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RESEARCH ARTICLE

Embryotoxicity and Teratogenicity of *Taraxacum officinale* Leaf Extract on the Developing Embryos of the Zebrafish, *Danio rerio*

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ABSTRACT

The present study aimed to detect the existence of Phytochemicals and to assess the toxic and teratogenic activities of aqueous leaf extract of *Taraxacum officinale.* Qualitative analysis of *T. officinale* leaf extract revealed the presence of several medicinally important phytochemicals. Based on microscopic observation, the survival and sublethal endpoint were assessed and calculated. Assays for toxicity of *T.officinale* leaf extracts on *Danio rerio* embryos confirmed that mortality, hatchability, heartbeat rate, teratogenicity, and embryogenicity were concentration-dependent. Lethal effects were pronounced in treatment groups whereas sub-lethal and teratogenic toxicity effects like yolk deformities, scoliosis, edema, reduced mobility, stunted tail, and tail malformation were expressed in 32% of the exposed models. Tail malformations were the most marked teratogenic effect of the plant extract. On the whole, *T.officinale* leaves contain bioactive components that exhibit toxicity and teratogenicity in the zebrafish embryos. This study will help in the early prediction of the potential health risk of using this plant as conventional medicine.

Keywords: *Taraxacum officinale*, Zebrafish, toxicity, teratogenicity, LC50.

INTRODUCTION

Plants are abundant in bioactive compounds and have been used as medicinal products [1] because of their richness of phytochemicals that can be used in the production and synthesis of drugs [2]. Additionally, due to their mild effects and very effective bioactivities, they are considered as an alternative medication [3]. Although plants possess enormous medicinal properties, it has been shown that a few of their chemical components are shown to possess mutagenic, carcinogenic, toxic and teratogenic effects. Hence forth, it is important to investigate the toxic and teratogenic effects of therapeutic plants.

Taraxacum officinale, commonly called dandelion is a globally distributed flowering herbaceous plant that has a position in the Asteraceae family. It is found to be a diuretic, cholerectic, and known to have anti-inflammatory, anti-oxidative, analgesic, anti-hyperglycemic, and anti-thrombotic activities, aside from the fact that dandelion is widely recognized as a weed [4,5]. Leaves are a rich source of an assortment of minerals and vitamins [6]. Leaves contain pharmacologically active compounds such as flavonoids (luteolin, apigenin, isoquercitrin, caffeic acid, and chlorogenic acid), terpenoids, and triterpenes [7]. The whole plant has been used to treat many diseases, including acne, eczema, high cholesterol, heartburn, gastrointestinal disorders, diabetes, and cancer. In Chinese, Arabian and Native American traditional medicine it is used to treat a variety of diseases related to liver, inflammation and cancer [8]. The aqueous leaf extract of this herb, however, has not yet been checked for its toxicity effects.

The capacity of substances to affect an organism is known as toxicity [9], while teratogenicity is known to be the malformation or unusual development of the shapes and forms caused by teratogens on the body of the developing embryo [10]. Therefore, toxicity testing is important to screen a sample's chemical compositions to ensure that they do not contain any life-threatening components. The embryonic and larval form of *Danio rerio* is increasingly used as a toxicological and teratological model due to the transparent larvae and embryo, fast development cycles, high fertility, ease of maintenance in the laboratory [11], high genetic homology to mammals [12], and its similarity tovertebrates of higher forms in embryonic development [13]. Zebrafishes can efficiently absorb small molecular compounds and thus serves as a promising model for drug screening and impact assessment of the teratogenic compounds [14,15]. Thus, this makes zebrafish embryo an advantageous assay in exploring many diversions of toxicology study yielding a prompt outcome [16]. The study was intended to evaluate the embryo-toxic and teratogenic effects of the *T.officinale* leaf extract using zebrafish embryos which could lead to the discovery of other potential bioactivities of this plant.

MATERIALS AND METHODS

Sample collection and preparation

The leaves of *T. officinale* were collected from CMST campus, Manonmaniam Sundaranar University. The leaves were washed with distilled water to expel any residue, chopped into pieces, and subjected to shade drying for 20 days. Dried leaves were made into a fine powder using a dry grinder. The powdered leaf material was processed for further extraction experiments in air-tight bags.

Extraction of *T. officinale*

The aqueous extract was prepared with the addition of 10g leaves powder of *T. officinale* in an Erlenmeyer flask containing 100 ml of distilled water in the extent of 1:10. The mixture was kept in a boiling water bath at 60ºC for 1 hour. The extract was further filtered through Whatmann No.1 filter paper and put away in refrigerated condition until further assessment.

Preliminary Phytochemical screening of aqueous leaf extract of *T. officinale*

To detect the existence of active secondary metabolites, the aqueous extract of *T. officinale* was subjected to preliminary phytochemical examination. Standard techniques were followed to test each extract separately with particular chemical reagents [17]. The presence (+) or absence (–) of specific active ingredients was determined by visible colour change or precipitate formation.

***In vivo* studies**

Zebrafish maintenance and embryo selection

Healthy adult Zebrafishes (*Danio rerio*) of wild-type (AB strain) were acquired from the Center for Marine Science and Technology, MS University, Tirunelveli. At a temperature of 27 ± 1 ºC, pH 7 (± 0.5), conductivity 650µS/cm and a 12:12-hour day/night cycle, male/ female zebrafishes in the ratio of 2:1 ratio were kept in aquariums in a dark room. For the mating purpose, a cycle of 14 hr light/10 hr dark was adopted. After exposure to light in the morning, zebrafish start laying eggs within 30 minutes. The eggs were carefully collected after spawning and immediately rinsed with 2 mg/L methylene blue to prevent contamination. Viable embryos were used in the assay while those unfertilized eggs were discarded [18].

Experimental design and treatment

The viable embryos were treated with various concentrations of *T. officinale* leaf extract up to 72 hpf (hour post-fertilization). For each group, thirty embryos were treated. In triplicates, all the tests were completed.

***In vivo* toxicity**

According to OECD 2013 guidelines, toxicity assays were performed using zebrafish embryos [19]. By diluting stocks of the aqueous leaf extract of *T. officinale,* the test arrangements were made. Thirty viable eggs were chosen randomly and introduced into each of the 6 well plates with five distinct concentrations (10 µl, 20 µl, 40 µl, 80 µl, and 160 µl) of *T. officinale* by dissolving in 3 ml embryo water. A compound microscope was used to visualize the development of embryos and larvae until 72 hours post-treatment. *D. rerio* embryos mortality was noted at 12h, 24h, 48h, and 72hpf. The mortality, hatchability, heartbeat rate of the embryos, and LC50 values were determined and photomicrographs were taken.

Teratogenicity assay

To assess if the compound has a teratogenic effect on the embryos, the teratogenicity assay was performed [20]. Thirty embryos were transferred to 6 well plate containing various concentrations of *T. officinale* (10 µl, 20 µl, 40 µl, 80 µl, and 160 µl) was inspected for distortions every 24h under a compound microscope. Frequencies of malformations were recorded until 72 hpf. Zebrafish embryos were assessed for delayed development, tail malformation, head malformation, scoliosis, limited movement, edema, and so on. The normal development of the embryo was compared with previously described by Kimmel et al. [21].

Statistical analysis

The experiment was carried out in triplicates and the obtained data was provided employing Mean ± Standard deviation (SD). The data thus obtained was analyzed statistically using SPSS version 22 software. The substantial differences between the experimental and control groups of embryos/larvae with a confidence interval of 95% were tested by one-way ANOVA with a Tukey comparison test.

RESULTS AND DISCUSSION

Phytochemical analysis

Table 1 lists the phytochemical elements found in *T. officinale*, including tannins, saponins, phenols, alkaloids, flavonoids, steroids, carbohydrates, and terpenoids. The reported health benefits of *Taraxacum officinale* leaves could

be attributed to these phytoconstituents (tannins, terpenoids, and phenolic compound) that may have medicinal value; tannins are plant metabolites well known for antimicrobial properties, and phenols retain antioxidant potential that could augment the body's resistance to pathology induced free radical generation [22]. Terpenoids, phenolics, and tannins have recently been discovered to be important components of several antibiotics used to treat prevalent pathogenic strains [23,24].

Mortality rate

Coagulation and the lack of heartbeat in zebrafish embryos are symptomatic of mortality [11]. The mortality of embryos was noted and the results were given in Mean ± SD and stated in Table 2. Treatment with lower leaf extract concentrations (10µl and 20 µl) resulted in mortality of 17.3 ± 16.43% and 30.6 ± 14.91% respectively, towards the end of the investigation at 72 hpf. Conversely, the embryos treated with a high dose of 160 µl displayed amortality rate of 100 % at 24hpf. Leaf extract concentration of 40 µl showed 47.3 ± 8.47% at 48 hpf and the end of 24hpf,50% mortality reached at the concentration of 80 µl in *Danio rerio* embryos indicating 69.6 ± 5.70% mortality.

Lethal concentration dose (LC50) of the aqueous leaf extract

Probit analysis was used to measure the LC50 value for *T.officinale* leaf extract, which is shown by the statistical calculation of the amount of toxicant per body weight needed to regulate the 50 percent mortality rate of zebrafish embryos [25].The logarithmic inference of the LC50 value using the embryo concentration and the mortality rate is shown in the figure1, and the LC50 was determined to be 29.75µl.

Hatchability of zebrafish embryos

Embryo dechorionization is the major indicator of hatchability. Zebrafish embryos begin hatching at 48 hpf, and in normal conditions, finish hatching at 72 hpf [26]. In the treatment groups, however, the embryo-hatching rate was significantly reduced with hatching rates of 82.6±4.81%, 75.6±6.23%, and 67.6±6.76 in the 10 µl, 20 µl, and 40 µl respectively. Eggs hatchability was greatly reduced in the 80 µland 160 µl showing 48 ± 8.50% and 11.3 ± 51.24% low hatching rates. These data showed a substantial dose-dependent decrease in the rate of hatching of treated groups of leaf extracts, which was significantly lower ($p < 0.05$) than that of the control groups. The percent hatchability for the control and leaf extract treated embryos at 72 hpf is shown in figure 2. A few embryos exhibited delayed hatching which could be because of formative anomalies in the developing embryos, bringing up the incompetence of the chorion to break; it also may well be explicated by the morphological anomalies detected in the embryos, which limit hatching [27].

Heartbeat rate

The normal heartbeat rate of zebrafish embryos ranges from 120 to 180 per min[28,29]. The mean heartbeat rates per minute of zebrafish embryos exposed to various concentrations of leaf extract at 72 hpf revealed that the control embryos had the highest heartbeat rate of 157.3 per min, followed by the embryos treated with concentrations of 10 µl, 20 µl, 40 µl, and 80 µl, having heartbeats of 147 ± 2.04%, 133 ± 2.55%, 129 ± 2.32%and 115. 3 ± 3.97% beats/min, respectively. No heartbeat was noticed in the *D. rerio* embryos as a result of early mortality at the concentration of 160 µl. While looking at the outcomes, the heartbeat rate is decreased, when the treatment concentration increases.

Teratogenicity of Zebrafish embryos

The teratogenicity recorded in zebrafish is critical as it reflects the predictive intensity of the bioassay for assessing formative toxicity in vertebrates [30]. Teratogenic effects were observed in zebrafish embryos after exposure to leaf extract. The teratogenicity chart (Table 3) and morphological defects (Fig. 3) indicated that all the embryos treated with the least concentration (10 µl) had fewer morphological defects, contrasted with the control. The treatment grouping 160 µl was found to be very toxic to the embryos, with all the embryos dying within 36hpf. Pericardial edema, yolk sac edema, tail malformation, tail curvature, growth retardation, delayed hatching, and scoliosis were observed in embryos treated with leaf extract at the concentrations of 20 µl, 40 µl,and 80 µl. A similar outcome is identified by Dulay et al. [31] in zebrafish embryos exposed to *G. Lucidum* extract showing tail malformation (bent tail and S-shaped tail) at 72 hpf as the most important morphological abnormality as seen in Fig 3I.

The most conspicuous malformations were tail malformation, tail curvature, and yolk sac edema. The frequency of tail malformation may be attributed to warfarin, an anticoagulant coumarin derivative being the fingerprint morphological endpoint of toxicity [32]. Yolk sac edema and tail malformations were intermittently detected endpoints of zebrafish embryos after contact with the known embryo-toxic and teratogenic compounds such as valproic acid, retinoic acid, hydroxyurea, methoxyacetic acid, and boric acid[33]. Thus, the yolk sac edema was likely due to the symptoms of hemostasis, hindered blood movement, or liquid incongruity. This hypothesis is upheld by a study suggesting that pericardial and yolk sac edema in zebrafish is associated with impaired blood flow and fluid imbalance [34]. The tail in Fig 3H showed a sharp kink in the center of the tail length coupled with a turn in the direction of the tail [35].

CONCLUSION

The study revealed that the toxic and teratogenic effects of *Taraxacum officinale* aqueous leaf extract triggered concentration dependent developmental toxicity in the embryos of zebrafish. Zebrafish embryos are intensely affected by high concentrations of treatment concentrations in terms of mortality, hatchability, and heartbeat rate. At 72hpf, the LC50 was found to be 29.75µl with teratogenic effects such as scoliosis, curved tail, twisted tail, and tail malformation. Advanced exploration is needed to define and elucidate the mechanisms acting specifically on particular teratogenicity in *D. rerio*embryos.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Table 1: Phytochemical Constituents of Aqueous Leaf Extract of *T. officinale*

Phytochemicals tested	Aqueous leaf extract of *T. officinale*
Tannins	+++
Saponins	++
Carbohydrates	++
Terpenoids	++
Steroids	+
Flavonoids	+++
Phenol	+
Alkaloids	++
Glycosides	+
Anthocyanin	-

Table 2: Mortality of *D. rerio* Embryos After 12, 24, 48, and 72 h of Exposure to Varying Concentrations of *T. officinale* Aqueous Leaf Extract (n=30)

Leaf extract Concentration	Mean mortality of the embryos (%)			
	12 hrs	24 hrs	48 hrs	72 hrs
Control	2.22	0.00	1.11	0.00
10 µl	5.55	7.77	2.22	0.00
20 µl	10.0	10.0	4.44	0.00
40 µl	22.22	8.88	4.44	2.22
80 µl	35.55	23.33	5.55	0.00
160 µl	55.55	37.77	8.88	0.00

Table 3: Teratogenic Effects of *T. officinale* Leaf Extract in Zebrafish Embryos

Leaf extract concentration	Types of Toxicity						
	Lethal		Sublethal				Teratogenicity
	Coagulation	No heartbeat	Pericardial edema	Yolk sac edema	Tail malformation	Scoliosis	Delayed hatch
Control	-	-	-	-	-	-	-
10 µl	*	-	-	*	*	-	-
20 µl	*	*	*	-	*	*	*
40 µl	*	*	*	*	*	-	*
80 µl	*	*	*	*	nd	*	nd
160 µl	*	*	nd	nd	nd	nd	nd

- nil, * - present, nd- not detected

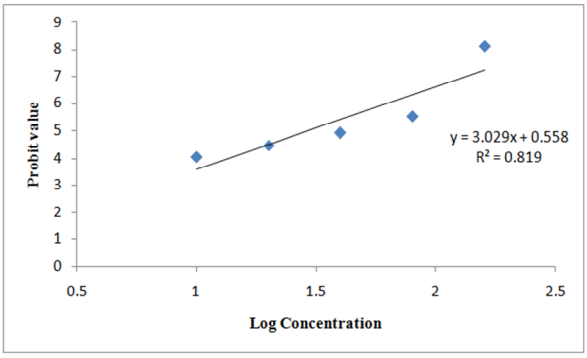

Figure 1: The linear regression curve of Log_{10} Concentration versus probit of aqueous leaf extract of *T. officinale* on zebrafish embryos

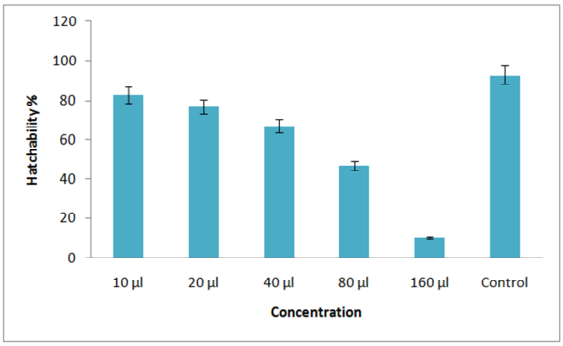

Figure 2: Effect of various concentrations of *T. officinale* leaf extract on hatchability in zebrafish embryos; *p <0.05 when compared to corresponding control.

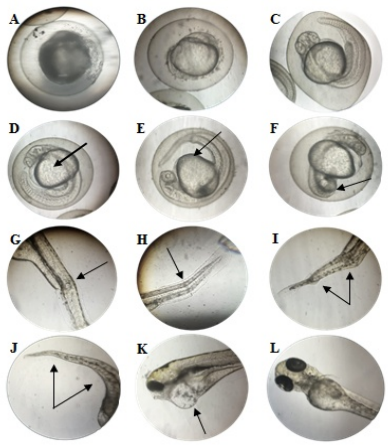

Figure 3: 10Xmagnification of Morphological malformations of zebrafish embryos exposed to *T. officinale* leaf extract from 6-72hpf: (A) Coaggulated egg at 40µl concentration hpf (B) delayed developed egg at 160 µl concentration (C) Oval shaped egg at 80 µl(D,E) embryo showing yolk sac edema and abdominal edema at 36h and 42 hpf (F) embryo with deformed tail (G-I) embryos with tail malformations (J) embryo showing scoliosis at 72h (K) pericardial edema at 72h (L) Normal embryo at 72 hpf