



Evaluation of Teratogenicity of *Semecarpus Anacardium* L. And *Trigonella Foenum-Graecum* L. In Zebrafish.

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ABSTRACT

Semecarpus anacardium L. and *Trigonella foenum-graecum* L. has been traditionally used in India. These drugs have a long history of use for the prevention and treatment of diseases. But due to the toxic potential of these drugs there is need to have an easy model which can be used for screening teratogenic potential of such plants. The zebrafish embryo is a promising alternative model in some fields of biomedical research, such as drug screening, safety pharmacology, and developmental toxicity assessment. This study is aimed at evaluating teratogenicity of aqueous extracts of *Semecarpus anacardium* L. and *Trigonella foenum-graecum* L. seeds on zebrafish embryos. The concentrations of the aqueous extracts of *Semecarpus anacardium* L. and *Trigonella foenum-graecum* L. used were 0.01, 0.02, 0.04 and 0.08%. Zebrafish embryos were exposed from 24-96 hpf to both the aqueous extracts. Malformations assessment was carried out at 96 hpf. They were evaluated for phenotype changes such as head malformation, pericardial edema, yolk sac edema and jaw malformation. There was dose dependent teratogenicity shown by both the extracts on zebrafish embryos. Based on these results, it was concluded that *Semecarpus anacardium* and *Trigonella foenum-graecum* seeds extracts may have potential teratogenic effects on fetuses.

Keywords: *Semecarpus anacardium*, *Trigonella foenum-graecum*, Teratogenicity, Zebrafish embryo, Fetus.

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INTRODUCTION

Teratogenesis refers to the production of defects in the fetus. A teratogenic agent is responsible for producing such a defect. The term teratogen is cited in the context of causing anatomical defects in an embryo that was previously differentiating normally. The thalidomide tragedy in the late 1950s and early 1960 brought into sharp focus the importance of rigorous and relevant testing of pharmaceuticals prior to their introduction into the marketplace (Kim and Scialli, 2011). Many phytomedicines are taken during pregnancy. These phytomedicines have a long history of use for the prevention and treatment of diseases. Throughout the world many pregnant women consume a great variety of herbs. Traditionally, 12-59% of women used herbal products during pregnancy for a variety of reasons, including pregnancy related conditions (nausea, vomiting, constipation), to prepare for labour, to induce abortion (Taloubi *et al.*, 2013). Medicinal plants form a large group of economically important plants which provide the basic raw material for indigenous pharmaceuticals. In Ayurveda, herbal drugs are thought to be safer in the treatment of variety of diseases and most of the Ayurvedic and herbal medicines are polyherbal preparations. Also some of the medicinal plants are mentioned not to be used during pregnancy. The teratogenic potential of any herbal drug may be dose dependent. Various herbal drugs have shown the teratogenicity as reported in the literature (Khalki *et al.*, 2010; Pandey *et al.*, 2012; Tahir and Khalid, 2007). Due to the teratogenic potential of medicinal plants there is need of testing medicinal plants which are consumed during pregnancy.

Semecarpus anacardium L. (Family: Anacardiaceae) is distributed in the sub Himalayan region, tropical and central parts of India. The nut is commonly known as 'marking nut' and the vernacular as 'Ballatak' or 'Bhilwa'. Traditionally it is used in asthma, ascites, epilepsy, neuralgia, tumours, warts, psoriasis and rheumatism; as abortifacient, anthelmintic and vermifuge (Shah and Seth, 2013). Studies have reported that the drug has anti-inflammatory, anti-arthritic, antioxidant and anticancer activities (Premalatha, 2000). Very few studies have been reported on hypolipidemic, hypoglycemic, anti-atherogenic, antifungal, antifertility and neuroprotective activities (Arul *et al.*, 2004; Mary *et al.*, 2003; Phukan *et al.*, 2015; Sharma *et al.*, 2002; Sharma *et al.*, 2003., Tripathi and Pandey, 2004). It is usually avoided in pediatric age group, pregnant women, and also in certain diseased conditions such as bleeding diathesis, renal function disorder, history of vesication and past history of intolerance to *Semecarpus anacardium* (SA; Raut *et al.*, 2007).

Trigonella foenum-graecum L. (fenugreek) is an annual plant belonging to the family of Papilionaceae. It is extensively cultivated in India and northern Africa. Seeds and leaves of this

plant have been used for centuries not only as food but also as an ingredient in traditional medicine. *Trigonella foenum-graecum* (TFG) seeds are known for various medicinal purposes. Many of these traditional uses of TFG are supported by data from pharmacological studies (Khalki et al., 2010). Traditionally, it has been used in the treatment of anorexia, dyspepsia, gastritis and convalescence (Shah and Seth, 2013). TFG is reported to have hypoglycemic (Deng, 2012), hypocholesterolaemic and antioxidant (Belguith-Hadriche et al., 2013), antifungal and antibacterial (Dharajiya et al., 2016), immunomodulatory (Bin-Hafeez et al., 2003), and anti-inflammatory (Pundarikakshudu et al., 2016) effects. However, TFG seeds have also been identified as potentially allergenic (Fæste et al., 2010) and have an anti-fertility effect in rabbit (Kassem et al., 2006). TFG has already shown developmental toxicity in mice (Khalki et al., 2010) and SA is to be avoided in pregnant women (Raut et al., 2007). Depend on this the present study was undertaken to evaluate teratogenic effects of aqueous extracts from SA and TFG seeds in zebrafish embryos.

Since testing on human being is irrational, a suitable model to screen the plants is necessary. Need for this investigation is to have an easy model which can be used for screening teratogenic potential of a medicinal plant. In the last decade assays using embryonic stages of the vertebrate zebrafish (*Danio rerio*) have attracted the attention of toxicologists due to their several advantages. Zebrafish are easy to maintain and produce large numbers of embryos that develop outside the mother. The transparency of their embryos allows the scoring of teratological and embryotoxic effects easily. In addition, the development is fast and has been well characterized, including morphological, biochemical and physiological information at all stages of early development (Hill et al., 2005). The development process is highly conserved across vertebrates and the zebrafish genome is completely characterized. Hence, zebrafish embryos represent an attractive model allowing reduction and refinement of animal use in research (Yang et al., 2009). Numerous studies have been reported exploring the capacity of zebrafish assays for the assessment of the teratogenic potential of chemicals showing a good concordance with in vivo results in mammals (Ali et al., 2014; Carlsson et al., 2011; Hermsen et al., 2011; Selderslaghs et al., 2009; Teixidó et al., 2012; Yang et al., 2009).

In this study the extracts were added to the water in which the embryos develop thus focused on aqueous extracts to avoid any confusing effects of carrier solvents.

MATERIALS AND METHOD

Plant collection and identification

SA and TFG seeds were collected in the area of Pune, India. The plant materials were identified in the Botanical Survey of India, SA (BSI/WRC/Cert./2015/175) and TFG (BSI/WRC/IDEN.CER./2016/453). Voucher specimens of both the plants were deposited at the herbarium of the Botanical survey of India.

Preparation of extract

SA and TFG seeds were ground to fine powder using grinding machine. Powdered seeds were agitated in distilled water for 10 h. The aqueous extracts obtained were centrifuged and the supernatants were washed with acetone to get dry powder. The yield of the aqueous extract for was 17% and for TFG 15%. Both the extracts were stored at -20°C until further use.

Ethics statement

The guidelines for zebrafish maintenance and experimentation, as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India, were followed. The animal care and experimental protocols were sanctioned by Institute Animal Ethics Committee (approval no. SKNCOP/IAEC-110-16).

Experimental Animals

Zebrafish (*Danio rerio*), wild-type were used for the experiment. All experiments were performed on zebrafish larvae at 24-96 hpf (hour post fertilization). All the embryos were incubated at 28.5°C in E3 medium. Fishes were maintained on a 14-h light: 10-h dark cycle. The fishes were fed with dry flake food and live brine shrimp. The day before eggs were required, males and females were placed in breeding tanks. The breeding tank with mesh egg trap used to prevent the eggs from being eaten by fishes. On the next morning, the eggs were collected 30 min after the light had been turned on. Eggs were transferred into plastic Petri dishes containing fresh E₃ medium. Eggs were washed repeatedly to remove debris. Unfertilized and dead embryos were identified under a stereomicroscope and removed.

Treatment of embryos

Zebrafish embryos of 24 hpf were transferred from the Petri dish using a sterile plastic pipette into 96-well plates. Single embryo was placed in each well, so that dead embryos would not interfere with others embryos, and also to observe proper growth of individual embryos for the whole duration of the experiment. Concentrations used for each aqueous extract were 0.01%, 0.02% 0.04% and 0.08%. We used E3 medium containing NaCl (5 mM), KCl (170 μM), CaCl₂ (330 μM) or MgCl₂ (330 μM), and methylene blue (0.6 μM) as a vehicle to prepare all the concentrations. Added 250 μL of either freshly prepared test compound, or vehicle, as a control in each well. 10 embryos for each concentration and 10 embryos as vehicle controls for each

compound were used. Experiment with each concentration of each extract was repeated 3 times ($n=30$ embryos per concentration of extracts and 30 embryos for vehicle).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism for Windows, to see the impact of extracts on zebrafish larvae development. We used one-way analysis of variance and Dunnett's multiple-comparison test with a probability level of 5% as the minimal criterion of significance.

RESULTS AND DISCUSSION

The effects of differing concentrations of aq. extracts of SA and TFG seeds on zebrafish embryos development were studied. Embryos were exposed continuously from 24 to 96 hpf. Zebrafish embryos were then assessed for malformations at 96 hpf. Both the extracts showed concentration dependent malformation. Analysis of larval morphology was carried out using stereomicroscope. The results of morphological analysis of larvae are summarized in Table no.1. The phenotypic effects that can be seen are illustrated in Figure 1.

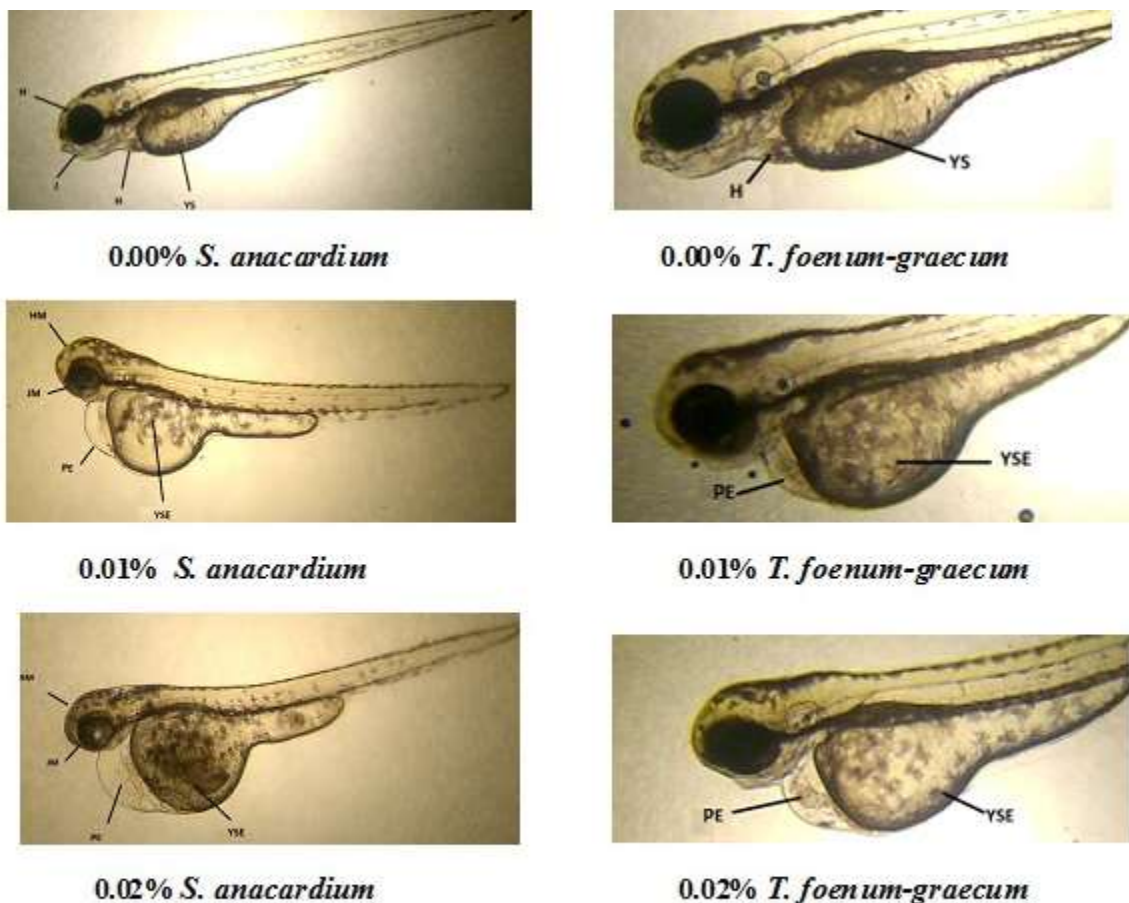


FIG. 1. Aqueous extract of *Semecarpus anacardium* L. and *Trigonella foenum-graecum* L. seeds showed phenotype changes at 96hpf. Where, HM-Head malformation, JM-Jaw malformation, PE- Pericardial edema, YSE- Yolk sac edema.

Table 1: Percent morphological abnormalities per extract concentrations used in Zebrafish larvae assay.

Concentration in percent	Head malformation	Pericardial edema	Yolk sac edema	Jaw malformation
0.00 %	0.00	0.00	0.00	0.00
0.01% SA	43.98±3.6 ^a	56.01±3.61 ^a	52.31±5.3 ^a	43.51±6.01 ^a
0.02% SA	58.92±1.78 ^a	63.68±4.16 ^a	63.68±4.16 ^a	49.99±4.125 ^a
0.01% TFG	No change	30.55±2.777 ^a	34.25±5.631 ^a	No change
0.02% TFG	No change	56.01±3.616 ^a	47.68±5.338 ^a	No change

The values given are the mean percentage morphological abnormalities from three replicates. Significance levels of all phenotype effects for each concentration were calculated from three independent experiments. Dose dependent phenotype changes were seen. Statistical analysis with one-way analysis of variance followed by Dunnett's multiple comparison test. ^a $p < 0.0001$ compared with 0.00% concentration. ($n=30$). TFG: *Trigonella foenum-graecum*, SA: *Semecarpus anacardium*.

The importance of the SA and TFG in folk medicine, as well as their potential pharmacological and toxicological properties underline the relevance of this study. Both the extracts have shown concentration dependent phenotype changes in zebrafish larvae. The phenotype changes were significant as compared to the vehicle group in both the types of extracts. The embryos were exposed from 24-96 hrs. This change became progressively more pronounced and was quite obvious by 96 hpf. So, the endpoint of phenotype changes in zebrafish larvae was at 96hpf. The concentration dependent phenotype changes with the aqueous extract of SA as head malformation, pericardial edema, yolk sac edema and jaw malformation were observed. With the concentration of 0.01%, SA extract showed mild phenotype changes which were prominent with the concentration of 0.02%. E3 medium used as a solvent to prepare concentrations of both the aqueous extracts as E3 medium showed better compatibility with zebrafish embryos. The phytochemical screening of the SA reveals the presence of biflavonoids, phenolic compounds, bhilwanols, sterols and glycosides (Premalatha, 2000). Further, the phytochemical fractionations of the aqueous extract of SA may provide the idea about the particular phytochemical constituent responsible for the teratogenic effect.

The aq. extract of TFG showed the phenotype changes as heart edema and yolk sac edema at various concentrations which were mild at 0.01% and prominent at 0.02%. Aq. extract of TFG seeds and aq. extract of SA seeds showed the phenotype changes at same concentrations. The phytochemical screening of TFG seeds reveals tannic acid, fixed oils and a bitter extract,

diosgenin, the alkaloids trigonelline, trigocoumarin, trigomethylcoumarin, and steroidal saponins such as gitogenin and traces of trigogenin and vitamin A (Petit et al., 1995). Khalki, 2010 has reported the developmental toxicity of the aq. extract of TFG seeds in mice, which reveals the developmental toxicity of aq. extract of TFG seeds may be due to the presence of steroidal saponins and alkaloids. Accordingly it can be suggested that these compounds could be responsible for the observed teratogenic effects in zebrafish.

Despite the advantages of zebrafish as a model for teratogenicity noted above there are shortcomings with the model that require further research. Extrapolation from data acquired with the zebrafish to humans remains an issue, as it does for other methods (Piersma et al., 2008). The exact dose of extract that reaches the embryo can not define itself since the chorion of the embryo can act as a biological protective barrier (Gellert and Heinrichsdorff, 2001) or the actual uptake of an extract by the embryo.

CONCLUSION

In conclusion the present study provides experimental evidence that aqueous extracts of *S. anacardium* and *T. foenum graecum* affects growth of zebrafish embryos and shows teratogenicity. Both the plants are used for the various medicinal purposes in Ayurveda. Both the drugs should be cautiously used in the pregnancy to avoid the teratogenic effects in the fetus. Further research on these specific issues and tests for an extended list of medicinal plants, will provide scientific information for this assay to be integrated into screening programs for the hazard identification of herbal drugs. However, future work in the validation of the zebrafish larval assay must include a wide range of medicinal plants, including those that are known teratogens in humans. The method described can offer a valid alternative for teratogenicity testing. In this way it can also contribute to a reduction of the use of mammalian organisms for teratogenicity assessment.

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