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Research Article

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Comparative Acute Toxicity Study of Syringodium Isoetifolium on Aquatic and Rodent Experimental Animals

Kavitha DHANARAJU[1,2],
ORCID: 0000-0003-3745-7939
R. PADMINI[1*],
ORCID: 0000-0002-0816-3600
Alekhya VERAMANENI[2],
ORCID: 0000-0002-7545-6569
Chandravadivelu GOPI[2],
ORCID: 0000-0001-7382-5474
Magharla Dasaratha DHANARAJU ,
ORCID: 0000-0003-4876-7051

[1] Department of Biochemistry, Vels institute of science, technology and advanced studies, Pallavaram, Chennai -600117, Tamilnadu, India

[2] Research lab, GIET School of Pharmacy, Rajamahendravaram-533296, Andhra Pradesh, India

[3] L. M. College of Pharmacy, Ahmedabad (Gujarat) India

Corresponding author:
Ramakrishnan PADMINI
Department of Biochemistry, Vels institute of science, technology and advanced studies, Pallavaram, Chennai -600117, Tamilnadu, India.
Email : padmini.sls@velsuniv.ac.in
Tel: +91 9941165319

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ABSTRACT

This study was designed to explore toxic potential associated with hydroalcoholic extract of Syringodium isoetifolium seagrass (HAESI) on brine shrimp, zebrafish and Wistar albino rats according to the OECD guidelines. The different concentrations of extract were applied to brine shrimp (0.01-1 mg/ml), zebrafish (12.5, 25, 50, 100, 200, 400 mg/ml) and Wistar albino rat (500, 1000, 2000, 2500 and 5000 mg/kg). The control group received distilled water and the studies were carried out as per the OECD guidelines [203 and 423]. The experimental subjects were observed individually for the first 24 hours, with special attention given during the first four hours, thereafter for a prescribed duration. The results of brine shrimp exhibited increased mortality with the increasing concentrations of the extract. Maximum mortality occurred at 1000 Microgram per milliliter (µg/ml) and least mortality happened at 1 µg/ml concentration. The toxic effect on brine shrimp due to the poor elimination of cytotoxic substances from the body at high concentrations and elimination freely occur at low concentrations. Whereas no mortality and behavioural change were observed in the zebrafish and Wistar albino rats irrespective of the concentration.

Keywords: Toxicological study, Syringodium isoetifolium, Hydroalcoholic extract, Aquatic animals, Rodent animals.

1. Introduction

Acute toxicity studies are performed to identify the short-term adverse effects of drug substances when administered in a single dose or in multiple doses during a period of 24 hours in rodent and non-rodent animals [1]. It provides information on the safety of the drugs, targets organ toxicity, drug-induced clinical observations and species differences in the toxicity study [2-3].There is a need for the use of herbal drugs amongst the general population, with these 'natural medicines' perceived as being safer with fewer incidences of adverse drug reactions and side effects than their synthetic counterparts [4]. A broad variety of herbal drugs are readily existing in the market all over the globe. With the burst in the use of herbal drugs globally as alternative and complementary medicines, either the safety and efficacy of herbal drugs have become a public health concern [5]. Adverse health effects and side effects associated with herbal products induced by contaminants/ adulterants [6,7]. Therefore, the study of toxicity in herbal medicine is unavoidable.

Acute toxicity of the drug substances refers to those adverse effects occurring following the administration of a single dose of a synthetic or a mixture of natural substances given to the experimental animals within a short span of time [8]. In this study, two classes of species such as aquatic and rodent were engaged. Brine shrimp and zebrafish are incredibly efficient and energy-rich aquatic species making a simple, high throughput cytotoxicity test of bioactive chemicals. Here, the lethality of the drug can be calculated from the lethal concentration 50 (LC_{50}) value. LC_{50} is a concentration of chemicals in the water that kills 50% of the tested animals during the observation period. Toxicity testing criteria was expressed as LC_{50} value is commonly valorized either by comparison to Meyer or to Clarkson's toxicity index in the brine shrimp toxic study [9]. They provide a preliminary toxicity screening for further experiments on mammalian animal models [10]. Rats are generally used for toxicity studies in synthetic and natural drug substances [11]. Lethal dose 50 (LD_{50}) value of the drug substances was calculated by causing the death of 50% of a group of albino Wistar rats tested.

Seagrasses are an important part of the marine ecosystem, growing in shallow, sheltered and soft-bottomed plants found in seawaters [12]. The biomass of seagrass has been employed as a food and therapeutic agent by people from coastal areas [13]. In folk medicine, seagrasses have been used for many therapeutic applications such as fever, skin diseases, stomach problems, wounds, muscle pains and as a remedy against different types of rays [14]. They also exhibit different pharmacological activities like anti-oxidant ([15], anti-microbial [16], anti-viral [17], stomach problems [18], anti-diabetic [19], wounds [20], tranquillizer [21] and anticancer activities [22] etc., *Syringodium isoetifolium* is a type of seagrass possessing different pharmacological activities. But, there are no toxicity studies on *Syringodium isoetifolium* seagrass so far. This study is to identify the toxicity level of hydroalcoholic extract of *Syringodium isoetifolium* seagrass on different animals such as brine shrimp, zebrafish and Wistar albino rats etc., The report suggested that no mortality and significant physical changes were not identified in most of the tested animals at low concentrations of the hydroalcoholic extract of *Syringodium isoetifolium*. There was lethality and morbidity detected in the brine shrimps at high concentrations (1, 10, 100 and 1000 µg/ml) of hydroalcoholic extract of *Syringodium isoetifolium* after administered with a single dose during the 48 hours of study. In addition to that, the acute toxicity of hydroalcoholic leaf extracts of *Syringodium isoetifolium* was tested in the zebrafish model for 96 hours. There is no death and visible signs of physical changes were observed in the tested animals. At last, an acute toxicity study was performed on the Wistar albino rat. There is no morbidity or mortality observed after the administration of different concentrations of hydroalcoholic leaf extracts of *Syringodium isoetifolium*. It is an important milestone for future researchers who will show an interest to study on seagrass. There is a need for a sub-acute toxicity study on hydroalcoholic leaf extracts of *Syringodium isoetifolium* to evaluate the toxicity for chronic conditions.

2. Materials and Method

2.1. Collection of plant leaves and preparation of hydroalcoholic extracts

The finest quality *Syringodium isoetifolium* leaves was from the family of Cymodoceaceae collected from Devipattinam, Ramanathapuram District, Tamilnadu. The collected seagrass leaves were authenticated by Dr. M.U. Sharief, Scientist E & head,

Botanical Survey of India, Southern Regional Centre, T.N.A.U Campus, Coimbatore – 641003. The Voucher specimen number of the *Syringodium isoetifolium* is BSI/SRC/5/23/2021/Tech/372. Leaves were rinsed thoroughly with sterile seawater to remove the extraneous filth & mud, dried in a shade, crushed and extracted with hydroalcoholic solvent (30:70) using the maceration technique [23]. Here, the powder material was soaked in 450 ml of cold solvents in a stopped flask with intermittent shaking for 24 hours. This extract was pooled, filtered and the solvent was removed at 60° in the rotary evaporator (model: rotavapor R-210 from Buchi). The dried extract was stored in a dark bottle until further use. The yield was calculated using the following formula [24].

Percentage Yield = (W_1 x 100) / W_2

Here W_1 -Weight of extract after removing the solvent; W_2-Dry weight of the sample.

This plant extract was utilized to carry out an acute toxicity study. All experimental studies were conducted after getting prior approval from the Institutional Animal Ethics Committee (GSP/IAEC/2021/12/02).

2.2. Experimental animals

2.2.1. Brine shrimp

The eggs of brine shrimp were obtained from the Andhra Pradesh state institute of fisheries technology, Kakinada. To hatch the eggs, artificial seawater was made with commercially available salt 38 gm/L [25]. The developing medium was prepared with artificial seawater in a small tank (Figure 1). A lamp was fixed above the water tank to attract the newly hatched Shrimps. After 24 hours, the shrimps have grown into nauplii (Artemia Salina). The shrimps were acclimatized in a tank under standard environmental conditions of light/dark cycles (12 hours) and temperature (23 ± 1°C). The shrimps had free access to a standard pellet diet and water, except fasting period of four hours before and after the administration of the hydroalcoholic extract of *Syringodium isoetifolium* [26].

2.2.2. Zebrafish

Zebrafishes (aged 3-6 months) were collected from a local aquarium, Kolathur, Chennai-600099, Tamil Nadu to conduct the acute toxicity study. They were kept under standard conditions of temperature (28.5°C), dark/light (14/10 hours), conductivity 400 Microseconds (µs) and pH (7.4). Fish were fed with commercial food and were housed in the zebrafish facility (Figure 2). It is a cost-effective model organism due to its little size, the capacity to redevelop many tissues and organs in a short period and easy maintenance. In addition, the genome sequences of zebrafish are 85% similar to human beings [27].

2.2.3. Wistar albino rats

The female albino Wistar rats were collected from GIET school of Pharmacy, Rajamahendravarm, Andhra Pradesh to perform the acute toxicity study of hydroalcoholic extract of *Syringodium isoetifolium* [28,29] (Figure 3). The rats approximately 12 weeks old, weighing 150–200 g were placed in polypropylene cages and maintained under standard environmental conditions (25±2^{0}C/12 hours dark/light cycle).

2.3. Assessment of acute toxicity

2.3.1. Brine shrimp

A bioassay of brine shrimp lethality is a simple, high throughput test used for testing the efficacy of hydroalcoholic extracts of *Syringodium isoetifolium*. The different concentrations of HAESI (0.1-1mg/ml in propylene glycerol/Tween 80/ water (4:1:4)) were evaluated in the test for lethality in brine shrimp. It was executed by following the reported procedure [30]. The study was conducted for 48 hours. The LC_{50} was calculated based on the mortality of 50% of brine shrimp in the container. The hydroalcoholic extract of *Syringodium isoetifolium* (20 mg) in 1 mL of propylene glycol/Tween 80/Water (4:1:4) was prepared as a stock solution. The positive control was prepared by admixing of Propylene glycol, Tween 80, and Water (4:1:4) in 5cc of saltwater. Test concentrations ranging from 0.1 to 1 mg/ml were prepared. Each test tube received 10 shrimps and was

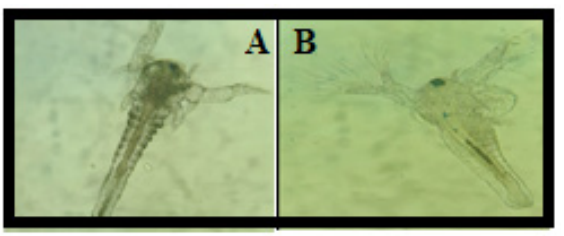

Figure 1. The image of brine shrimp before (A) and after (B) administration of hydroalcoholic extract of *Syringodium isoetifolium* seagrass.

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Figure 2. The image of Zebrafish treated with different concentrations of hydroalcoholic extract of *Syringodium isoetifolium* seagrass.

Figure 3. The image of Wistar albino rats treated with different concentrations of hydroalcoholic extract of *Syringodium isoetifolium* seagrass.

cultured in artificial seawater. Every 6, 12, 24 and 48 hours, the number of dead shrimps in each test tube was counted and recorded. The experiment was carried out three times in each concentration (n=3). Statistical analysis was used to calculate the proportion of deaths and the lethal concentration (LC_{50}).

Percentage of death (%) = (Total nauplii – Alive nauplii)/ Total nauplii X100%

2.3.2. Zebrafish

Toxicity tests of hydroalcoholic extract of *Syringodium isoetifolium* were carried out to investigate the safety of zebrafish at various concentrations such as 12.5, 25, 50, 100, 200, and 400 mg/L as per OECD guidelines 203. Each container cultured 7 fish in fresh water. The fishes were exposed to various concentrations of hydroalcoholic extracts for 96 hours and mortalities and physical changes were recorded every 24, 48, 72, and 96 hours. Fresh water was used as a treatment in the control group. The fishes were anaesthetized with an ice bar of 2-5°C, the liver and intestine tissues were dissected and stained using standard hematoxylin protocols [31]. Manipulations of the fish were kept to a minimum and were achieved gently to avoid damage to the zebrafish. After euthanasia, the organs of the fish were placed in rinses of fixatives (20× fish volume) and then incubated. All organs were serially sectioned on a Leica RM2125RT automated rotary microtome and set to 5 µm thickness.

2.3.3. Wistar albino rats

A sum of eighteen Wistar albino female rats was randomly allocated into six groups containing 3 rats for each group as per OECD guidelines 423 (annexe-3). The hydroalcoholic extracts of *Syringodium isoetifolium* were administered to all the animals except group 1. Group 1 (Control), Group 2 (*500mg/kg*), Group 3 (*1000mg/kg*), Group 4 (*2000mg/kg*), Group 5 (*2500mg/kg*) and Group 6 (*5000mg/kg*). Each group of rats was given a fixed dose of the respective hydroalcoholic extract of *Syringodium isoetifolium* in a stepwise procedure. The group I rats received distilled water. After dosing the rats are fasted for 4hrs and observations were recorded twice a day for up to 14 days. Mortality, behavioural changes such as irritability, restlessness, fearfulness, changes in the eye, hair, colour of the skin, food and water intake etc., were observed [32].

2.4. Statistical analysis

To analyze the findings a one-way analysis of variance (ANOVA) was used. Data were expressed as the mean ± SEM. Results with *$p < 0.05$ and **$p < 0.01$ were considered statistically significant [33].

3. Result

3.1. The percentage yield of the extract

Among different solvents tested, the hydroalcoholic solvent (30:70) extracted 90% of bioactive phyto-constituents from *Syringodium isoetifolium* seagrass using the maceration technique (Table 1).

3.2. Acute toxicity of hydroalcoholic extract of Syringodium isoetifolium using Brine shrimp

Symptoms and mortality were identified in the group treated with a high dose of hydroalcoholic extract of *Syringodium isoetifolium*. There is no such type of symptoms and lethality that were observed in the brine shrimp at 0.1 µg/ml during the 48 hours. The percentage of mortality was transformed using Finney's table to obtain Probit values (Analyst Soft Inc., Canada) to calculate LC_{50} of brine shrimp. The report of acute toxicity on brine shrimp was furnished in Table 2.

3.3. Acute toxicity of hydroalcoholic extract of Syringodium isoetifolium using Zebrafish

There was no death and behavioural changes were not observed in the zebrafish (*Danio rerio*) exposed at various concentrations (12.5, 25, 50, 100, 200, 400 mg/L). The fishes were actively moving the hydroalcoholic extract of *Syringodium isoetifolium*. This suggested that the extract does not contain any toxic materials in it. The report of acute toxicity in fish was illustrated in Tables 3 & 4.

3.4. Acute oral toxicity of hydroalcoholic extract of Syringodium isoetifolium using albino Wistar rats

The hydroalcoholic extract of *Syringodium isoetifolium* upto the dose of 5000 mg/kg produced no toxic effect on the tested Wistar albino rats during the entire period of study. Neither mortality nor behavioral changes were detected. The result of acute toxicity on female albino Wistar rats was furnished in Tables 5 & 6.

Table 1. Qualitative phytochemical analysis of leaves extracts of *Syringodium isoetifolium.*

S. No	Phytochemicals	Hydro-alcoholic Extract (70:30)
1	Tannin	++
2	Saponin	++
3	Flavonoids	++
4	Steroids	+
5	Terpenoids	+
6	Triterpenoids	+
7	Alkaloids	+
8	Anthroquinone	+
9	Polyphenol	++
10	Glycoside	+
11	Coumarins	+
12	Emodins	-
13	Anthocyanins	-
14	Carbohydrate	+
15	Carboxylic acid	++

("+" point out the presence of the phytochemicals; "-" indicates the absence of the phytochemicals, "++" point out the high concentration of phytochemicals).

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Table 2. Results of Brine shrimp lethality assay

Plant Extracts	Concentration (µg/ml)	Number of surviving Nauplii												Percentage of Death	LC_{50} µg/ml
		6hrs			12hrs			24hrs			48hrs				
		T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3		
HAESI	0.1	10	10	10	10	10	10	10	10	10	10	10	10	0	79.615
	1	10	10	10	10	10	09	09	08	09	08	08	08	9.16	
	10	10	10	10	10	09	09	10	09	09	07	08	07	10.83	
	100	08	09	09	07	09	08	06	07	08	05	06	05	30	
	1000	07	05	06	06	05	06	06	03	04	04	03	04	53.33	
Positive control	----	10	10	10	10	09	10	10	09	10	10	09	10	2.5	---
Negative control	----	10	10	10	10	10	10	10	10	10	10	10	10	0	---

Positive control- propylene glycol/Tween 80/Water (4:1:4); Negative control- Saltwater

Table 3. Results of Zebrafish lethality assay

Plant Extracts	Concentration (mg/L)	Number of surviving Zebrafish												Percentage of Death
		24hrs			48hrs			72hrs			96hrs			
		T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	
HAESI	12.5	07	07	07	07	07	07	07	07	07	07	07	07	0
	25	07	07	07	07	07	07	07	07	07	07	07	07	0
	50	07	07	07	07	07	07	07	07	07	07	07	07	0
	100	07	07	07	07	07	07	07	07	07	07	07	07	0
	200	07	07	07	07	07	07	07	07	07	07	07	07	0
	400	07	07	07	07	07	07	07	07	07	07	07	07	0
Artificial Seawater (Control)	----	07	07	07	07	07	07	07	07	07	07	07	07	0

Table 4. Behavioral responses of zebrafish after administration of a single dose of hydroalcoholic extract of Syringodium isoetifolium

S.No	Behaviour	Response	
		Before	After
1	Distress	Normal	Normal
2	Skin depigmentation	Absent	Absent
3	Lethargic movements	Normal	Normal
4	Swimming performance	Normal	Normal
5	Pain response	Normal	Normal

Table 5. Results of albino Wistar rats lethality assay

Plant Extracts	Concentration (mg/kgbw)	Number of surviving albino wistar rats									Percentage of Death
		1st day			7th day			14th day			
		T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	
HAESI	500	03	03	03	03	03	03	03	03	03	0
	1000	03	03	03	03	03	03	03	03	03	0
	2000	03	03	03	03	03	03	03	03	03	0
	2500	03	03	03	03	03	03	03	03	03	0
	5000	03	03	03	03	03	03	03	03	03	0
Artificial Seawater (Control)	----	03	03	03	03	03	03	03	03	03	0

Table 6. Behavioral responses of female Wistar albino rats after administration of a single dose of hydroalcoholic extract of *Syringodium isoetifolium*

S.No	Response	Head		Body		Tail	
		Before	After	Before	After	Before	After
1	Alertness	NR	NR	NR	NR	NR	NR
2	Grooming	AB	AB	AB	AB	AB	AB
3	Touch response	NR	NR	NR	NR	NR	NR
4	Gripping strength	NR	NR	NR	NR	NR	NR
5	Torch response	NR	NR	NR	NR	NR	NR
6	Corneal reflux	PR	PR	PR	PR	PR	PR
7	Pain response	NR	NR	NR	NR	NR	NR
8	Pupils	NR	NR	NR	NR	NR	NR
9	Convulsion	AB	AB	AB	AB	AB	AB
10	Pinna reflux	PR	PR	PR	PR	PR	PR
11	Tremors	AB	AB	AB	AB	AB	AB
12	Urination	NR	NR	NR	NR	NR	NR
13	Lacrimation	AB	AB	AB	AB	AB	AB
14	Righting reflux	NR	NR	NR	NR	NR	NR
15	Salivation	AB	AB	AB	AB	AB	AB
16	Writhing	AB	AB	AB	AB	AB	AB
17	Skin colour	NR	NR	NR	NR	NR	NR

NR-Normal; PR- Present; AB-Absent.

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3.5. Histopathological parameters of zebrafish

The liver and intestine of zebrafish were extracted from the group treated with a high concentration (400 mg/L) of hydroalcoholic extract of *Syringodium isoetifolium* and transverse sections of these organs were studied histopathologically. The result revealed that these organs did not have any clear differences when compared to the control group during the entire period of study. The result is presented in Figure 4.

4. Discussion

There are many reports in the literature suggesting that unnecessary or reckless use of herbal drugs can lead to problems [34]. Therefore, the study of toxicity in herbal medicine is unavoidable [35]. The biomass of seagrass has been employed as a food and therapeutic agent by people from coastal areas [36]. There is no toxic studies were not performed on hydroalcoholic extracts of *Syringodium isoetifolium* seagrass so far. The main aim of evaluating the toxicity study on hydroalcoholic extract of *Syringodium isoetifolium* is to identify safety and establish the exposure level at which the toxicity effect is observed. Therefore, the toxicity studies of hydroalcoholic extracts of *Syringodium isoetifolium* were performed on different animal models such as brine shrimp, zebrafish and Wistar albino rats. There was lethality and morbidity detected in the brine shrimps at different concentrations of hydroalcoholic extract of *Syringodium isoetifolium* after being administered with a single dose during the 48 hours of study. The shrimps were observed after 6, 12, 24 and 48 hours.

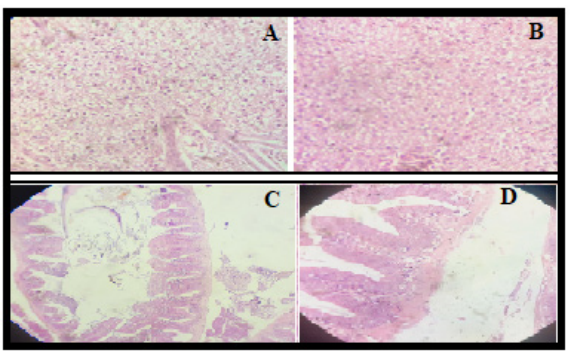

Figure 4. Histopathological sections of the liver, and intestine of control (A, C) and the group treated (B, D) with hydroalcoholic extracts of *Syringodium isoetifolium* in Zebrafish.

The higher concentration of hydroalcoholic extracts showed good larvicidal activity than the lesser concentrations. Therefore, the maximum number of deaths was observed at 1000 µg/ml concentration and the least at 1 µg/ml concentration. There are known to possess toxic effects on or above the concentration of 1 µg/ml due to the poor elimination of the toxic substances from their body. Hence, the hydroalcoholic extract derived from *Syringodium isoetifolium* has LC_{50} value of 79.615 µg/ml. Scientist [37] had reported a similar report from brine shrimp after the administration of methanolic extract of *Swietenia mahagoni (Linn.)* Jacq. seed and mortality rate of animals increased with the increasing concentration of fruit seed in the methanolic extract. In addition to that, the acute toxicity of hydroalcoholic leaf extracts of *Syringodium isoetifolium* was tested in the zebrafish model (*Danio rerio*) as per the OECD guidelines 203. The fish were exposed to different concentrations of extract preferably for 96 hours. In this toxicity study, no mortality was observed up to 400 mg/L till 96 hrs. Behavioral responses are not altered and there are no signs of distress, skin depigmentation, lethargic movements or no disturbances in swimming performance. At last, hydroalcoholic extracts of *Syringodium isoetifolium* were administered to rats at fixed doses such as 500, 1000, 2000, 2500 and 5000 mg/kg body weight as per the OECD guidelines 423. There is no mortality and visible signs of physical changes, *i.e.*, water and food consumption; aggression, salivation, rising furs and writhing were not changed for 14 days after administration of different concentrations of extracts to female albino Wister rats. During the 14 days period, all animals were observed for functional and behavioral examinations such as home cage activity and handheld activity. Home cage activities like respiration, tonic involuntary movement, body position, palpebral closure, clonic involuntary movement, approach response, touch response, sound responses, tail pinch response, pinna reflex were observed. Handheld activities like handling, salivation, reactivity, lacrimation, palpebral closure, pupillary reflex, piloerection, limb tone and abdominal tone were observed. Functional and behavioural examinations were normal in all treated and control groups. Histopathological result of Zebrafish showed the nontoxic effects of hydroalcoholic extracts of *Syringodium isoetifolium* during the study. Transverse sections of the liver and intestine were prepared and were studied histopathologically. The results suggest that leaf extract can protect or-

gans such as the liver and intestine etc., The histopathology of the liver does not show much difference when compared to the control group except vacuolization was observed. The intestine has little ruptured in villi and detachment of villi from the basal membrane. There is no other complication was not observed in the zebrafish. Similar results were also obtained in the research work conducted by scientists who were involved in the identification of toxicity of seagrass at various concentrations[38]. The results of acute toxicity confirm that hydroalcoholic extracts of *Syringodium isoetifolium* is nontoxic and hence safe to use as herbal medicines.

5. Conclusion

The proposed methods seem to be accurate, exploring the acute toxicity of hydroalcoholic extracts of *Syringodium isoetifolium* in Brine shrimp, Zebrafish and Wistar albino rats. Animal usage may rise the volume of information in the early stages of new medicines. The result of brine shrimp findings showed that the increases in mortality and morbidity were observed with the increasing concentrations of the hydroalcoholic extract of *Syringodium isoetifolium* due to the poor elimination of toxic substances at its high dose. Whereas, the drug metabolized freely at low doses. There was no mortality and morbidity in the zebrafish and albino Wistar rats after the administration of different concentrations of the extract. The histopathology of the liver and intestine of zebrafish did not show much difference when compared to the control group. The experimental methods are accurate, reliable, inexpensive and therefore can be employed by both individuals and organizations. Further studies of chronic toxicity are needed to support preclinical results.

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Conflict of Interest

The authors declare that there is no conflict of interest in this study.

Statement of Contribution of Researchers

Concept – R.P., K.D.; Design – K.D., M.D.D.; Supervision – R.P; Resources K.D., M.D.D.; Materials – K.D; Data Collection and / or Processing – K.D., V.A.; Analysis and / or Interpretation – K.D., C.G., R.P.; Literature Search – K.D.; Writing – K.D., C.G.; Critical Reviews – M.D.D.

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