in flavonoids and phenolic acids (Chua *et al*., 2019).

Several studies have been reported on the pharmacological, medicinal, and folkloric uses of *P. nodiflora*. However, the teratogenic and embryonic effects on zebrafish have not been studied, thus, the present study was thus carried out to address some aspects of this need by evaluating the toxic and teratogenic effects of the plant extract on the embryonic development of zebrafish (*Danio rerio*).

MATERIALS AND METHODS

Preparation and Extraction of Plant Material:

The leaves of the plant "*Phyla nodiflora*" were collected from the Western Ghats, approximately located at 8.4721º N and 78.0398º E in Tamil Nadu, India. Fresh and healthy leaves were collected and rinsed thoroughly with tap water followed by distilled water to remove all the dust and unwanted foreign particles, cut into small pieces and dried at room temperature. About 10 g of the dried leaves were weighed and transferred into 250 mL beakers containing 100 mL distilled water and boiled at a temperature of 60ºC for one hour. The extracts were then filtered through Whatman No. 1 filter paper to remove particulate matter and to get clear solutions which were then refrigerated (4°C) in 250 mL Erlenmeyer flasks for further experiments.

Fish Husbandry:

Adult zebrafish (AB strain), all (> six months old), were maintained under 10:14

h of the dark: light cycle with ambient temperature at 28.5 °C in 3 L aquarium tanks. The adult fishes were originally purchased from the SNP Aquarium, Nagercoil. Adult males and females were used for this experiment. Only 3 fish with a female-to-male ratio of 1:2 were placed per tank to ensure a stress-free environment for the highly sensitive fish. The tanks were continuously supplied with water by a recirculation water system. The fish were fed with brine shrimps (*Artemia salina*) four times per day to ensure healthy and high fecundity. The volume of brine shrimps fed to the fish was approximately 4 mL/3 L tank for each feeding.

Spawning, Collection, and Selection of Embryos:

Healthy (visual assessment of body condition scoring according to Clark *et al*., 2018), active, and well-fed adult zebrafish (> six months old) were selected for breeding. Five fishes were maintained in a 3 L aquarium equipped with a recirculation water system maintained under 10:14 h of the dark: light cycle at 28.5 °C, with a female-to-male ratio of 1:2. Artificial aquarium plants, were placed in the spawning tank together with a spawn trap for egg collection to stimulate spawning. Three spawning tanks were set up for the experiment to have an adequate supply of fish eggs. Fertilization usually occurs in the morning, within 30 minutes after the light is turned on. Fish eggs were collected, washed with distilled water, rinsed with methylene blue, transferred into clean Petri dishes containing embryo water, and incubated at 28 °C. According to the guideline by Organisation for Economic Co-operation and Development (OECD 2013), the fertilization rate should be more than 50%, while in our laboratory standard protocol, the experiment will be conducted only when the rate of fertilization is more than 70%. Fertilized embryos were rinsed with methylene blue, and any dead or unfertilized eggs were removed (to eliminate fungal growth).

Embryonic Exposure Experiments:

The exposure experiment was performed in 6 well plates according to the method described by OECD (2013). After initial range-finding experiments, six concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/ml) of the extract were selected as the final test concentrations. Ten embryos were transferred into each well containing the different treatment concentrations. For the control group, embryos were exposed to embryo water. The maximum volume per well was kept at 3 ml. The plate was incubated at 28°C for the exposure experiment. Three independent replicates were performed for each treatment concentration.

Evaluation of Toxicity Effects:

A series of toxicity parameters such as mortality rate, heartbeat rate, and hatching rate was observed. Upon completion of the early developmental process, a zebrafish larva is normally released from the chorion because of chorion breakdown. Normally, the hatching process is completed by 72 hpf; however, this biological process is interrupted in toxic conditions. The hatching rate was determined by quantifying the number of successfully hatched embryos at 72 hpf. All observations were made and recorded after viewing the embryos under a compound microscope. The mortality rate data obtained was then used to determine the median lethal concentration (LC_{50}) of the extract using probit analysis (Finney, 1971) in Microsoft Excel. The heartbeat of the individual embryo was determined by manually counting the embryo's heartbeat over 1 minute. No anesthetic drug was used while measuring the heartbeat.

Evaluation of Teratogenic Effects:

Several parameters of teratogenicity such as the abnormal shape of the head, eyes, and heart, bent body axis, growth retardation, pericardial edema, and deformity of yolk were assessed for 72 hours by viewing under a compound microscope.

Ethical Consideration:

It is to declare that there is no need for ethical approval for the study involving zebrafish embryos up to 120 hours post-fertilization (HPF). In the present study, observation proceeded with 72 HPF which is <120 HPF. Further, no human subjects have been involved in this investigation, and hence, Institutional Ethics Committee approval was not considered mandatory for this study.

Statistical Analysis:

All results obtained were expressed as mean ± standard deviation (SD) from three independent replicates and calculated. In addition, the *P* values obtained from analysis of variance (ANOVA) analysis using the posthoc Tukey's test were *($P \leq 0.05$) was significantly different from the control group.

RESULTS AND DISCUSSION

Effect on Mortality Rate:

The effect of the extract on zebrafish embryos mortality rate was evaluated over a range of concentrations (6.25-200 µg/ml). As shown in Figure 1, no mortality was recorded for the control and low concentration groups (6.25 and 25 µg/ml) observed least mortality. However, the mortality rate of the embryos was significantly increased with exposure to higher concentrations starting from 50 µg/ml, inducing a significant increment in mortality rate from 26.67% (50 µg/ml) to 60% (200 µg/ml). In particular, 12.5 and 25 µg/ml concentrations induced 13.3% and 20% mortality within 72 hpf, respectively. Meanwhile, it was observed that the highest test concentrations of 100 µg/ml induced 40% mortality in the 72 hpf and 200 µg/ml induced 50% mortality before reaching 48 hpf. During early developmental stages, the weakened protective layer (chorion) around the zebrafish embryo will be easily affected by an influx of external solutes, including the aqueous extract of *Phyla nodiflora (*Ali *et al*., 2017). Prolonged exposure to the extract may lead to increased accumulation of the extract until a concentration may induce toxicity in the embryos (Alafiatayo *et al*., 2019). Similarly, exposure to *P. nodiflora* extract at higher doses and for longer periods (2-72 hpf) also increased the mortality of zebrafish embryos in our study.

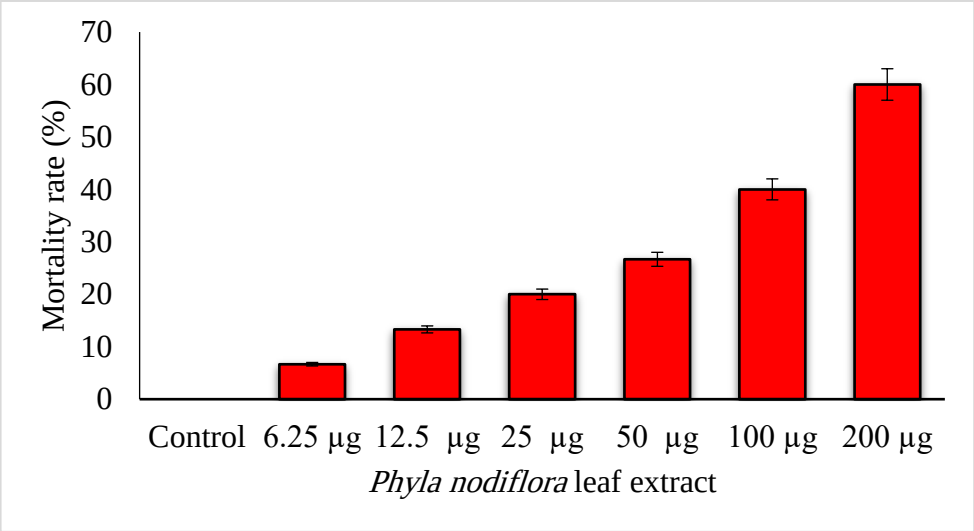

Fig. 1: Mortality of *D. rerio* Embryos After 72 h of Exposure to Varying Concentrations of *P. nodiflora* Aqueous Leaf Extract.

LC_{50}:

The percentage mortality data were used to determine the LC_{50} value of the test extract using probit analysis. Consequently, the LC_{50} value of the extract was calculated to be 147.02 µg/ml. The logarithmic estimation of the LC_{50} value is displayed in Figure 2. Generally, higher LC_{50} values imply less test chemical toxicity as greater concentrations are required to elicit 50% mortality in the test organisms (Thiagarajan *et al*., 2019). Meanwhile, according to the OECD (2013), any toxicants are categorized as 'harmful', 'toxic', and 'highly toxic' if the value of LC_{50} ranges between 10–100 mg/L, 1–10 mg/L, and < 1 mg/L, respectively. Since the LC_{50} value of the extract was higher than the OECD values, it could be concluded, at this stage, that this aqueous leaf extract is non-toxic and safe for consumption, at least for concentrations lower than its LC_{50} value. However, the mortality rate is not the final decisive criterion for the safety of a plant extract. Its effect on the overall development of an organism must also be considered.

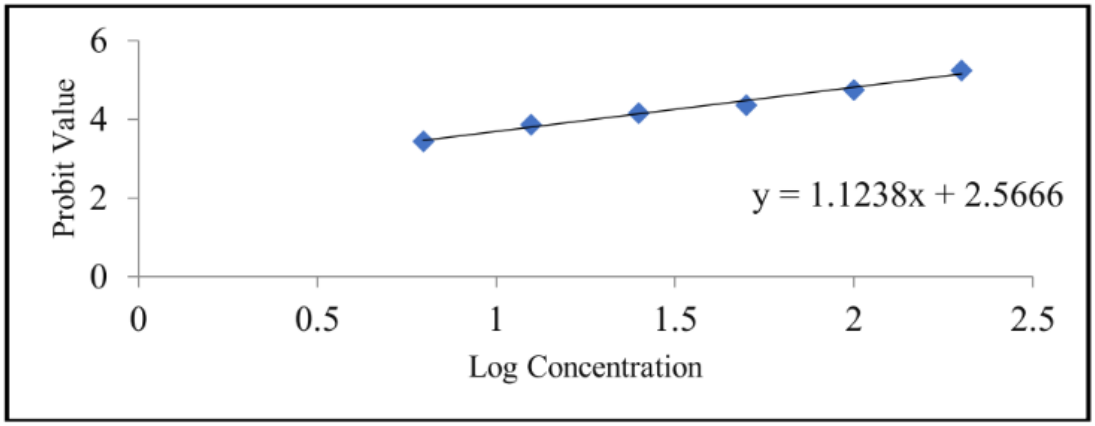

Fig..2: The linear regression curve of Log_{10} concentration versus probit of *P. nodiflora* leaf extract on zebrafish embryos

Effect on Hatchability:

During normal embryogenesis of zebrafish, the hatching process is characterized by the breakdown of the chorion, releasing the free-living larvae. This process usually occurs within 48-72 hpf (Thiagarajan *et al*., 2019). Therefore, the hatchability rate of zebrafish embryos exposed to varying concentrations was evaluated. As presented in Figure 3, the hatchability rate of the exposed embryos was strongly dependent on the concentration of the test extract. At higher concentrations of 50, 100 and 200 µg/ml, hatching rate was reduced at 72 hpf due to the delayed growth and 50% mortality was recorded even before 48 hpf, at the concentration of 200 µg/ml. In contrast, 93.3%, 86.67% and 80% hatching was recorded for concentrations of 6.25, 12.5, and 25 µg/ml, which was comparable to the control group. Hatchability in higher concentrations may have been affected by the weakening of the chorionic membrane or due to the induced chorionase enzyme activity (Pamanji *et al*., 2015).

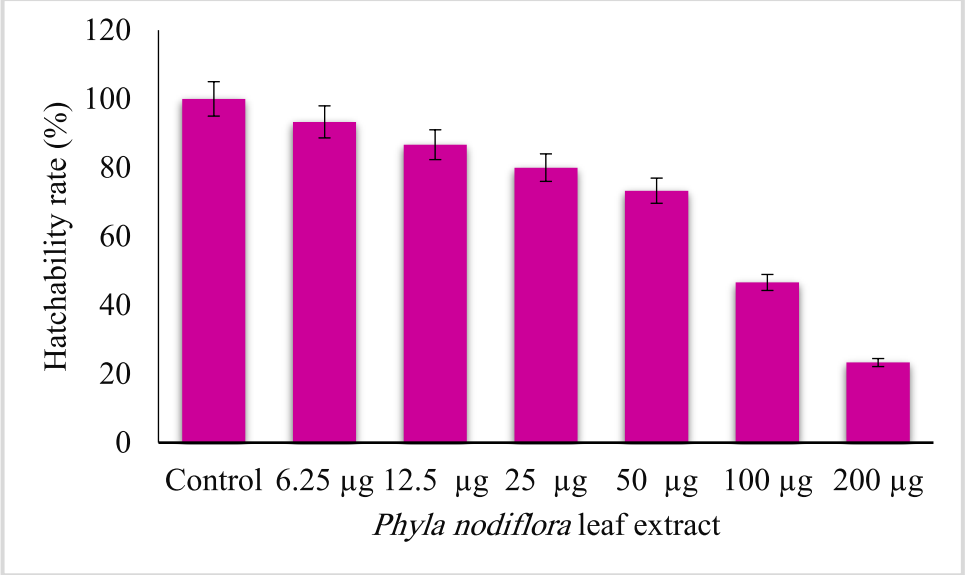

Fig. 3: Effect of different concentrations of *P. nodiflora* leaf extract on hatchability in *D. rerio* embryos

Effect on Rate of Heartbeat:

The normal heartbeat rate of zebrafish embryos ranges from 120 to 180 beats per minute (bpm) (De Luca *et al*., 2014). Therefore, the effect of the varying concentrations of the extract on the embryo's heartbeat rate was evaluated at 72 hpf; values were expressed as several beats per minute (bpm). The results are shown in Figure 4. There was no significant difference in the mean heartbeat rate between the control group and groups with 6.25-25 µg/ml concentrations. In contrast, embryos exposed to 200 µg/ml showed a significant decrease in their heartbeat rate with a mean value of 107.7 bpm, compared to the control and the 6.25-25 µg/ml treatment groups. Heartbeat decrement rates were also reported in zebrafish embryos exposed to zearalenone, naproxen, hexabromocyclododecane and caffeine (Muthulakshmi *et al*., 2018, Rana *et al*., 2010). This result suggests that exposure to *P. nodiflora* extract at higher concentrations may affect embryo cardiac function.

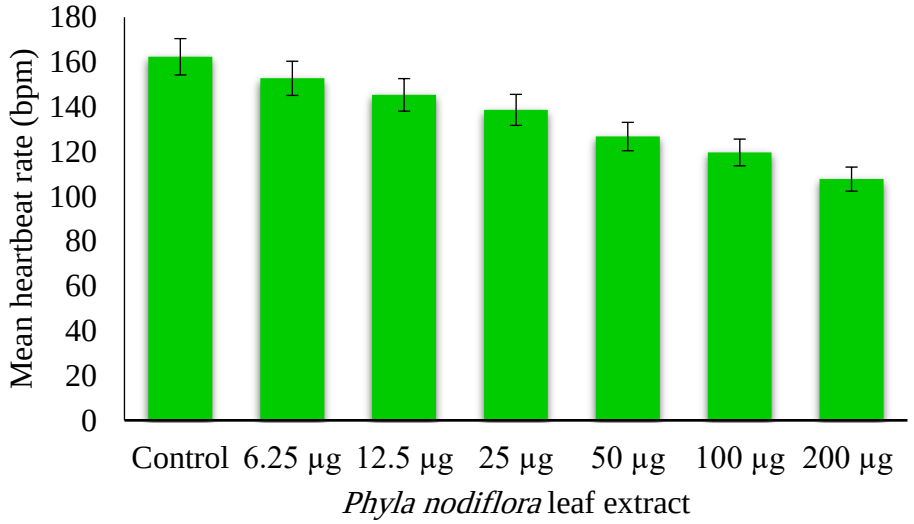

Fig..4: Effect of different concentrations of *P. nodiflora* leaf extract on heartbeat rate in *D. rerio* embryos

Teratogenic Effects:

As shown in Figure 5, embryos exposed to high concentrations exhibited multiple signs of developmental abnormalities, including delay in development, bent or undetached tail, spinal column curving, pericardial sac edema, yolk sac edema, abnormal head shape, and uninflated swim bladder. Delayed growth was noted at 24 hpf in the surviving embryos at 100 and 200 µg/ml concentrations. In contrast, active embryos with complete detachment of the tail from the yolk sac were observed at concentrations of 6.25, 12.5 and 25 µg/ml, comparable with the normal embryos in the control group. Pericardial edema, yolk sac edema, scoliosis, and bent tail were observed at 50 and 100 µg/ml concentration after 72 hpf. Also, at a higher concentration of 200 µg/ml teratogenic effects in the form of deformities in body development was recorded, displaying malformations such as kink tail, bend trunk, physiological curvature, and yolk sac edema after 48hpf. Exposure to toxicants can also lead to egg coagulation and undeveloped organs such as the spine, tail, and heart (Ismail *et al*., 2017).

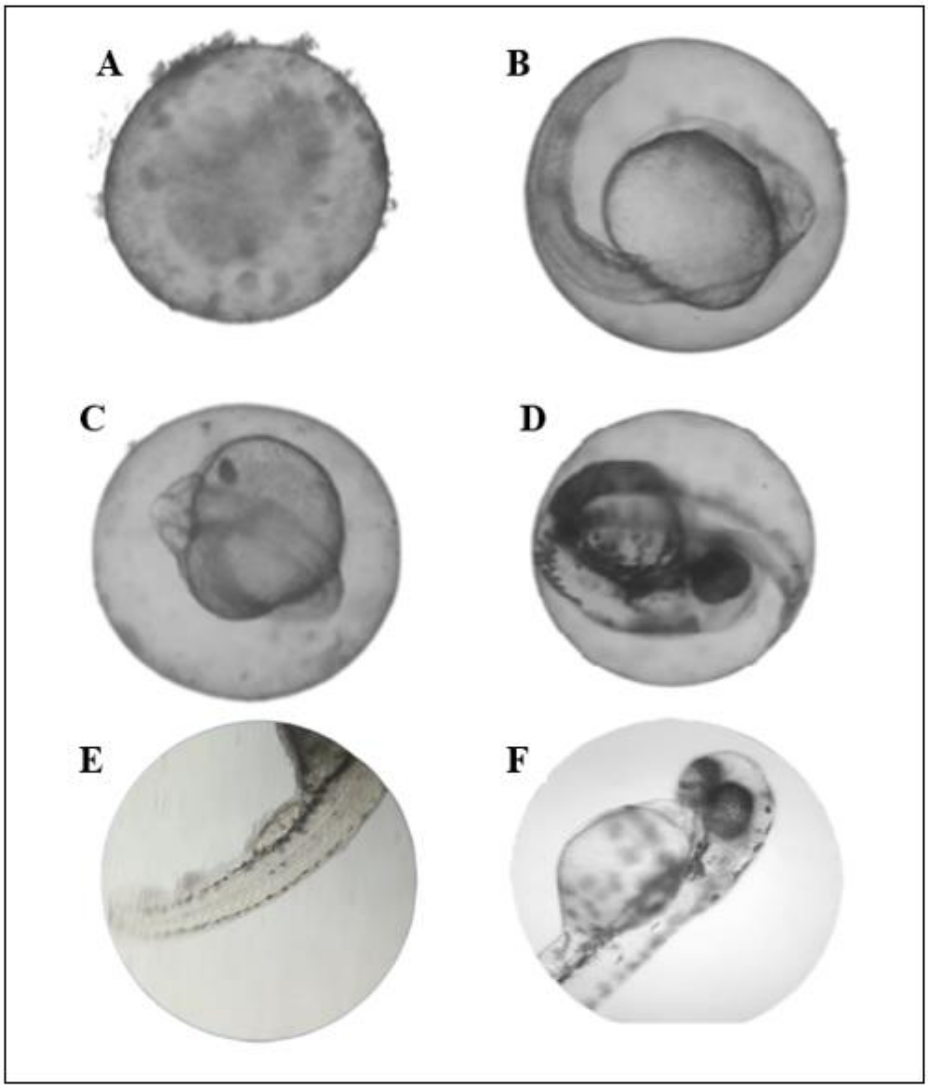

Fig. 5: Morphological malformations of zebrafish embryos exposed to *P. nodiflora* leaf extract: (A) Coagulated egg at 50 µg concentration (B) delayed developed egg at 200 µg concentration (C) Oval shaped egg at 100 µg (D) Coiling of embryo (E) embryo with tail malformation (F) embryo showing pericardial edema and yolksac edema

Conclusion

Based on the aquatic toxicity classification of OECD, *P. nodiflora* aqueous leaf extract was classified as safe. However, high concentration of leaf extract showed developmental toxicity in zebrafish embryos such as increased mortality, low percentage hatchability, decreased heartbeat rate, delayed growth, slightly detached tail, abnormal head-trunk angle, scoliosis/flexure, yolk sac edema, and coagulation of embryos. The results showed a significant concentration-response relationship between toxicity endpoints and *P. nodiflora* leaf extract concentration. However, the mechanism of action of *P. nodiflora* constituents and the investigation of the individual compound responsible for the described malformations are needed to be studied.

Conflicts of Interest: The authors declare no conflict of interest.

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