***Original Research Article***

# Toxicity of methanolic extract of fruits of *Catunaregamspinosa* (Rubiaceae) on *Daniorerio* embryos


## ABSTRACT

**Aims:** *Catunaregamspinosa* is an exotic plant in Sri Lanka. Fruits of this plant contain saponins, steroids, flavonoids possessing piscicidal property. Since years ago *C. spinosa* employs in the fishery industry, especially in rural areas. This study was established to evaluate toxicity and teratogenic effects of fruits of *C. spinosa* on *D. rerio* embryos.
**Methodology:**Semi-static renewal method was conducted to determine the median lethal concentration. Concentrations of 15.0, 17.0, 19.0, 21.0, 23.0, and 25.0 mg $L^{-1}$ were tested with twenty embryos per treatment. Each concentration was triplicated. Dilution water and 3, 4- Dichloroaniline at 4.0 mg $L^{-1}$ were tested for negative and positive control respectively. Four apical observations and teratogenic effects were examined at 24, 48, 72 and 96 h time intervals.
**Results:**Embryos exposed to 19.5 mg $L^{-1}$ concentration exhibited 50 % mortality at $p = 0.05$ significance level. Embryos exposed to high concentrations exhibited more teratogenic deformities with a high mortality rate. Negative control recorded >90 % survival rate and positive control 95.0% lethality after 96 h exposure. Hatchability was negatively correlated with the concentration of extract.
**Conclusion:**Methanolicfruits extract of *C. spinosa* showed concentration-dependent mortality and teratogenic effectson *D. rerio*embryos. It could be concluded that the fruits of *C. spinosa* shows moderate piscicidal activity.



*Keywords: Catunaregamspinosa, piscicide, Daniorerio, embryo, lethality*


## 1. INTRODUCTION

Plants are source of the multiple applications in different aspects.*C. spinosa* belongs to family Rubiaceae which possesses profound pharmacological profile. Anti- oxidant, anthelminthic, anti- inflammatory, cytotoxicity, insecticidal, antimicrobial and piscicidalactivities are some of characterized functions of*C. spinosa.*Fruits of *C. spinosa* are reported as folklore remedies for different therapeutic ailmentssuch as sedative activity and dermatological treatments like eczema, abscess and wounds(Kapoor, 2001; Singh *et al*., 2010; Mridula*et al*., 2018).Despite of all the productive pharmacological properties, extensive studies need to be carried out to ensure the major concerns regarding their effectiveness and safety. Piscicidal activity of *C. spinosa* has been literalized in many archives mostly its practical use in fish harvesting. Leaves, unripe fruits, fruits, stem bark and root have been used by ancient people for fish harvesting (Ignacimuthu*et al*., 2006; Negi*et al*., 2009; Dominic, R. and Ramanujam, 2012; ENVIS Centre,2017; Jawale, 2018). Currently, piscicides and their indiscriminate use in fishery industry are causing hazardous side effects in the environment. In contrast to that, ancient people used plant species called “piscicidal” or “ichthyotoxic” plants which possess naturally occurring piscicidal compounds. In present,piscicides are not only used in fishery industry but also in pond culturing for artisanal fish breeding.

Phytochemicals found in crushed or macerated parts of these plants thrown into stagnant or slow flowing water bodies act up on stupefying fish. It eases the fishermen to harvest the crop.

Teratogens are substances that cause defects in embryonic development. Due to their suppressive effect on cancer cells via reactivating the normal embryonic pathways, teratogens are considered as anti-cancer agents (Murugesu*et al*., 2019).Therefore it is crucial to study toxicity of crude extract of *C. spinosa*. Zebra fish (*Daniorerio*)embryonic assay is a popularly known testing tool of screening teratogenicactivity that has been currently advocated for vertebrate studies in preference to mice. Transparent embryos, rapid and similar embryonic development to mammalians affirm the reliability of *D. rerio* embryos in neurotoxicity testing.Fruits of *C. spinosa*possess chemical attribute contained withalkaloids, flavonoids, tannins,oleanolic acid, triterpenoid, saponinsand phenolic compoundsetc. (Movalia*et al*., 2009; Senthamarai*et al*., 2011).While triterpenoid,saponins and rotenone are well known functional agents(Cannon *et al*., 2004), cardiac glycosides, alkaloids and tannins also escort synergetic effect in fish poisoning (Rodriguez 1990). Crushed parts aid to eradicate invasive and dominant fish species in pond preparing prior introduction of new fish population to the pond. Scientific approaches of *C. spinosa* in its piscicidal activity have been evaluated in several aspects. Shirgur (1975) studied time taken to stupefy fish using seeds, whole fruit and pulp of *C. spinosa* and revealed it as 10 min, 30 min and 90 min respectively. A research carried out in Nepal on piscicidal activity of fruits of *C. spinosa* reported LC50 as 0.0036% (w/v) within 5 h on *Heteropneustesfossilis*(Kulakkattolickal 1989). Most of the literatures have only listed *C. spinosa* as a fish poisoning plant (Neuwinger 2004;Murthy*et al*., 2010)and only few have conducted the quantification studies regarding the activity which are many years ago. Sri Lanka is abundant with diverse plant species. *C. spinosa* as a plant with potent pharmacological activities is still underrated in its versatile applications. *C. spinosa* can play a vital role as a natural source to address theraising issue of organic and chemical agricultural products and their hazardous impacts. However, there are lacks of studies regarding analysis of piscicidal activity of methanolic fruit extract of *C. spinosa* in Sri Lanka. Thus this study intended to study the toxicity of fruit of *C. spinosa*found in Sri Lanka in order to provide a scientific proof of its piscicidal activity.

## 2. METHODOLOGY

### 2.1. Preparation of plant extract

Four to five months old mature fruits were collected from Ayurveda Herbal Garden, Haldumulla, Sri Lanka. The extraction protocol was developed with the combination of studies byAlafiatayo*et al*., (2019) and Xavier and Kripasana, (2020) with slight modifications. A weight of 50.0 g of dried fruits was ground in an electric grinder. The weight was soxhletextracted with 200.0 mL analytical grade methanol (Merck, Germany) for 4 h at 45℃. Extract was dried in rotary evaporator at 50℃ at 100 rpm. The crude was stored in -20℃ for further use.

### 2.2. Range finding test

Healthy *Dania rerio* wild type fingerlings with weight of 5.8±1.5 g and length of 4.5±2.0 cm were obtained from Aquarium at Karadiyana, Piliyandala. Males and females were conditioned in two separate glass tanks with the loading capacity of 1 L per fish under a photoperiod of 12 – 16 h over a month prior using for mating (Organization for Economic, Co- operation and Development (OECD). 2013). Fish were fed twice per day at a 5 % of body weight (Mohottiand Epa, 2016). Surplus feed and feces were siphoned out after 1 h. Tanks were continuously aerated. Water quality and cleanliness were maintained thoroughly. Stock solution was prepared dissolving 31.0 mg of mature fruit extract in conditioned water and top upped in 1 L volumetric flask. Concentration range of 1.0-31.0 mg $L^{-1}$ was prepared mixing required volume of stock solution and conditioned water up to 200.0 mL which volume enough to cover the embryos completely.

### 2.3. Definitive test

Semi- static renewal 96 h embryo toxicity test was conducted followed by guidelines of Organization for Economic Co-operation and Development (OECD), 236, adapted on 26 July 2013. Based on the results of range finding test, definitive test was conducted at concentrations of 15.0, 17.0, 19.0, 21.0, 23.0 and 25.0 mg $L^{-1}$. Positive control was tested with 4.0 mg $L^{-1}$ of 3, 4- Dichloroaniline and negative control with dilution water. Assay was conducted following completely randomized design exposing 20 embryos in each treatment vessel. Four apical observations and teratogenic effects were evaluated at 24, 48 and 72 and 96 h post fertilization (hpf) intervals. Temperature, pH, conductivity and dissolved oxygen level were measured at the start of the exposure time of freshly prepared test solutions on daily basis. Median lethal concentration (LC50) after 96 h and 95 % confidence limits were calculated using regression analysis (Agresti 1990).

Physico-chemical parameters of test solutions and controls were expressed in mean ± SD and compared by one – way ANOVA at significant level $p = 0.05$.

## 3. RESULTS AND DISCUSSION

Embryo-toxicity using *D. rerio* embryos has been a model in assessing hostile effects of particular substances on cell structure and functioning. Physico-chemical parameters of pH, temperature, dissolved oxygen level and conductivity of controls and treatments are tabulated in Table 1. It showed no significant different between and among controls and treatments ($p>0.05$). According to De Castro*et al*., (2015) dissolved oxygen level required to be ranged between 7.15-3.33 mg $L^{-1}$to ensure there is no significant effect on zebra fish embryonic development. In our study the parameter laid in between the acceptable range.

Table 1: Physico-chemical parameters of solutions [mean ± standard deviation (SD)]

| Parameter | Concentration of plant extract (mg $L^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | 15.0 | 17.0 | 19.0 | 21.0 | 23.0 | 25.0 |
| pH | 7.45±0.01 | 6.84±0.06 | 7.10±0.04 | 7.18±0.02 | 7.31±0.05 | 7.6±0.06 | 7.86±0.04 |
| Temperature (℃) | 26.22±0.12 | 26.2±0.27 | 26.4±0.42 | 26.3±0.08 | 26.37±0.01 | 26.42±0.12 | 26.5±0.08 |
| Dissolved oxygen(mg $L^{-1}$) | 5.6825± 0.20 | 5.66± 0.20 | 5.3525± 0.11 | 5.44± 0.25 | 5.2825± 0.22 | 5.175± 0.19 | 5.1175± 0.09 |
| Conductivity (µS $cm^{-1}$) | 10.09±0.01 | 11.14±0.04 | 11.37±0.06 | 11.84±0.10 | 12.26±0.10 | 12.76±0.1 | 13.43±0.07 |

Values in rows are not significantly different ($p>0.05$) as indicated by one – way ANOVA

Concentration dependent mortality was observed which is depicted in Table 2. Toxicity of mature fruit extract was initially observed with coagulation of embryos after 24 h (Plate 1a). Lack of somite formation (Plate 1b), non- detachment of tail bud (Plate 1c) and lack of heart beat were observed in embryos during the exposure time.

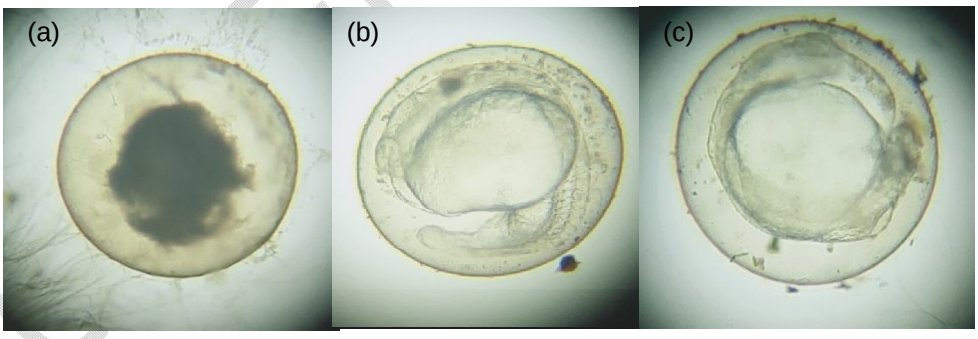


Plate 1: (a) Coagulated embryo (b) Lack of somite formation (c) Non detachment of tail bud

Positive control recorded more than 85.0 % mortality just after 24 h and 95.0 % mortality at the end of 96 h. Overall survival of embryos in negative control recorded 95.0 % (>90%). Higher mortality in positive control and higher survival in negative control validates the reliability of the experiment.

Table 2: Concentration dependent mortality and hatchability of *D. rerio* embryos

| Concentration ($mgL^{-1}$) | Cumulative mortality | Mortality (%) | Hatching percentage (%) |
|---|---|---|---|
| 15 | 0.1 | 10 | 90 |
| 17 | 0.15 | 15 | 75 |
| 19 | 0.5 | 45 | 55 |
| 21 | 0.6 | 55 | 45 |
| 23 | 0.65 | 65 | 35 |
| 25 | 0.8 | 80 | 20 |
| Positive control(3, 4-Dichloroaniline, 4.0 mg $L^{-1}$) | 0.95 | 95 | 5 |
| Negative control (Dilution water) | 0.05 | 5 | 95 |

### 3.1. Hatchability

Hatchability of embryos after 48 hpf or 72 hpf is an indicator of successful development of embryo in to a larva. Hatching rate was 95.0% (>80%) in the negative control at the end of exposure time. Increasing concentration decreased the hatching percentage significantly ($p$ = 0.0106, <0.05). Normal embryos hatched after 48-72 hpf. At low concentrations (15.0 and 17.0 mg $L^{-1}$) well somite formation and normal heart beat were observed. Hatched nauplii were observed after 72 hpf. Half of the embryos were died at concentration of 19.0 mg $L^{-1}$ after 96 h (Plate 2a). Most of them were coagulated at first 24 h. The rest of embryos exhibited other three apical observations by the end of exposure time. All survived eggs exhibited delayed hatching and more teratogenic malformations at concentration of 19 mg $L^{-1}$. Percentage of hatching was significantly decreased at increasing concentration starting from 19 mg $L^{-1}$. At high concentrations (21.0, 23.0, 25.0 mg $L^{-1}$) some of fully developed nauplii were trapped inside the chorion even after 96 h (Plate 2b).

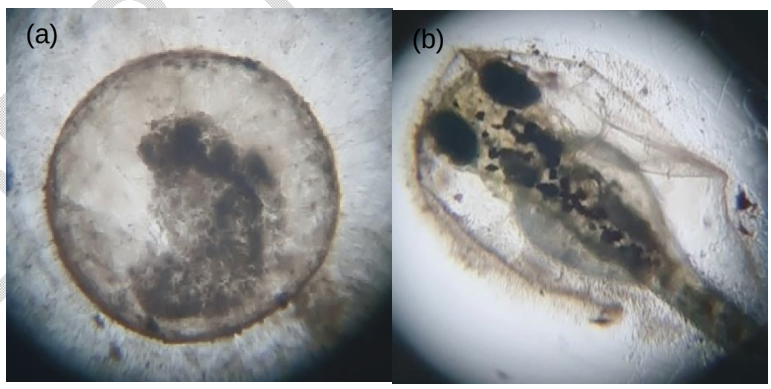

Plate 2: (a) Dead larvae inside the embryos (b) Trapped larvae inside embryos after 96 h

These results indicate the increasing concentration of mature fruit extract of *C. spinosa*affects the hatchability of embryos (Figure 01). Delayed hatching of naupliiis one of important teratogenic effects that can be affected by toxic compounds found in fruit extract hence causing inhibition of enzymes and their activities attributed to the breaking chorion(Streckeret *al*., 2011) Further hatching rate can be suppressed due to lack of energy of juveniles caused by delayed growth and malformations.

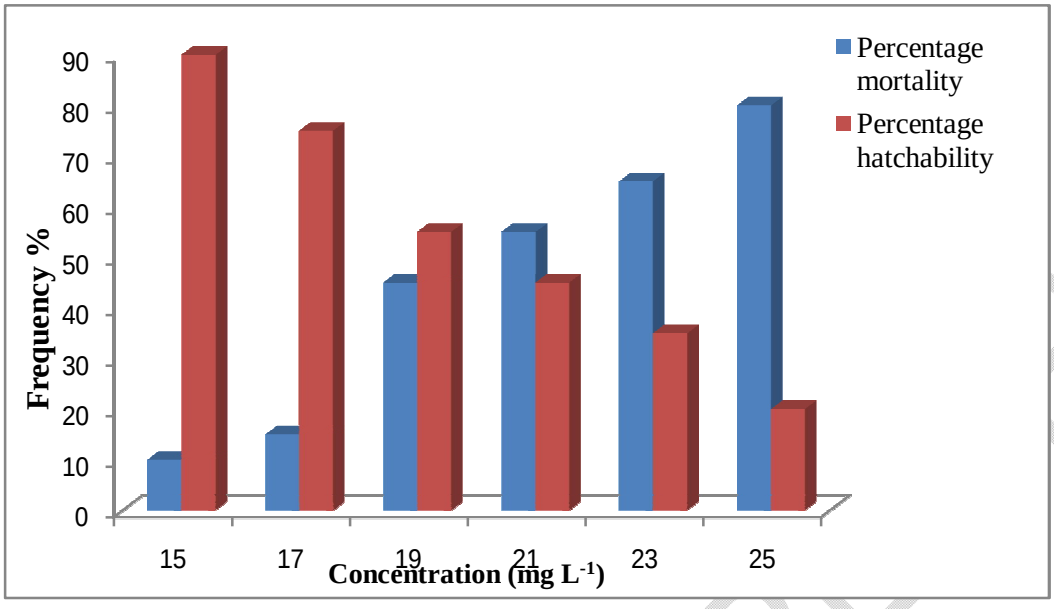


Figure 1: Graphical representation of mortality and hatchability of zebra fish embryos at 96 hpf

### 3.2. Lethality

The 96 h LC50 value of mature fruits extract of *C. spinosa* at 95% confidence level was 19.50 mg $L^{-1}$. Significantly high mortality rate was observed at 23.0 and 25.0 mg $L^{-1}$ concentrations whereas survived larvae also exhibited different developmental abnormalities. Most of them included lack of spatial movements, abnormal caudal peduncle (Plate 3a), yolk sac edema (Plate 3b), slow down heart rate, scoliosis where the tail is bent (Plate 3c) etc. None of these were observed in embryos tested in negative control and normal embryonic development.

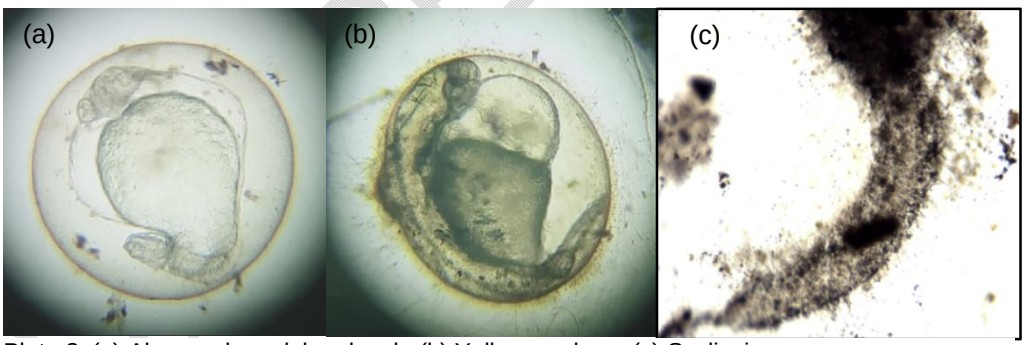


Plate 3: (a) Abnormal caudal peduncle (b) Yolk sac edema (c) Scoliosis

Based on the results and observations of *C. spinosa* causes developmental defects and significant mortality on *D. rerio* embryos. Triterpene, cyanogenics, rotenone and saponins are mostly active compounds responsible piscicidal activity of plants (Andel 2000). Saponins affect both physiological and behavioral activities of nauplii. It disturbs the normal growth of nauplii and suffocates them by lowering water surface tension causing them to excess use of respiratory organs (Shirgur 1975; De Vera*et al*., 2016).Saponins lyse red blood cells hence quick spread of toxins in the bloodstream (Jawale 2018). Many literatures support the presence of saponins in fruits of *C. spinosa* namely Dumetoronin A, B, C, D, E and F (Patel*et al*., 2011; Ghante*et al*., 2012;Nooraniand Kale, 2012; Atlas of Poisonous Plants in Hong Kong, 2021). Organic fatty acids are reported in inhibition of the process of metamorphism and later to death of nauplii. Organic acids are also found in seeds of *C. spinosa*(Kumar*et al*., 2014). Also Kedi*et al*., (2009) has identified several fatty acids vtz. 11,14-eicosadienoic acid, methyl ester, palmitic acid, stearic acid, myristic acid, hexadecanoic acid, ethyl ester in fruits of *C. spinosa*.Palmitic acid is a reported agent of apoptosis induction(Murugesu*et al*., 2019).In our study 96 h LC50 values of methanolicfruit extract of *C. spinosa* was 19.50 mg $L^{-1}$ at 95% confidence level. Toxicity of available phytochemicals regulates the piscicidal activity of plants. *Derris elliptica* and *Tephrosia candida* are well reputed fish poisoning natural sources with high

content of rotenone. Melo*et al*., (2015) reported 12.2 µg $L^{-1}$ as 96 h LC50 for rotenone induced mortality. Akinbulumo*et al*., (2004) revealed 24 h LC50 value of ethanolic extract of dried *D. elliptica* roots as 139.5 mg $L^{-1}$ and Guerrero and Guerrero, (1986) reported 96 h LC50 of ethanolic extract of dried *D. elliptica* roots as 10-20 mg $L^{-1}$ on *Oreochromisniloticus* fingerlings. According to a study by Mohotti and Epa (2016) *T. candida* reported 6.43 mg $L^{-1}$ of 96 h LC50 on *O. niloticus* fingerlings. There were no evidences regarding presence of rotenone in fruits of *C. spinosa*. It can be predicted as a reason of low toxicity of fruits of *C. spinosa* compared to *D. elliptica* and *T. candida*. Xia et al., (2017) revealed LC50 of *Carthamustinctorius* L. (safflower) as 345.6 mg $L^{-1}$ which contains hydroxylsafflor yellow A, flavonoids and Quinochalcones as active agents. Singh *et al*., (2010) reported presence of flavonoids (Apigenin-5-methyl ether) and triterpenoid glycosides as possible agents for piscicidal activity of leaf and bark extract of *Thevetiaperuviana*.A study of embryo-toxic and teratogenic effect of *Tinosporacordifolia* leaf and bark extracts on zebra fish embryos mentioned di-terpenoid lactones, steroids, sesquiterpenoid and glycosides as toxicants whereas absence of early mentioned crucial constituents for piscicidal activity (Romagosa*et al*., 2016). That can be one of the reasons *T. cordifolia* recording low piscicidal activity compared to *C. spinosa*. Based on the results methanolic extract of fruits of *C. spinosa* showed moderate toxicity on *D. rerio* embryos which is less toxic compared to rotenone induced mortality. This study would help in unveiling more details about the piscicidal activity of *C. spinosa* in order to develop timely necessity of biopiscicides as a part of green revolution. It will encourage using natural products over chemical synthetics to cease overpopulation of fish species such as *Poecilia reticulate* (Guppy) and*Hypostomusplecostomus* (Tank cleaners) which interrupt harvesting of edible fishand increasing competition among fish individuals in rivers and other water resources in Sri Lanka (Pradeep 2013). Further the exhibited teratogenic potential of fruits of *C. spinosa* would aid discovering and developing new anti-cancer drugs.

## 4. CONCLUSION

The 96 h LC50 value of mature methanolic fruit extract of *C. spinosa* at 95% confidence level was 19.50 mg $L^{-1}$. It can be concluded fruit extract of *C. spinosa* possesses potential piscicidaland teratogenic activity affecting hatchability and embryonic development of *D. rerio* embryos. Further studies needs to identify more unique compounds found in fruits causing fish poisoning. It would drive to develop environment friendly biopiscicides followed by appropriate isolation and mechanism development for sustainable growth in fishery industry.

## DISCLAIMER

Commonly and predominantly used products in Sri Lanka have been used for this research. This research was conducted solely for advancement of knowledge. Thus there is no conflict of interest between authors and companies of products supplied. Further, research is completely funded by University of Sri Jayewardenepura under the grant no: ASP/01/RE/2019/15 not by any other product producing company.

## CONSENT

It is not applicable

## ETHICAL APPROVAL

Ethical approval is not required as the embryos of *D. rerio* are used up to 96 hours post fertilization concurrently they are not free feeding.

## SIGNIFICANCE OF THE STUDY

This study enlightens the ancient application of *C. spinosa* in fish poisoning in scientific manner. Further in case of getting better idea about suitability of fruits of *C. spinosa* in application of present environment.

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