Original Research Article

Toxicity of methonolic extract of fruits of *Catunaregam spinosa* (Rubiaceae) on *Danio rerio* embryos

ABSTRACT

Aims: *Catunaregam spinosa* 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: Methanolic extract of fruits of *C. spinosa* showed concentration-dependent mortality on embryos of *D. rerio*. It could be concluded that the fruits of *C. spinosa* shows moderate piscicidal activity.

Keywords: Catunaregam spinosa, piscicide, Danio rerio, embryo, lethality

1. INTRODUCTION

Plants are source of multiple applications in different aspects. *C. spinosa* belongs to family Rubiaceae which possesses different pharmacological activities. Among the activities of anti- oxidant, anthelminthic, anti-inflammatory, cytotoxicity and insecticidal, piscicidal property of *C. spinosa* has been occupied among the people since decades ago. 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. However, currently piscicides are not only used in fishery industry but also in pond culturing for artisanal fish breeding. Numerous articles have mentioned the piscicidal activity of different parts such as unripe fruits, fruits, leaves, stem bark and root of *C. spinosa* [1, 2, 3, 4, 5]. Phytochemicals found in crushed or macerated parts of this plant thrown into stagnant or slow flowing water bodies act up on stupefying fish. It ease the fishermen to harvest the crop. Triterpenoid saponins and rotenone are well known functional agents in fish poisoning [6]. Other than those cardiac glycosides, alkaloids and tannins escort synergetic effect in fish poisoning [7]. 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 by seeds, whole fruit and pulp of *C. spinosa* and revealed it as 10 min, 30 min and 90 min respectively

[8]. A research carried out in Nepal on piscicidal activity of *C. spinosa* reported its LC50 as 0.0036% (w/v) within 5 hrs on *Heteropneustes fossilis* [9]. Most of the literatures have only listed *C. spinosa* as a fish poisoning plant [10, 11] whereas few have carried out the quantification studies again also 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 important applications. *C. spinosa* can play a vital role as a natural source amidst of raising issue in the world about organic and chemical agricultural products and their hazardous impacts. However, there are lacks of studies regarding analysis of piscicidal activity of methanolic extract of fruits of *C. spinosa* in Sri Lanka. Thus this study would be helpful in unveiling more details about the piscicidal activity of this plant found in Sri Lanka.

2. METHODOLOGY

2.1. Preparation of plant extract

Four to five months old mature fruits were collected at Ayurveda Herbal Garden, Haldumulla. A weight of 50.0 g of dried fruits was ground electrically and extracted with methanol in soxhlet extraction at 45℃ over 4 h. 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 [12]. Fish were fed twice per day at a 5 % of body weight [13]. 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 examined at 24, 48 and 72 and 96 h post fertilization (hpf). Temperature, pH, conductivity and dissolved oxygen level of test solution were measured at the start of the exposure time and freshly prepared test solutions on daily basis. Median lethal concentration (LC50) after 96 h and 95 % confidence limits were calculated using probit analysis [14]. 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

Physico-chemical parameters of pH, temperature, dissolved oxygen level and conductivity recorded during the test duration (Table 1). It showed no significance between and among controls and treatments ($p > 0.05$). Dissolved oxygen level laid between 7.15-3.33 mg L^{-1} which ensures no significant effect on zebra fish embryonic development [15].

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

Concentration dependent mortality was observed (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 by the time of exposure.

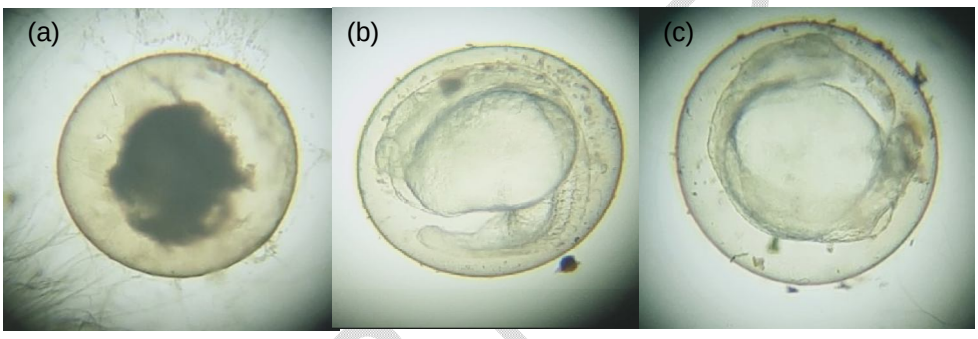

Plate 1: (a) Coagulated embryo (b) Lack of somite formation (c) Non detachment of tail bud

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 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

Hatching rate was 95.0% (>80%) in the negative control at the end of exposure time. Increasing concentration decreased the hatching percentage. 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}) fully developed nauplii were trapped inside the chorion even after 96 h (Plate 2b).

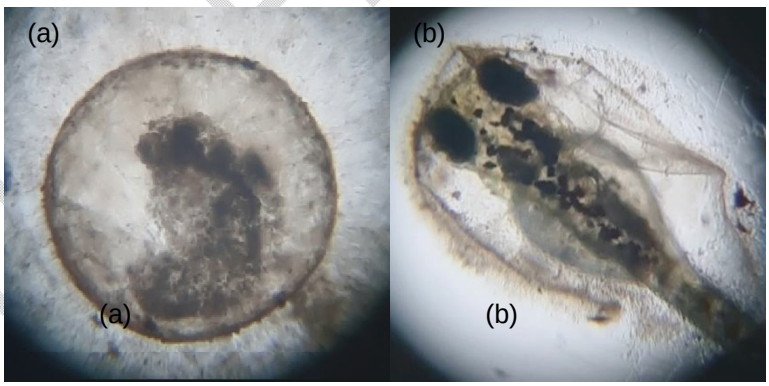

Plate 2: (a) Dead larvae inside the embryos (b) Trapped larvae inside embryos after 96 h

These results indicate the increasing concentration of mature fruit extract of C. spinosa decrease the hatchability of embryos (Figure 01). Delayed hatching of nauplii can be affected by toxic compounds found in fruit extract hence causing

inhibition of enzymes and their activities attributed to the breaking chorion [16](Strecker et al., 2011). Further hatching rate can be suppressed due to lack of energy of juveniles caused by delayed growth and malformations.

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

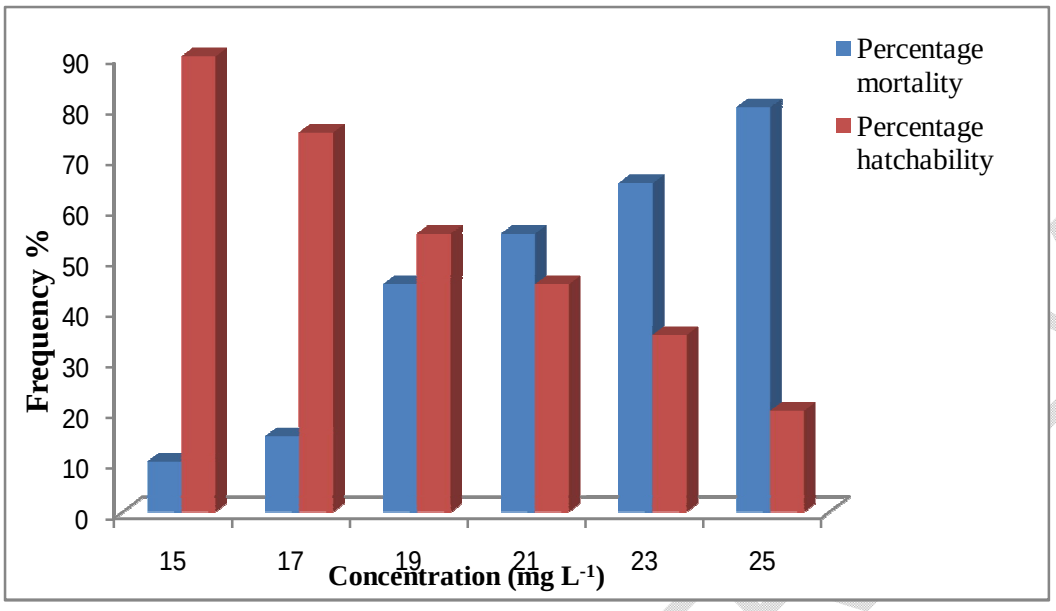

Figure 1: Graphical representation of mortality and hatchability of zebra fish embryos at 96 hpf

3.2. Lethality

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) and yolk sac edema (Plate 3b), slow down heart rate, scoliosis where the tail is bent (Plate 3c) etc. None of those were observed in embryos tested in negative control and normal embryonic development was recorded.

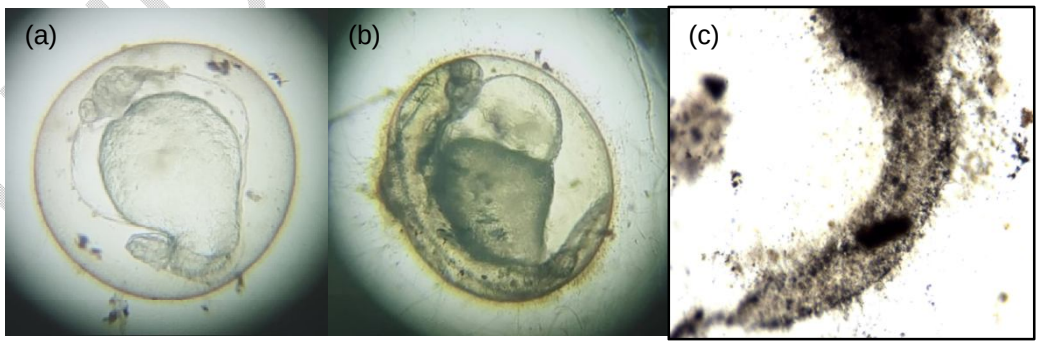

Plate 3: (a) Abnormal caudal peduncle (b) Yolk sac edema (c) Scoliosis

The 96 h LC50 value of mature fruits extract of *C. spinosa* at 95% confidence level was 19.50 mg L^{-1}. Concentration dependent mortality was graphed in Figure 2.

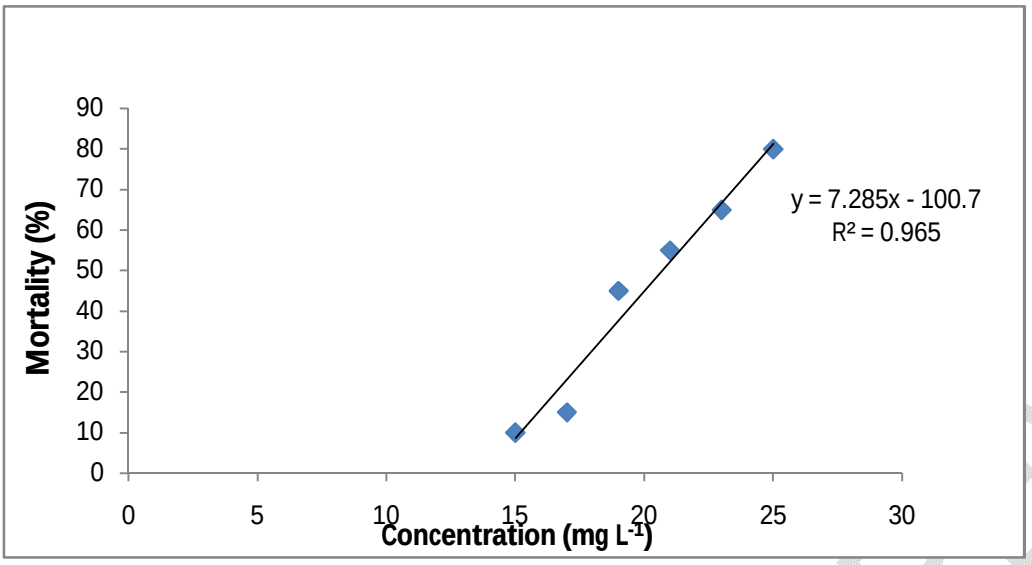

Figure 2: Concentration dependent mortality of zebra fish embryos after 96 hpf

Based on the results and observations *C. spinosa* causes developmental defects and significant mortality on zebra fish embryos. Triterpene, cyanogenics, rotenone and saponins are mostly active compounds responsible piscicidal activity of plants [17]. Saponins affect both physiological and behavioral activities of nauplii. It disturbs the normal growth of nauplii and suffocate them by lowering water surface tension causing them for excess use of respiratory organs [8, 18] De Vera et al., 2016). Saponins lyse red blood cells hence quick spread of toxins in the bloodstream [5]. Many literatures support the presence of saponins in fruits of *C. spinosa* namely Dumetoronin A, B, C, D, E and F [19, 20, 21, 22]. 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* [23]. In our study 96 h LC50 values of methonolic fruit 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 [24]. 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 *Oreochromis niloticus* fingerlings [25, 26]. 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 [13]. 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 *Carthamus tinctorius* L. (safflower) as 345.6 mg L^{-1} which contains hydroxylsafflor yellow A, flavonoids and Quinochalcones as active agents [27]. 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 *Thevetia peruviana* [28]. A study of embryo-toxic and teratogenic effect of *Tinospora cordifolia* 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 [29]. 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.

4. CONCLUSION

The 96 hrs LC50 value of mature fruits extract of *C. spinosa* at 95% confidence level was 19.50 mg L^{-1}. It can be concluded fruit extract of *C. spinosa* possesses significant piscicidal potential affecting hatchability and embryonic development of zebra fish 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 are not required due to the embryos of *D. rerio* are used up to 96 hrs post fertilization.

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.

References

[1] Ignacimuthu, S., Ayyanar, M. and Sankara, S.K. 2006. Ethnobotanical investigations among tribes in Madurai District of Tamil Nadu (India). Journal of Ethnobiology and Ethnomedicine. 2: 25.
[2] Negi, K.S. and Kanwal, K.S. 2009. Plants used as fish toxins in Garhwal region of Uttarakhand Himalaya. Indian Journal of Traditional Knowledge. 8(4):535-538.
[3] Dominic, R. and Ramanujam, S.N. 2012. Traditional knowledge and ethnobotanical uses of piscicidal plants of Nagaland, North east India. Indian Journal of Natural Products and Resources. 3(4):582-588.
[4] ENVIS Centre, Manipur. List of poisonous plants http://www.manenvis.nic.in/Database/PoisonousPlants_2935.aspx. 2017. [Accessed 16 May 2020].
[5] Jawale, C.S. 2018. Piscicidal Plants of India. Trends in Fisheries Reserach. 7(2):2319–4758.
[6] Cannon, J.G., Burton, R.A., Wood, S.G. and Owen, N.L. 2004. Naturally Occurring Fish Poisons from Plants. Journal of Chemical Education. 81(10):1457-1461.
[7] Rodriguez, P.A. 1990. The occurrence of piscicides and stupificants in the plant kingdom. Advances in Economic Botany. 8:1-23.
[8] Shirgur, G.A. 1975. Indication of safe poison materials from indigenous plants for clearing unwanted fishes from nursery ponds. 126-132.
[9] Kulakkattolickal, A.T. 1989. Piscicidal Plants of Nepal: Ripe Fruit of *Catunaregam spinosa* (Thunb.) (Rubiaceae) and Leaves of *Polygonurn h ydropiper* L. (Polygonaceae) as Fish Poisons. Aquaculture. 78:293-301.
[10] Neuwinger, H.D. 2004. Plants used for poison fishing in tropical Africa. Toxicon. 44:417–430.
[11] Murthy, E.N., Pattanaik, C., Reddy, C.S., Reddy, K.N. and Raju, V.S. 2010. Piscicidal plants used by Gond tribe of Kawal wildlife sanctuary, Andhra Pradesh, India. Indian Journal of Natural Products and Resources.
[12] Organization for Economic, Co- operation and Development. 2013. OECD guidelines for the testing of chemicals, in Fish Embryo Acute Toxicity (FET) Test.22.
[13] Mohotti, C.R.W.C. and Epa, U.P.K. 2016. Toxicity of aqueous extract of white hoary pea, *Tephrosia candida* (Papilionoideae) on Nile tilapia, *Oreochromis niloticus* (Cichlidae) fingerlings. Sri Lanka Journal of Aquatic Sciences. 105-112.
[14] Agresti, A. Categorical Data Analysis. 1990. John Wiley and Sons, New York, 710.
[15] De Castro, M.E.G., Dulay, R.M.R. and Alfonso, N.F. 2015. Teratogenic Effect of Papaya (*Carica papaya*) seed extracts on the embryonic development of Zebrafish (*Danio rerio*). Advances in Environmental Biology. 9(18):91-96
[16] Strecker, R., Seiler, T.B., Hollert, H. and Braunbeck, T. 2011. Oxygen requirements of zebrafish (*Danio rerio*) embryos in embryo toxicity tests with environmental samples. Comparative Biochemistry and Physiology, Part C 153. 318–327.
[17] Andel, T.V. 2000. The diverse uses of fish-poison plants in northwest Guyana. Economic Botany. 54(4):500–512.
[18] De Vera, J.S., De Castro, M.E.G. and Dulay, R.M.R. 2016. Phytochemical constituents and teratogenic effect of lyophilized extracts of *Bixa orellana* L. (Achuete) and *Piper betle* L. (Ikmo) Leaves in *Danio rerio* Embryos. Der Pharma Chemica. 8(18):432-437.

[19] Patel, J.R. and Bhatted, S. 2011. A comparative study on Vamana Karma with Madanaphala and Krutavedhana in Ekakushtha (Psoriasis). Ayu. 32(4):487-93.
[20] Ghante, M.H., Bhusari, K.P., Duragkar, N.J., Jain, N.S. and Warokar, A.S. 2012. Bronchorelaxant, mast cell stabilizing, anti-inflammatory and antioxidant activity of *Randia Dumetorum* (Retz.) Lamk. extracts. Acta Poloniae Pharmaceutica - Drug Research. 69(3):465-474.
[21] Noorani, A.A. and Kale, M.K. 2012. Pretreatment of albino aats with methanolic fruit extract of *Randia dumetorum* (L.) protects against alcohol induced liver damage. The Korean Journal of Physiology & Pharmacology. 16:125 ー 130. http://dx.doi.org/10.4196/kjpp.2012.16.2.125
[22] Atlas of Poisonous Plants in Hong Kong - A Clinical Toxicology Perspective. 2021. *Catunaregam spinosa* 山石榴, Rubiaceae 茜草科. [Cited date: 12.12.2021]. https://www3.ha.org.hk/toxicplant/en/catunaregam_spinosa.html
[23] Kumar, Y.R.S., Ram, T.S., Selvam, N.T., Parekar, S.S., Venugopalan, T.N. and Nair, P.K.S. 2014. Toxicological study of *Randia dumetorum* Linn seeds in Wistar Albino rats. International Journal of Phytotherapy Research. 4(1):19-28.
[24] Melo, K.M., Oliveira, R., Grisolia, C.K., Domingues, I., Pieczarka, J.C., Filho, J.D.S. and Nagamachi, C.Y. 2015. Environmental Science and Pollution Research, 22 (18):13926-13938. DOI 10.1007/s11356-015-4596-2.
[25] Akinbulumo, M.O., Fagbenro, O.A. and Fasakin, E.A. 2004. Acute toxicity of ethanolic extract of *Derris elliptica* roots to *Oreochromis niloticus* fingerlings.
[26] Guerrero, R.D. and Guerrero, L.A. 1986. Uses of *Derris* root power for management of fresh water ponds. Aquatic Biosystems, Bay, Laguna, Philippines :125-127.
[27] Xia, Q., Ma, Z., Mei, X., Luo, J., Wang, Y., Li, T., Feng, Y., Ni, Y., Zou, Q., Lin, R. 2017. Assay for the developmental toxicity of safflower (*Carthamus tinctorius* L.) to zebrafish embryos/larvae, Journal of Traditional Chinese Medical Sciences. doi: 10.1016/j.jtcms.2017.05.004.
[28] Singh, S.K., Yadav, R.P. and Singh, A. 2010. Piscicidal activity of leaf and bark extract of *Thevetia peruviana* plant and their biochemical stress response on fish metabolism. European Review for Medical and Pharmacological Sciences. 14:915-923.
[29] Romagosa, C.M.R., David, E.S. and Dulay, R.M.R. 2016. Embryo-toxic and teratogenic effects of *Tinospora cordifolia* leaves and bark extracts in Zebrafish (*Danio rerio*) embryos. Asian Journal of Plant Science and Research. 6(2):37-41.