

RESEARCH PAPER

Embryotoxicity of Alkaloidal and Flavonoidal Extract of *Plumeria obtusa* in zebrafish (*Danio rerio*) Embryo Model

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ABSTRACT

Background: *Plumeria obtusa* L. is a tropical ornamental plant, belongs to family Apocynaceae, which has been traditionally employed in the control of diabetes, wounds and skin ailments. Traditionally the crude extracts from leaves, flowers and bark of this plant have been found to be antioxidant, antidiabetic and antimicrobial, etc.

Objective: This study showed the embryotoxic potential of an alkaloid enriched fraction and a flavonoid enriched fraction sequentially extracted from the same plant material was assessed and compared, for developmental safety in the zebrafish (*Danio rerio*) embryo model.

Methods: Standard confirmatory tests were used to confirm both fractions as being positive for their respective phytochemical class. The six concentrations (3.125-100 µg/mL) of each fraction were administered to the zebrafish embryos for 96 h, following OECD Test Guideline 236 and the endpoints of mortality, hatching and morphological endpoints were recorded every 24 h.

Results: Probit analysis yielded 96-h LC₅₀ values of 128.600 µg/mL (95% fiducial limits: 50.096-330.126) for P1 and 160.673 µg/mL (95% fiducial limits: 63.460-406.804) for P2. Pericardial edema was observed as a significant developmental abnormality in both fractions, and this was most clearly related to concentration, and was largely reversible as shown by survivors which developed normal morphology and swimming behaviour by 96 hpf; additional abnormalities related to the yolk sac, tail and the spinal cord were seen at higher concentrations.

Conclusion: The results give for the first time a concentration-referenced, fraction-specific developmental toxicity profile of *P. obtusa* and a helpful concentration window for the design of additional pharmacological studies on this plant.

Keywords: *Plumeria obtusa* L., Apocynaceae, zebrafish (*Danio rerio*), developmental toxicity, morphological endpoints

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INTRODUCTION

Medicinal plants are still one of the most important potential sources of pharmacologically active secondary metabolites and the interest of scientists in the bioactive properties of plants has been stimulated by the structural diversity and variety of biological activities exhibited by these compounds. Among these large classes of metabolites, the alkaloids and flavonoids are particularly significant the traditional medicinal plants are well represented in these classes. Alkaloids have analgesic, antimicrobial and anticancer properties, and flavonoids have been found to be good broad-spectrum antioxidants and anti-inflammatory agents, etc. It is often not possible to predict or assign the response to a particular class of phytochemicals in a crude plant extract, as the response is likely to be due to a combination of several phytochemicals. Separation of the alkaloid-rich and flavonoid-rich fractions and their studies separately will provide a more accurate perception of the chemistry of the plant responsible for a specific biological action [1,2].

Plumeria obtusa L. (Apocynaceae) also known as Singapore graveyard flower, frangipani is a tropical, deciduous ornamental tree. It is widely cultivated for its fragrant flowers in the gardens, parks and graveyards. The leaves, flowers, bark and latex of the plant are used in traditional medicine for a variety of purposes such as treating diabetes mellitus, wounds, and skin diseases, as well as serving as a purgative and diuretic, etc. The phytochemical analysis of the leaves and the stem bark of *P. obtusa* have revealed the presence of pentacyclic triterpenoids, iridoids, cardiac glycosides, flavonoids, phenolic acids and alkaloids [3,4]. Traditionally, the leaves of *P. obtusa* are used for medicinal purposes, which align with its reported pharmacological effects and activities, such as antioxidant, antidiabetic and antimicrobial activities, including the amelioration of hyperglycaemia-associated behavioural changes in alloxan-induced diabetic rats [5], free-radical scavenging activities of leaves extract. Although the number of phytochemical and pharmacological studies on *P. obtusa* has been done but there is still limited toxicological information available [6].

The presence of a beneficial pharmacological effect in one assay is not necessarily a developmental safety, since the secondary metabolites which give the beneficial effect to the plant may also interfere with normal developmental processes at high enough concentrations [6]. The concentration-dependent effect of purified plant alkaloids, such as aconitine and berberine, is also well illustrated in the zebrafish, where they caused pericardial edema and cardiac looping changes, although other compounds, like caffeine, did not. This body of evidence is used to assess an alkaloidal fraction and a flavonoidal fraction from the same plant separately, as the developmental profile of one is not necessarily the same as the other, and cannot be safely assumed to be the same [7].

The zebrafish (*Danio rerio*) embryo is a widely used vertebrate model for developmental toxicity screening due to its developmental toxicity characteristics, including external fertilization, fast and well

characterized embryogenesis, optical transparency and genetic homology to other vertebrates [8]. It uses newly fertilized zebrafish eggs and exposes them to test substance for a standardized period of 96 hours. The developmental phases of Zebrafish Embryo, as shown in Figure 1. These enable direct in vivo microscopic observation of developmental stages without invasive manipulations. The Fish Embryo Acute Toxicity (FET) test was formally established by the Organization for Economic Co-operation and Development (OECD) in Test Guideline 236 (OECD, 2013) that quantifies coagulation, somite formation, tail detachment and heartbeat during a 96-h exposure. This is frequently expanded to incorporate sub-lethal informative endpoints like hatching delay, pericardial/yolk sac changes and tail/spinal morphology. Like any screening model, the zebrafish results should be considered as hazard screening evidence that should be used to guide, but not prohibit, further safety assessment [9].

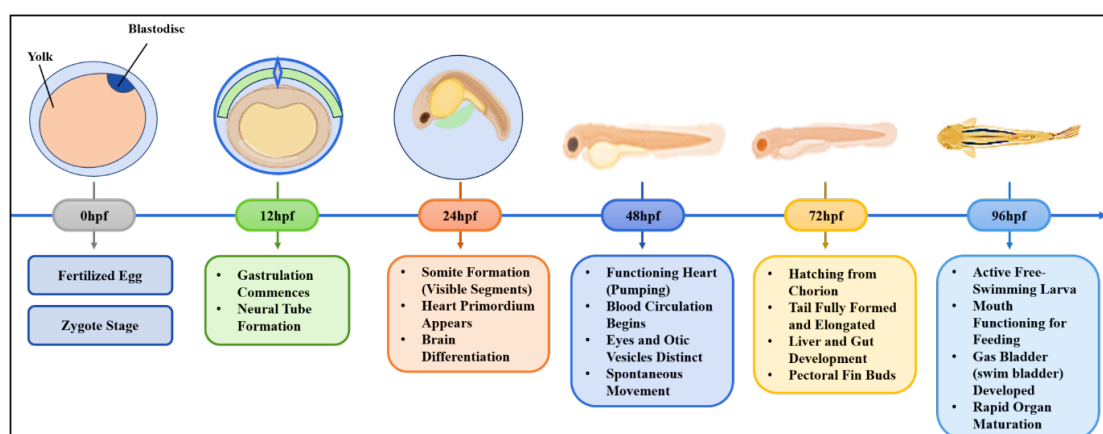


Figure 1: Developmental Phases of Zebrafish Embryo

Based on the literature, there was a gradual rise in the interest in the phytochemistry and pharmacology of *P. obtusa* [3-5]. To the best of our knowledge, no published report has assessed and compared the developmental toxicity of an alkaloid-enriched fraction and a flavonoid-enriched fraction of *P. obtusa*, which were independently prepared and tested, using zebrafish embryos. The pharmacological studies carried out on this species so far have been carried out on the crude or the total extracts, it is difficult to correlate the effect observed with a class of secondary metabolite. The present study, therefore, focused on characterizing and comparing the developmental effects of the alkaloidal fraction (P1) and the flavonoidal fraction (P2) of *P. obtusa* in the zebrafish embryo model, which will provide concentration-referenced information about the effects of the two plant fractions, useful for their further rational and safe development.

MATERIALS AND METHODS

Collection and Authentication of Plant Material

Fresh, healthy, disease-free leaves, flowers and bark of *Plumeria obtusa* L. were collected from mature and

actively growing plants of Paramedical campus, Uttar Pradesh University of Medical Sciences (UPUMS), Saifai, Etawah, Uttar Pradesh, India in early morning hours. Only fully expanded leaves, as well as freshly opened flowers and medium thickness bark from mature branches were selected since the amount of secondary metabolites is stage dependent. The collected plant material was authenticated by Prof. (Dr.) Rajvir Singh, Head, Department of Botany, K.K.P.G. College, Etawah, Uttar Pradesh, India. The plant material was washed under running tap water, blotting and shade drying at ambient temperature ($25 \pm 2^\circ\text{C}$) for 10-15 days ensured the protection of heat and light sensitive phytoconstituents. The dried material was coarsely ground by mechanical grinding method and stored in an air tight and light proof container room temperature till further use [10].

Extraction of the Alkaloidal Fraction (P1)

The coarsely ground plant material was defatted with petroleum ether using Soxhlet extraction method. The defatted material was basified with ammonia solution (pH ~8-9) to change the alkaloidal salts to their free-base

form and re-extracted by Soxhlet with chloroform. The chloroform extract was then acid-base partitioned. The alkaloids were washed repeatedly with acid to an aqueous acidic phase. This was then basified (pH ~9-10) and extracted again with fresh chloroform for 3 time to get back the liberated free alkaloids. The combined chloroform fractions gave the crude alkaloidal fraction P1 which was confirmed with Mayer's, Hager's, Wagner's and Dragendorff's test [10].

Extraction of the Flavonoid Fraction (P2)

The coarsely powdered plant material was defatted with petroleum ether by Soxhlet extraction and then marc was air dried. Defatted plant material was subjected to acid hydrolysis with dilute hydrochloric acid with gentle and controlled heating then kept for 24 hours at room temperature to release the less polar aglycones of the flavonoids present in the plant. The cooled hydrolysate was filtered, then partitioned with ethyl acetate and n-amyl alcohol, respectively to give the flavonoid aglycone rich and glycoside rich fraction of the hydrolysate. Both the fractions were subjected to preliminary phytochemical screening test for flavonoids by Shinoda, alkaline reagent and sulfuric acid test [11].

Zebrafish Embryo Acute Toxicity Testing

Acute embryotoxicity was confirmed in wild type *Danio rerio* embryos using the protocol agreed between the sponsor and testing laboratory (Whizbang Bioresearch Pvt. Ltd, Chennai, India, Study No. WBR 1230/2026). The test was conducted based upon the principles of OECD Test Guideline 236. Embryos were allowed to develop until 90 min. post fertilization (hpf) and were individually transferred to 24-well plates containing 2 mL of the test solution, which were then continuously exposed for 96 h. Twenty embryos per test item were

exposed to each of the six nominal concentrations (100, 50, 25, 12.5, 6.25 and 3.125 µg/mL) with one embryo per well. A positive control (4 µg/mL 3,4-dichloroaniline), a common vehicle control (0.01% v/v ethanol in E3 medium, 24 embryos in 12 wells) and an internal negative control (dilution water only) were also included. All groups were sampled for water quality parameters at the start and finish of the exposure. Embryos were examined at 24 hours intervals for the four OECD TG 236 apical endpoints (coagulation, lack of somite formation, non-detachment of the tail, absence of heartbeat) and hatching rate and gross morphological abnormalities were recorded at each interval [8,9,12].

Statistical Analysis

The percentage mortality for each fraction after 96 hpf was analyzed using probit analysis to obtain the median lethal concentration (LC50) and 95% fiducial limits (FL) for each of the LC50 estimates. Mortality, hatching rate and morphological/teratogenic endpoints were pooled and descriptively compared between concentrations and between the four 24h observation intervals. The vehicle control and positive control were used as negative and positive reference points for the validity of the assays [13].

RESULTS

Preliminary Phytochemical Test

Alkaloidal fraction (P1) was found positive to all the three alkaloid confirmatory tests performed (Mayer's, Wagner's and Dragendorff's tests), confirming the presence of alkaloids. The flavonoids confirmatory tests carried out for flavonoid fraction (P2), included the Shinoda test, alkaline reagent test and sulfuric acid test was positive in all three tests suggesting presence of flavonoids (Table 1, Figure 2).

Table 1: Phytochemical screening of the alkaloidal (P1) and the flavonoidal (P2) fractions

Fraction	Confirmatory test	Result
P1- (Alkaloidal Extract)	Mayer's test	Positive
	Wagner's test	Positive
	Hager's test	Positive
	Dragendorff's test	Positive
	Shinoda test	Positive
P2- (flavonoidal Extract)	Alkaline reagent test	Positive
	Sulfuric acid test	Positive

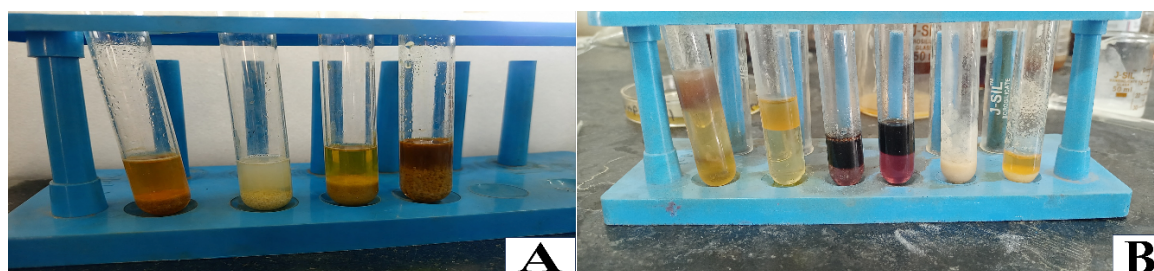


Figure 2: Preliminary Phytochemical Tests of (A) Alkaloidal and (B) Flavonoidal Extract

Median Lethal Concentration (LC50)

Probit analysis of cumulative mortality at 96 hpf gave a 96-h LC50 of 128.600 µg/mL (95% fiducial limits: 50.096-330.126 µg/mL) for P1 and 160.673 µg/mL (95% fiducial limits: 63.460-406.804 µg/mL) for P2 (Table 2,

Figures 3 and 4). The results indicated that the alkaloidal fraction was slightly more effective than the flavonoidal fraction in the 96-hour exposure period, albeit in both fractions a relatively high concentration was needed to achieve 50% lethality.

Table 2: LC50 after 96 h of alkaloidal (P1) and flavonoidal (P2) fractions

Test item	LC50 (96 h) (µg/mL)	95% Fiducial limits (µg/mL)	Relative sensitivity
P1 (alkaloidal fraction)	128.6	50.096 – 330.126	More potent of the two
P2 (flavonoidal fraction)	160.673	63.460 – 406.804	Less potent of the two

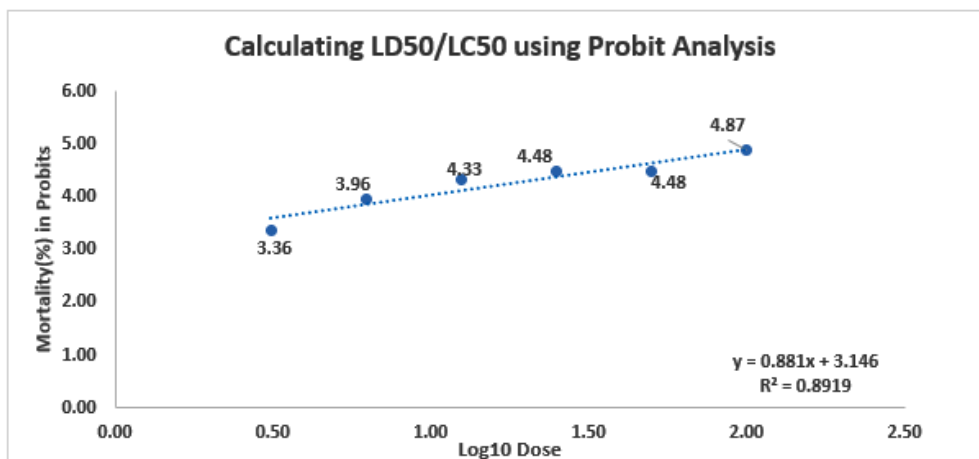


Figure 3: Probit plot of LC50 of the alkaloidal fraction (P1)

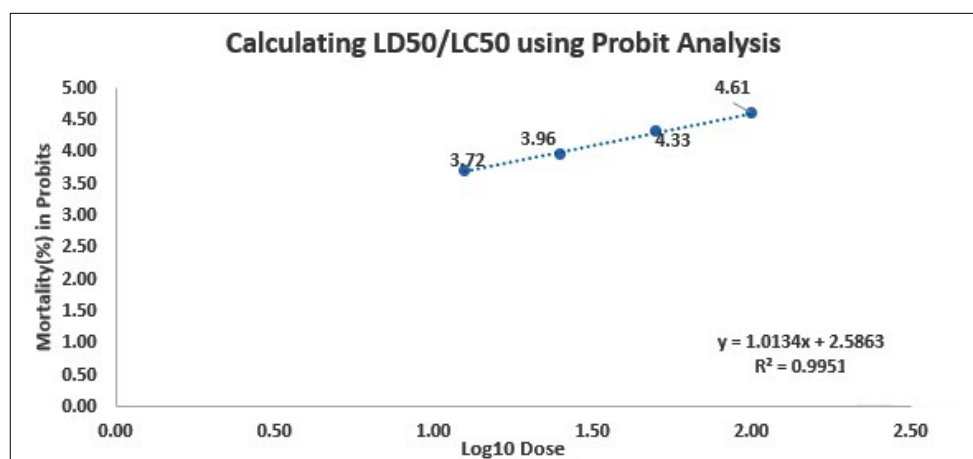


Figure 4: Probit plot of LC50 of the Flavonoidal fraction (P2)

Mortality

Both fractions (Table 3) showed a concentration-dependent increase in cumulative mortality by 96 hpf. The mortalities for the highest concentration (100 µg/mL) were 45% for P1 and 35% for P2, and 5% for P1 and 0% for P2 at the lowest concentration (3.125 µg/mL). There was no mortality in the vehicle control and the

positive control (3,4-dichloroaniline, 4 µg/mL) produced 90% mortality indicating the sensitivity and validity of the assay. The majority of deaths in both fractions occurred within 24 to 48 hpf, with little additional mortality after 72 hpf, suggesting little overall mortality of surviving embryos after that time.

Table 3: Cumulative mortality (%) of P1 (alkaloidal) and P2 (flavonoidal) fractions for 96 h

Fraction	100	50	25	12.5	6.25	3.125	Control
P1 (alkaloidal)	45	30	30	25	15	5	0
P2 (flavonoidal)	35	25	15	10	0	0	0

Hatching Response

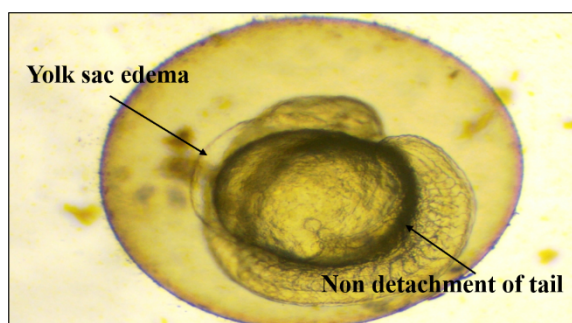
Both fractions delayed hatching in a concentration dependent manner when compared to the vehicle control (80% hatching by 48 hpf). In P1, the hatching became more concentrated at higher concentrations in the 72-96 hpf window and more embryos hatched at 72 hpf at 6.25 and 3.125 $\mu\text{g/mL}$. P2, which hatched at 12.5 and 6.25 $\mu\text{g/mL}$, was relatively less developed at these concentrations at 120 hpf compared to the higher concentrations by 96/60 hpf, whereas at 3.125 $\mu\text{g/mL}$, 90% of the embryos hatched by 48 hpf, which was similar to that of the control.

Morphological and Developmental Observations

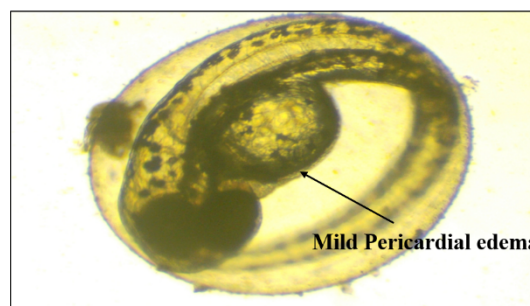
Both fractions produced a range of concentration dependent morphological changes, the most consistent being mild to moderate pericardial edema which was the main finding across most treatment groups and time points for both P1 and P2 (Table 4; Figure 5). Other alterations included yolk sac oedema/elongation, decrease in eye size, retardation of growth, tail bending/curling and, less frequently, tail bending/curling of the spinal cord. Both groups showed a high degree of recovery of the surviving larvae, as most had normal or near-normal morphology and swimming at the end of the exposure period, with the greatest change in both populations occurring at 24 hpf and the fewest larvae at 96 hpf.

Table 4. Developmental/Morphological observations at 96 hpf

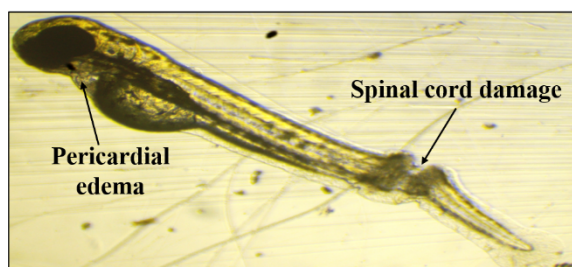
Conc. ($\mu\text{g/mL}$)	P1 (alkaloidal) — 96 hpf	P2 (flavonoidal) — 96 hpf
100	Pericardial edema	Pericardial edema, yolk sac elongation
50	Pericardial edema	Pericardial edema, tail curling, stunted growth, tail break
25	Pericardial edema, spinal cord bending	Pericardial edema
12.5	Pericardial edema	Pericardial edema
6.25	Pericardial edema	Pericardial edema
3.125	Pericardial edema, yolk sac damage	Pericardial edema
Control	Nil	Nil



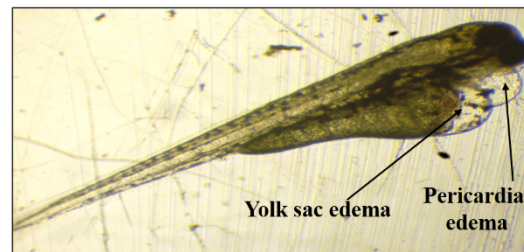
24hpf- Non detachment of tail, Elongation of yolk sac



48 hpf- Unhatched, Pericardial edema



72hpf- Hatched, Pericardial edema, Spinal cord damage



96hpf- Hatched, Pericardial edema, Yolk sac edema

Figure 5: Representative Photomicrographs from 24-96-hours observation period

DISCUSSION

The two fractions from *Plumeria obtusa*, which were enriched in phytochemicals, namely P1 (alkaloidal fraction) and P2 (flavonoidal fraction) were tested in the zebrafish embryo model, and showed a concentration dependent developmental response. However, both fractions are in a relatively high concentration range compared to their respective lethality thresholds with 96-

h LC50 of 128.600 $\mu\text{g/mL}$ for P1, and 160.673 $\mu\text{g/mL}$ for P2. P1 is a little more potent than P2. Pericardial edema, a well-documented and reported sub-lethal endpoint in the zebrafish developmental toxicity literature, was the most consistent finding in both fractions and typically is a transient response to the exposure of a xenobiotic, and not a specific structural injury. Encouragingly, the rate of morphological changes

was also reduced for both fractions from 24 to 96 hpf and the majority of the surviving larvae had normal morphology and swimming activity at the end of the exposure period indicating that most embryos had a transient, rather than permanent, developmental effect on their morphology. The recovery curve is a convenient and comfort factor feature of the concentration-response curve for both fractions.

The positive confirmatory test for alkaloid for P1 and flavonoid for P2 confirmed that both the extracts P1 and P2 are phytochemically enriched, multi-component fraction extracts and not single, chemically pure compound. The embryotoxic effect of P1 and P2 on zebrafish was found may be due to the presence of quinoline-type alkaloids and flavonoid/flavonoid-glycoside components in *Plumeria obtusa* since compounds of these chemical classes have already been reported to cause developmental toxicity and pericardial oedema in zebrafish embryos.

These results, taken together, indicate that the alkaloidal & flavonoidal components of *P. obtusa* have biological activity over a range of relatively high concentrations in Zebrafish embryo model. Moreover, a lower degree of mortality and morphological change was found in both fractions under the lower concentrations tested (3.125-12.5 µg/mL). This concentration-response relationship is useful data that can be used in selecting safer working concentrations for any future pharmacological and/or formulation studies of this plant and is consistent with the well-known toxicological rule that the safety of a substance is dependent upon the dose administered and is not fixed and binary. Like any single-model screening study, these data can only be used as a first step in a developmental-hazard assessment and should not be interpreted as a definitive assessment of developmental hazard in other systems such as mammal.

CONCLUSION

The alkaloidal fraction (P1) and the flavonoidal fraction (P2) of *Plumeria obtusa* showed concentration dependent developmental responses to the zebrafish embryo model after 96 hours of exposure with LC50 values of 128.600µg/mL and 160.673µg/mL respectively. Pericardial edema, the most consistent sub-lethal effect for both fractions, was largely reversible as the majority of these effects disappeared by 96 hpf in surviving larvae within the range tested. The results presented here are a concentration-referenced, fraction-specific developmental toxicity profile of *P. obtusa* that has not been reported previously, and allow for a practical basis to select working concentrations for future

pharmacological, formulation or mechanistic studies of this traditionally used plant.

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