Teratogenic effects of aqueous extract of *Ficus glomerata* leaf during embryonic development in zebrafish (*Danio rerio*)

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**ABSTRACT**

Over the past few decades, many reports were published in scientific journals describing medicinal properties of *Ficus glomerata* (FG). However, its effects on embryonic development and its safety characteristics have not been studied. The purpose of this investigation was to determine lethal concentration 50 (LC₅₀) and study the effect of aqueous extract of FG leaf (AEFG) on developmental abnormalities in zebrafish embryos. LC₅₀ value of AEFG was calculated by using probit analysis. Effect on percentage hatchability, heartbeat rate, total body length, and developmental morphological abnormalities, i.e., delayed growth, abnormal movement, tail detachment, abnormal head-trunk angle, scoliosis/flexure, and yolk sac edema were recorded. AEFG revealed LC₅₀ of 239.88 ppm. The result showed a significant reduction in percent hatchability (*p* < 0.05), heartbeat rate (*p* < 0.001), total body length (*p* < 0.001), and developmental morphological abnormalities in the embryos treated with AEFG. This research can be used in considering the safety of an AEFG extract for their use during pre-conception or early pregnancy period.

**INTRODUCTION**

Plants have been a source of medicine over centuries and been part of the traditional healing practices. *Ficus glomerata* (FG) leaf has been traditionally used for the treatment of bronchitis, bowel syndrome, wound healing, and piles (Joseph and Raj, 2010). The leaves of FG contained active constituents such as kaempferol, rutin, arabinose, bergapten, psoralenes, ficusin, and coumarin (Baruah and Gohain, 1992; Deraniyagala et al., 1998). Scientific study on aqueous extract of FG leaf (AEFG) revealed its antioxidant (Abusufyan et al., 2018; Eshwarappa et al., 2015), anthelmintic (Divakar et al., 2017), antidiabetic (Divyash et al., 2017a), and antiulcer activity (Divyash et al., 2017b). However, its teratogenic effect should be investigated to obtain safety characteristics of extract prior to its development as a potential pharmacological agent. Despite possessing several therapeutic benefits by the medicinal plants, some of their phytoconstituents have been known to cause potent mutagenicity, carcinogenicity, and teratogenicity (Akintonwa et al., 2009). Teratogens are any substances responsible for the formation of anatomical abnormalities or defects in the developing embryos.

Developing embryos of zebrafish is transparent which permits imaging of the internal organs (Rajaretinam et al., 2015). In addition, this model system has attracted the attention of the researchers for the study of the teratogenic activity of the drugs due to external fertilization, development, and rapid organogenesis of embryonic zebrafish. The present study was undertaken to investigate LC₅₀ value and effects of AEFG on embryonic development using zebrafish embryo as a model system.
MATERIALS AND METHODS

Preparation of Ficus glomerata leaf extract

The leaves of FG were collected from Sindhudurg district of Maharashtra, India and identified with authentication no. 15-023 by botanist Dr. A. S. Upadhye of Agharkar Research Institute, Pune, India. The powdered leaf material was extracted by hot water extraction and concentrated by using rotary evaporator.

Preliminary phytochemical screening

The AEGF extract of FG was characterized by its preliminary qualitative phytochemical screening for alkaloids, amino acids, flavonoids, glycosides, phenolics, tannins, triterpenoids, and steroids by using the standard methods (Khandelwal, 2013).

Zebrafish maintenance and embryos collection

Adult zebrafish were collected from local pet shop located in Mumbai, India. These fish showed characteristics as described by Wixon (2000). All fish were maintained, breed and eggs were collected according to standard procedure (Dulay et al., 2012). The research protocol was approved by the Institutional Animal Ethics Committee with reference No. AIKTC/SoP/IAEC/2017/01.

Zebrafish embryo toxicity test

Zebrafish embryo toxicity test of AEGF was studied by using zebrafish embryos with test time of 96 hours in accordance with Organization for Economic Co-operation and Development (OECD) TG No. 236, 2013. The procedures were adopted with slight modification from several standard methods studied earlier (Indrash et al., 2018; Ingrid et al., 2019; OECD, 2013; Thangavel et al., 2012). After initial range-finding experiments, five concentrations of AEGF 125, 250, 500, 1,000, and 2,000 ppm were selected as the final exposure concentrations. Three replicate treatment groups (3 × 30 embryos) were assayed in 96 well microplates incubated at 27.0°C ± 1.0°C. Evaluation was conducted by using parameters such as delayed growth, limited movement, abnormal head-trunk angle, scoliosis/flexure, and yolk sac edema (Nagel, 2002). These effects were examined and images were taken after 24, 48, and 72 hpte by using a Labomed digital microscope.

Statistical analysis

Data were analyzed by one-way analysis of variance, followed by post-hoc test, paired sample t-test, and descriptive statistic by using JASP 0.9.1.0 software.

RESULTS AND DISCUSSION

Preliminary qualitative phytochemical screening was performed to investigate the nature of chemical constituents present in AEGF. The results of preliminary phytochemical screening are listed in Table 1. Alkaloids, flavonoids, phenolics, and saponins were identified in AEGF.

The laboratory zebrafish is an established animal model for in vivo drug discovery and toxicology studies (Sipes et al., 2011; Strahle and Grabher, 2010; Zon and Peterson, 2005). Fish embryo toxicity test of AEGF was studied on zebrafish embryos exposed to different concentrations of test solution for 96 hours. This test was done to determine the LC50 value of AEGF. The LC50 value is the concentration of extract which causes death in 50% of test animals. The zebrafish embryo was considered as dead if it showed any one parameter of lethality, i.e., coagulation, non-detachment of the tail, somite disruption, and lack of heartbeat. No mortality was observed in embryos exposed to 0–100 ppm concentrations of extract. The data of percentage mortality after 96 hpte with selected concentration of test solution were analyzed by probit analysis with 95% confidence interval and 5% alpha. The LC50 value of AEGF was found to be 239.88 ppm (Fig. 1). Higher LC50 values indicate less toxicity of test drug as greater concentrations are required to produce 50% mortality in organisms (Basha and Rani, 2003). As per OECD guidelines, toxicity of pollutants against zebrafish is categorized as harmful (10 mg/l < LC50 < 100 mg/l), toxic (1 mg/l < LC50 < 10 mg/l), and highly toxic (LC50 < 1 mg/l). Based on this, AEGF extract was found to be relatively safe. Previous study also suggested that AEGF extract was found to be safe in acute oral toxicity test with a single oral dose of 2,000 mg/kg in Wistar rats (Divyash et al., 2017b).

Study of developmental abnormalities in zebrafish embryos

Developmental morphological abnormalities were studied by using parameters such as delayed growth, limited movement, abnormal head-trunk angle, scoliosis/flexure, and yolk sac edema (Nagel, 2002). These effects were examined and images were taken after 24, 48, and 72 hpte by using a Labomed digital microscope.

<table>
<thead>
<tr>
<th>Chemical constituents</th>
<th>AEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids</td>
<td>−</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>−</td>
</tr>
<tr>
<td>Phenolics</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>−</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>−</td>
</tr>
<tr>
<td>Steroids</td>
<td>−</td>
</tr>
</tbody>
</table>

* = positive, − = negative.
Hatching was completed after 72 hours post fertilization in control embryos. However, a significant decrease ($p < 0.05$) in the hatching rate was observed at all selected concentrations of AEFG as compared to control (Fig. 2). Low hatchability and high mortality rate at a high concentration of AEFG could be attributed to the delayed embryogenesis of zebrafish embryos and can, therefore, be one of the important aspects of its sublethal properties. It has been reported that the slimy coating around embryos due to the high concentration of extract might contribute to the death and decrease in overall hatchability (Asharani et al., 2008). Similar slimy coating was seen around embryos with a high concentration of AEFG.

Heartbeat is an important sublethal endpoint which is routinely measured in zebrafish embryos as an index of toxicity. Control embryos show normal heartbeat after 96 hours post fertilization. Significant dose-dependent decrease ($p < 0.001$) in the heart rate was observed at selected concentrations of AEFG as compared to control (Fig. 3). Impaired cardiac functioning in the underdeveloped heart may induce an abnormal heartbeat and failure of blood circulation with subsequent retardation of body growth caused by deficiency of nutrients (Majewski et al., 2017). Control larvae showed total mean body length of 3.72 ± 0.035 mm which is in accordance with the length of normal larvae mentioned in zebrafish developmental staging series (ZFIN, 2018). A significant decrease ($p < 0.001$) in total body length of zebrafish larvae was observed in selected concentrations of AEFG as compared to control (Fig. 4). Concentration-dependent decrease in total body length in AEFG treated zebrafish larvae might be due to the failure of blood circulation and nutritional deficiency associated with a reduction of heartbeat rate. Therefore, it should be noted that the developing zebrafish heart might be a potential target of the high concentration of AEFG toxicity.

Developmental effects of the different concentrations of AEFG were studied after 24, 48, and 72 h pte and presented in Tables 2 and 3. Active embryos with spontaneous movement and completely detached tail were observed at 0 and 125 ppm concentrations of AEFG. However, high concentration (250, 500, 1,000, and 2,000 ppm) of AEFG extract showed delayed growth, limited movement, and slightly detached tail at 24 and 48 hpte (Table 3, A1 to B6). Yolk sac edema, scoliosis/flexure,
and abnormal head-trunk angle were the three most commonly occurring morphological changes observed at 250 and 500 ppm concentration after 72 hpte of AEFG (Table 3, C3 and C4).

This study is the first investigation of AEFG extract on developmental toxicity in zebrafish embryos. Previous studies suggested that chemicals and drugs have similar toxicological effect in developing embryos (Driever et al., 1996; Lam et al., 2005). Preliminary qualitative phytochemical investigation showed the presence of alkaloids, flavonoids, phenolics, and saponins which have been reported for their cytotoxic actions (Romagosa et al., 2016). This strong cytotoxic activity can be significantly considered for the future evaluation of its anticancer, antitumor, and apoptotic properties.

**CONCLUSION**

Based on aquatic toxicity classification of OECD, AEFG was classified as safe. However, high concentration of AEFG showed developmental toxicity in zebrafish embryos.

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**Table 2. Teratogenic effects of AEFG in zebrafish embryos.**

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Delayed growth</th>
<th>Limited movement</th>
<th>Slightly detached tail</th>
<th>Abnormal head-trunk angle</th>
<th>Scoliosis/Flexure</th>
<th>Yolk sac edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>250</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>500</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1,000</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2,000</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

+ = positive, − = negative; NA = not applicable as embryo died at the time of observation

**Table 3. Representative images of teratogenic effects of AEFG in zebrafish embryos.**

<table>
<thead>
<tr>
<th>Concentrations ppm</th>
<th>24 hpte</th>
<th>48 hpte</th>
<th>72 hpte</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>125</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>250</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>500</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>1,000</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td>2,000</td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
</tbody>
</table>

(A3 to C6): Indicate delayed embryogenesis; (C3, C4): White arrow showing yolk sac edema at 250, 500 ppm after 72 hpte of AEFG; (C4): Red arrow showing scoliosis/flexure at 500 ppm after 72 hpte of AEFG; (C4): Black arrow showing abnormal head-trunk angle at 500 ppm after 72 hpte of AEFG; (C5, C6): Blue arrow showing coagulation of zebrafish embryos at 1,000, 2,000 ppm after 72 hpte of AEFG.
such as low percentage hatchability, decreased heartbeat rate, decreased total body length, delayed growth, slightly detached tail, abnormal head-trunk angle, scoliosis/fixure, yolk sac edema, and coagulation of embryos. The results showed a significant concentration-response relationship between toxicity endpoints and AEFG concentration. However, the mechanism of action of AEFG constituents and investigation of individual compound responsible for the described malformations are needed to be studied.

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CONFLICT OF INTERESTS
The authors declare that they have no conflicts of interest.

REFERENCE


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