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Isolation, Characterization and *in vivo* toxicity of *Delonix regia* Raf. Petals on *Danio rerio*

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Abstract

Natural pigments and food colorants have shown a positive impact on human health compared to the use of synthetic pigments and colorants. We are reporting the oral toxicity of *Delonix regia* Raf. It has natural pigments with various pharmaceutical properties. The crude extract was obtained from the *D. regia* petals and the functional groups in the extract were characterized with UV, FTIR and the compounds were identified using GC-MS. To analyze the toxicity of the crude extract, Zebrafish was used as a model organism. Different concentrations of the extract (5 µg/mL to 2000 µg/mL) were used in the zebrafish embryo. The results revealed that the toxic effect was only seen at higher concentrations (1000 µg/mL and beyond). In lesser concentrations, no toxicity was observed. This preliminary study forms a basis for further animal studies. Also, it adds a new safe food colorant.

Keywords: *Delonix regia*, Food Colorants, Zebra Fish, Oral Toxicity, Petal Extract

Introduction

Delonix regia is widely known as Flame tree or Gulmohar, an ornamental tree that produces bright red to orange, yellow-colored flowers [1]. They have many medicinal properties like antipyretic, anti-inflammatory, anti-diabetic, anti-diarrheal, hepato-protective, photo-protective and cytotoxic properties alongside anti-microbial and anti-oxidant properties [1-4]. At present, there is vast emergence of new diseases, but there are not many solutions available. One of the better ways to deal with this problem is to come up with new drugs that can be evaluated and used as a curative drug. Many carotenoids, flavanoids and phenols were reported from the flowers of *D.regia* [5,6]. Although many bioactivities are known in various research studies, the systematic evaluation of toxicology is rarely reported. Earlier, toxicity studies on in-vivo animal models have been studied with extracts of pigment-yielding plants. For example, flavonoids and anthraquinones from *Allium sativum L.* [7].

Recently, most of the *in vivo* studies for toxicology and clinical studies are carried out using Zebrafish [8], being a model organism since early drug discovery and toxicology screening; it is a preferred organism for biological, physiological and molecular studies [8,9]

Danio rerio is a simple organism with high fecundity, low maintenance and easy to observe distinct eggs and experimenting in all stages. The OECD (Organization for Economic Co-operation and Development) guidelines have given a methodology for FET (Fish Embryo acute Toxicity) to evaluate toxicity in Zebrafish embryo

developmental stages [11,12]. An earlier report on Brine shrimp's lethal toxicity was reported in *Delonix sp*. However, there have been very little to no studies on the toxicological effects of *Delonix regia*, particularly in Zebrafish. Here, we report the details of extraction, characterization, and toxicity level of *D. regia* petals extract in *Danio rerio*. We believe this study will be useful in the food dyeing [13,14] and pharmaceutical industries.

Materials and Methods

Plant material: *Delonix regia*Raf. flower petals were collected from the VIT campus during flowering seasons (April-May). The petals were kept in shadow to dry for two weeks and taken for characterization.

Chemicals and reagents: Chemicals and solvents used in the study were analytical standards purchased from Himedia Pvt. Ltd, India. Throughout the study, double distilled and millipore water were used.

Extraction: In a continuous soxhlet extraction approach, thirty grams of dried petals were taken and extracted using ethanol (100%). Along with pigments; many compounds get eluted in alcohol. The resulting extract was then subjected to rotavapor (BUCHI Labortechnik AG, Switzerland) for vaporization at 335mbar and pressure reduction of 42° C. The *Delonixregia* petals extract (DRPE) obtained was then calculated for the measurement of percentage yield and stored at 4° C for further characterization [15].

Characterization of DRPE: The crude extract was subjected to UV spectroscopy for the confirmation of the compounds in the crude extract. The range was from 200 nm to 800 nm (Shimadzu UV-2401 PC). The absorbance spectrum of DRPE was recorded. To study the functional group of this petal extract, FTIR was used. FTIR spectrophotometer model Magna 750 with DTGS detector, NI-chrome source and KBr beam splitter reported a spectrum with a maximum of 100 scans and a resolution of $4cm^{-1}$ was used. Spectra were collected and treated using OMNIC software offered by the manufacturer of the spectrophotometer. To identify the compounds and their mass the extract was subjected to GC-MS and predicted through library search available with the instrument.

Fish breeding and embryo collection: Adult, wild type, *Danio rerio* (> 6 months old) was obtained from Kamaraj University, Madurai, Tamil Nadu, India. Fish treatment was conducted in compliance with OECD No.236. In a normal setting, the adult fishes were maintained in a tank with a standard environment, water temperature 26±2 °C, light period 16:8 hr ratio of light: dark and pH 6.8-7.2. Frozen Artemia and boiled chicken liver were fed to the broodstock.

Standard breeding method was followed. Females with big bellies and males that are active and healthy were chosen. The ratio was 3 females: 5 males. The brooders were separated in the breeding tank till early morning and allowed to mate by 5.30 am with artificial lights. The tanks were fitted with artificial plants and the floor bottom was covered with mesh net for eggs to be protected and easy collection. When the light was on, spawning happened within 30 minutes at 5.30 am and collection of the eggs was done carefully; the expended fish were then returned to their tanks and instantly given food. In the embryo E3M medium, the embryos were collected subsequently. Further, they were rinsed with the same medium; the embryos which are fertilized were separated and kept at 28°C for natural development (6hrs period).

DRPE FET Test: The embryos of *Danio rerio* were exposed to the crude extract at various concentrations in a 24- well plate according to the method described in OECD guidelines. At 6hrs post-fertilization, stable embryos were washed and tested under the microscope. Samples of fertilized eggs (n=10) at 6 (hours after post-fertilization) was treated with different DRPE concentrations. The experiment was

performed in triplicate. Embryo media (2 mL) with 0.1 percent DMSO (Dimethyl Sulfoxide) was added in each well with varying concentrations of DRPE (5, 10, 25, 50, 100, 250, 500, 1000 and 2000 µg/mL). The control (untreated group) had only 0.1 percent DMSO and 2mL of E3M. Using a bright-field microscope the developments were assessed for three days, microscope was purchased from Meiji Techno Pvt. Ltd. Japan; model Meiji MT4300H). The embryos and larva were continuously examined to record their body formation for a three-day exposure period.

Statistical analysis: One-way ANOVA was used to compare different concentration groups followed by the multiple comparisons through the test of Dunnett's was treated to be significant (p<0.05). All statistical studies were conducted using the programme version 5 of Graphpad Prism (Graphpad Prism, USA).

Results and Discussion

DRPE characterization

Different spectroscopic and analytical techniques were used to characterize DRPE. A total of 5.76g of ethanol extract was obtained from a 30g of shade-dried petals. The extract had a characteristic reddish-brown colour that changed to yellow when diluted in ethanol. The ethanol extract of DRPE was analyzed in UV visible spectrometry revealed peaks at 272, 290, 378 and 451 nm with wavelength of 1.902, 0.974, 0.496 and 0.321 respectively (Fig. 1).

FTIR data of DRPE revealed unique functional group of band 3325 cm^{-2} indicating the existence of an alcohol group (OH), 2974 cm^{-2} and 2885 cm^{-2} displays alkane group (C-H stretch), 1653 cm^{-2},1381 cm^{-2} displays alkene group (-C=C- stretch)and aldehyde group; similarly 1328 cm^{-2}, 1274 cm^{-2}, 1087 cm^{-2}, 1045 cm^{-2} indicate aromatic, ester groups (C-O stretch) and 879 cm^{-2}, 802 cm^{-2}, 650 cm^{-2} show vibrations of alkyne groups (C-H bend) (Fig. 2). GC-MS results for the given DRPE were shown in Fig. 3 along with the predicted compounds through analyzing the retrieved compounds with the known over library search.

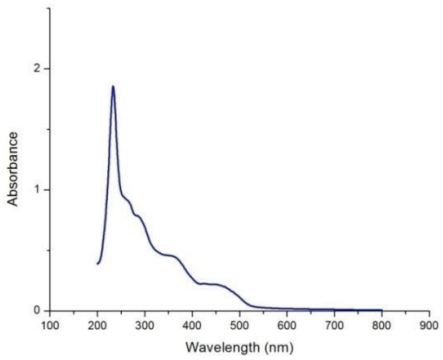


Fig.1: UV Visible spectrum of DRPE wavelength ranging from 200 to 800 nm.

DRPE effect on danio rerio fish embryos

Danio rerio embryological changes to the concentration of DRPE were recorded at 24, 48 and 72 hpf are shown in Fig. 4. Embryo and larva percentage viability at 24, 48 and 72 hpf were calculated and plotted as graph in Fig. 5. Embryo mortality rates were higher at 1000 µg/mL and 2000 µg/mL at 48 and 72 hrs. In controls, there was no mortality at 24 hpf, 10% mortality at 48 hpf and 20 % mortality at 72 hpf. In comparison with the controls, all the treatment groups were analyzed. From 5 µg/mL to 100 µg/mL, there is no significant change in mortality at 24, 48 and 72 hpf. But at 250 µg/mL and 500 µg/mL a significant mortality was recorded (p> 0.05). Above this concentration at 1000 µg/mL and 2000 µg/mL, no live embryos were recorded even at 24 hpf.

Morphological abnormalities

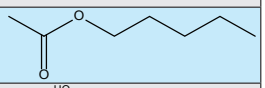
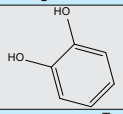
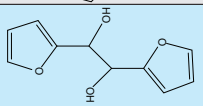
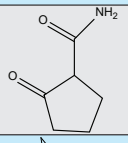
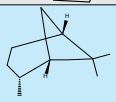
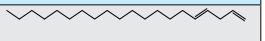
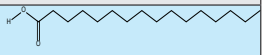
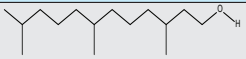
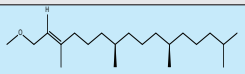
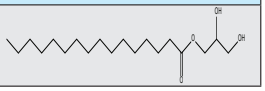
Stable and malformed status of *Danio rerio* embryos in each treatment can be easily seen in Fig. 4. The untreated control group did not show any abnormality. Despite that, the treated groups (1000 and 2000 µg/mL) showed higher rates of malformation relative to the control group (p<0.05). There was no

abnormality in the embryos exposed to lower concentrations (< 250 µg/mL) of DRPE.

As the concentration of DRPE increased, the rate of malformation increased during the later developmental stages. At 48 hpf and 72 hpf in 1000 µg/mL abnormalities like bended notochord (BN), spinal curvature (SC), twisted caudal fin (TCF) were observed. Whereas, at 48hpf and 72 hpf in 2000 µg/mL spinal curvature (SC) and dead (D) embryos, larva were observed in Fig. 4.

In an earlier report, *A. squamosa* extract showed low levels of embryo survival at higher concentrations. In addition to that, our study also showed high levels of dosage, there is a decrease in embryo survival [8]. Based on the previous article, the LD_{50} is estimated. DRPE LD_{50} on *Danio rerio* is calculated to be 234 µg/mL, similar to *A. carmichaeli* [16].

Table 1. List of compounds present in DRPE, conformed through GC-MS library

S.No	Retention Time	Compound Similarity	Molecular Formula	Molecular weight (g/mol)	Structure
1.	9.927	Pentyl acetate	$CH_3COO(CH_2)_4CH_3$	130.18	
2.	11.068	Catechol	$C_6H_6O_2$	110.11	
3.	12.100	Hydrofuroin	$C_{10}H_{10}O_4$	194.18	
4.	13.702	Cyclopentene 1 - carboxyamide	$C_6H_9NO_2$	127.14	
5.	16.143	Bicyclo [3.1.1] heptanes, 2,6,6- trimethyl	$C_{10}H_{18}$	138.25	
6.	16.663	1 , 4 - Eicosadiene	$C_{20}H_{38}$	278.5	
7.	18.316	Palmitic acid	$CH_3(CH_2)_{14}COOH$	256.42	
8.	19.306	1-Dodecanol, 3,7,11- trimethyl	$C_{15}H_{32}O$	228.41	
9.	19.465	Phytol	$C_{20}H_{20}O$	296.5	
10.	23.827	1-Palmitoyl glycerol	$C_{19}H_{38}O_4$	330.5	

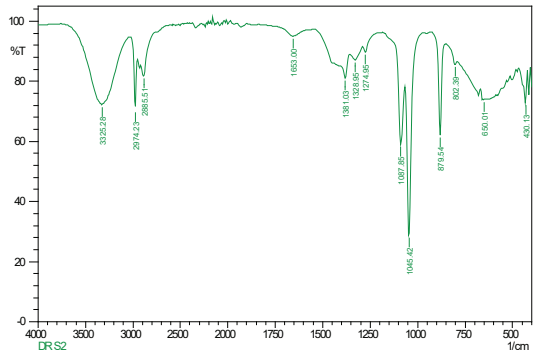


Fig. 2: FTIR spectrum of DRPE in 4000–400 cm^2 region

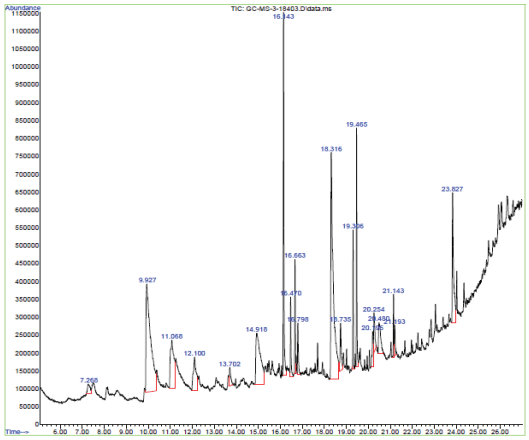


Fig. 3: Chromatographic representation of DRPE showing the presence of ten major peaks.

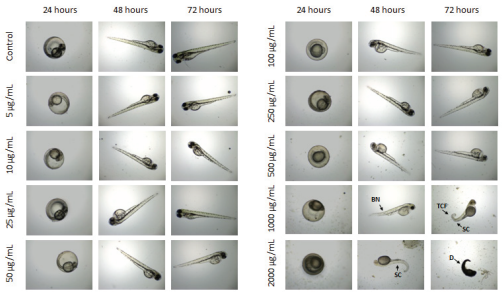


Fig. 4: Effect of DRPE on *danio rerio* embryos. The microscopic observation of *Danio rerio* embryos exposed with various concentrations (5, 10, 25, 50, 100, 250, 500, 1000, 2000 µg/mL) of DRPE at different time intervals (24, 48 and 72 hours). [Abbreviations: BN (bended notochord), SC (spinal curvature), TCF (twisted caudal fin), D (death)].

D. regia petals were collected and extracted similar to the previously reported extraction procedures as they have also used ethanol as a solvent for the high yield of metabolites [17,18]. The extracts yield is identical to the report where they have tried using different extraction procedures. The maximum yield mentioned was around 20% which again is matching to our extraction yield [19]. There have mentioned various compounds from many *Delonix* sp. extracted with different solvents in an earlier article [20], similarly, we have reported some of the compounds from *D. regia* petals extracted with ethanol.

UV characterization, FTIR and GC-MS compounds predicted in our study are similar to the early reports where they used *M. merculiaris* flower extract. The UV spectrum showed absorption peaks at 272, 290, 378 and 451; this is similar to Dhivya report [21]. The major peaks in the GC-MS results of the present study were matched with the compounds retrieved using the GC-MS library especially palmitic acid was in higher proportions [22]. In *P. pulchellum*, the levels of phytols (Abundance: 800000, RT: 19.645) when compared to Velmurugan report [23] with other organic acids and decane substrates.

The most responsive process against external stimulus is the early maturation of embryos from toxic chemicals, mechanical stress and other toxicants [24]. Findings from our study revealed a substantial rise in the impact of toxicity after 48 hrs post- fertilization, which led to a decline in the rate of survival; organ malformation and delayed hatching rate. In comparison, heart and blood supply formation is completed within 28- 48hrs post-fertilization and it is the earliest internal organ to form and operate properly. The vulnerability of Zebrafish embryos to plant extracts has been seen in many articles. Ethanolic extract of *C.*

dactylon and *S. acuta* revealed altered blood flow velocity and heartbeat rate when treated on Zebrafish embryos cardiovascular study with LC_{50} of 32.6 and 20.9 mg/mL [25]. Similarly, *J.curcus* extract showed LC_{50} of 1.61 g/L in Zebrafish embryo toxicity study [26]. During the three-day study of Zebrafish toxicity, there was no sign of mortality in all the concentrations at 24 hpf, there was 50% to 60% death occurrence in 100- 250 µg/mL concentration at 48 hpf; whereas there was 60- 100% death in 250- 500 µg/mL concentration. LD_{50} calculated was as less toxic when compared to curcumin standard LD_{50} value mentioned [27].

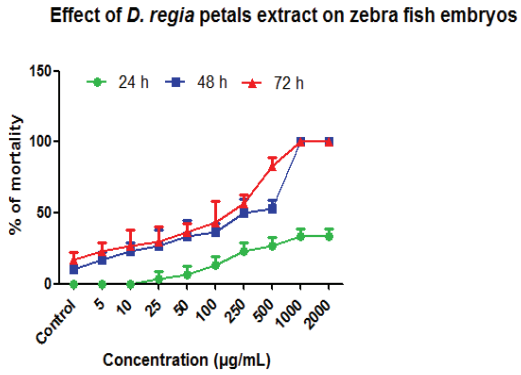


Fig. 5: Graph showing mortality rate of DRPE on *Danio rerio* embryos at different time intervals (24, 48 and 72 hpf) and its data were represented as Mean ± SD.

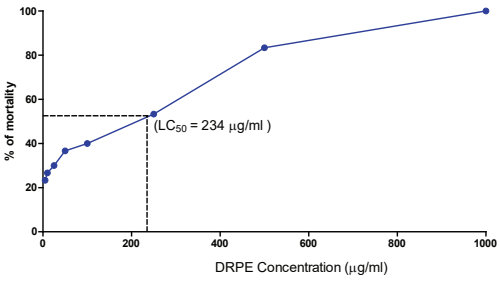


Fig. 6: LD_{50} value of DRPE on *Danio rerio* embryos at the end of the experiment (72 hrs)

Conclusion

As we look forward to using DRPE in the food and pharmaceutical industry, our primary concern is to study its toxicity. The compounds are characterized using UV, FTIR and GC-MS. Many organic compounds are found to in large quantities like phenylacetate, catechol, hydrofuroin, palmitic acid and phytol. The toxicity study was conducted with Zebrafish (*Danio rerio*). Different concentrations (5 µg/mL to 2000 µg/mL) were used and the concentration above 1000 µg/mL was found to be exhibit toxic effects (delayed hatchling and malformation). Thus it is proved that 250 µg/mL would be safe in the food and pharma industry. This work is the first paper to examine the toxicity of *Delonix regia* petals extract.

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