Journal of Applied Pharmaceutical Science Vol. 9(05), pp 107-111, May, 2019
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2019.90514
ISSN 2231-3354

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

Abusufyan Shaikh[1*], Kalidas Kohale[2], Mohammed Ibrahim[3], Mohib Khan[4]

[1]School of Pharmacy, Anjuman-I-Islam's Kalsekar Technical Campus, New Panvel, Maharashtra, affiliated to Mumbai University, Mumbai, India.
[2]Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India.
[3]PNR College of Pharmacy, Hyderabad, India.
[4]Oriental College of Pharmacy, Mumbai, India.

ARTICLE INFO

Received on: 25/01/2019
Accepted on: 11/03/2019
Available online: 08/05/2019

Key words:
Ficus glomerata, toxicity test, teratogenesis, embryogenesis.

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 (LC_{50}) and study the effect of aqueous extract of FG leaf (AEFG) on developmental abnormalities in zebrafish embryos. LC_{50} 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 LC_{50} 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 kampferol, 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 LC_{50} value and effects of AEFG on embryonic development using zebrafish embryo as a model system.

*Corresponding Author
Abusufyan Shaikh, School of Pharmacy, Anjuman-I-Islam's Kalsekar Technical Campus, New Panvel, Mumbai University, Mumbai, India.
Email: abusufyan.shaikh @ aiktc.ac.in

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 AEFG 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 AEFG 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 (Indra *et al*., 2018; Ingrid *et al*., 2019; OECD, 2013; Thangavel *et al*., 2012). After initial range-finding experiments, five concentrations of AEFG 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 of lethality, i.e., coagulation, non-detachment of the tail, somite disruption, and lack of heart-beat. LC_{50} value was calculated using probit analysis in MS-Excel.

Hatchability

A zebrafish embryo is considered as hatched when the entire body of larvae from tail to head is out of the chorion. Percent hatchability after 72 hours post-treatment exposure (hpte) was calculated using the formula:

$$\%\,\text{Hatchability} = \frac{\text{No. of hatched embryos}}{\text{Initial no. of embryos}} * 100$$

Heartbeat rates and total body length of zebrafish

Numbers of heartbeat per minute of anesthetized zebrafish embryos after 72 hpte were recorded under Labomed digital microscope. For determining the total body length, images of anesthetized zebrafish larvae after 96 hpte were taken and measured by using image J software.

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.

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 AEFG. The results of preliminary phytochemical screening are listed in Table 1. Alkaloids, flavonoids, phenolics, and saponins were identified in AEFG.

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 AEFG was studied on zebrafish embryos exposed to different concentrations of test solution for 96 hours. This test was done to determine the LC_{50} value of AEFG. LC_{50} 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 LC_{50} value of AEFG was found to be 239.88 ppm (Fig. 1). Higher LC_{50} 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 < LC_{50} < 100 mg/l), toxic (1 mg/l < LC_{50} < 10 mg/l), and highly toxic (LC_{50} < 1 mg/l). Based on this, AEFG extract was found to be relatively safe. Previous study also suggested that AEFG 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).

Table 1. Phytochemical screening of AEFG.

Chemical constituents	AEFG
Alkaloids	+
Amino acids	−
Flavonoids	+
Glycosides	−
Phenolics	+
Saponins	+
Tannins	−
Triterpenoids	−
Steroids	−

+ = positive, −= negative.

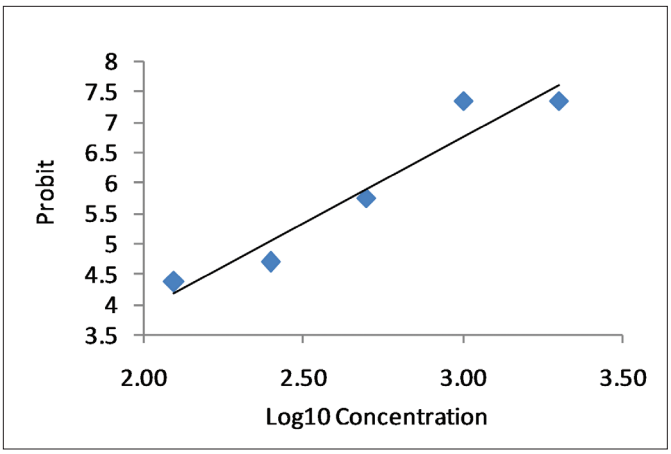


Figure 1. The linear regression curve of Log10 Concentration versus probit of AEFG on zebrafish embryos. $y = 2.850 * X - 1.803$. $R^2 = 0.92592$.

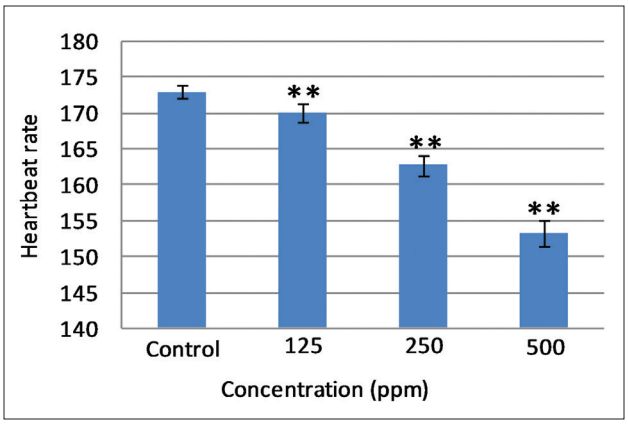


Figure 3. Effect of different concentrations of AEFG on heartbeat rate in zebrafish embryo. Values are expressed as mean ± SD ($n = 10$ embryos); $^{**}p < 0.001$ when compared to corresponding control.

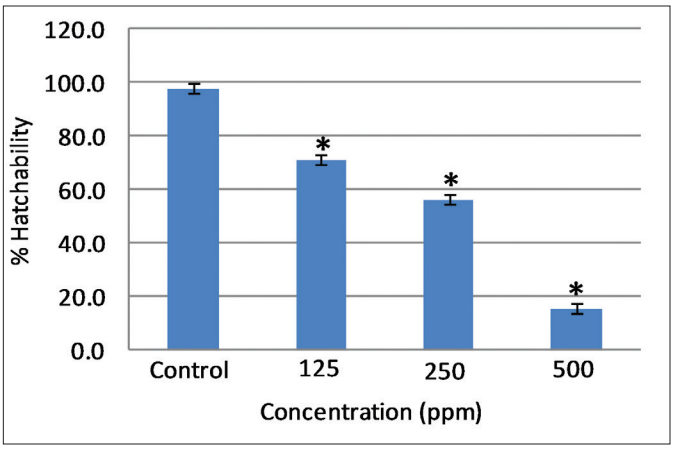


Figure 2. Effect of different concentrations of AEFG on % hatchability in zebrafish embryo. Values are expressed as mean ± SD ($n = 30$ embryos); $^{*}p < 0.05$ when compared to corresponding control.

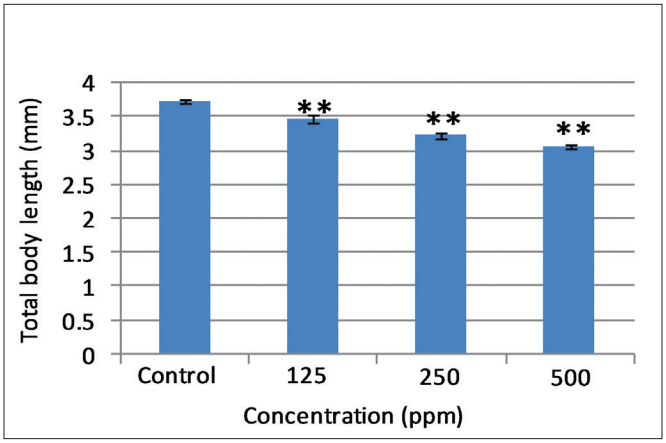


Figure 4. Effect of different concentrations of AEFG on total body length (mm) in zebrafish embryo. Values are expressed as mean ± SD ($n = 10$ embryos); $^{**}p < 0.001$ when compared to corresponding control.

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.

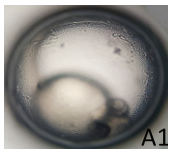
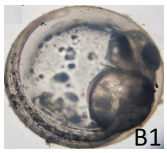
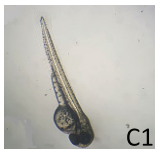
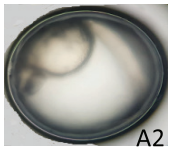
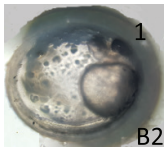
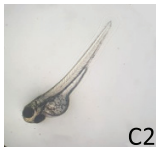
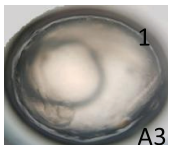
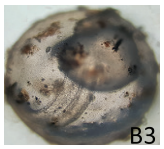
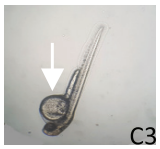
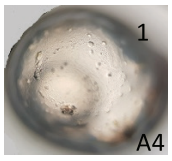
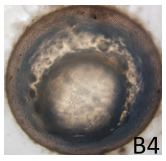
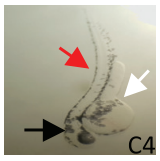
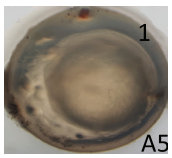
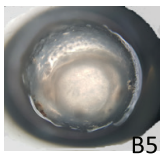
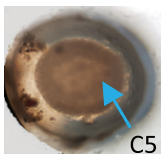
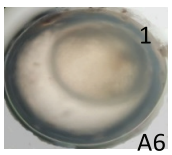
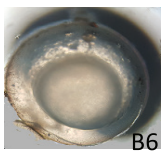
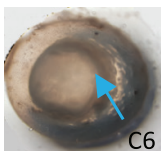
Developmental effects of the different concentrations of the AEFG were studied after 24, 48, and 72 hpte and presented in Tables 2 and 3. Active embryos with spontaneous movement and completely detached tail were observed at 0 and 125 ppm concentrations of AEFG. However, high concentration (250, 500, 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,

Table 2. Teratogenic effects of AEFG in zebrafish embryos.

Concentration (ppm)	Delayed growth	Limited movement	Slightly detached tail	Abnormal head-trunk angle	Scoliosis/ Flexure	Yolk sacedema
125	−	−	−	−	−	−
250	+	+	+	−	−	+
500	+	+	+	+	+	+
1,000	+	+	+	NA	NA	NA
2,000	+	+	+	NA	NA	NA

+ = 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.

Concentrations ppm	24 hpte	48 hpte	72 hpte
0	 A1	 B1	 C1
125	 A2	 B2	 C2
250	 A3	 B3	 C3
500	 A4	 B4	 C4
1,000	 A5	 B5	 C5
2,000	 A6	 B6	 C6

(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.

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

such as low percentage hatchability, decreased heartbeat rate, decreased total body length, 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 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.

ACKNOWLEDGMENTS

The author wishes to thank Dr. Abdul Razak Honnutagi, Director, AI-Kalsekar Technical Campus and Dr. Shariq Syed, Dean, School of Pharmacy, AI-Kalsekar Technical Campus for providing facilities to carry out this work.

FINANCIAL SUPPORT AND SPONSORSHIP

None.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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How to cite this article:
Shaikh A, Kohale K, Ibrahim M, Khan M. Teratogenic effects of aqueous extract of *Ficus glomerata* leaf during embryonic development in zebrafish (*Danio rerio*). J Appl Pharm Sci, 2019; 9(05):107–111.