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Original Article

Pharmacology Section

Evaluation of Preclinical Toxicity of Methanolic Extract of *Sargassum tenerrimum* using the Zebrafish Model

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ABSTRACT

Introduction: In the field of biomedicine, marine algae have gained significant attention due to the abundance of bioactive compounds they contain. However, studies on the chronic toxicity of algae are limited.

Aim: To evaluate the in-vitro, in-vivo acute, and subchronic toxicity of *Sargassum tenerrimum* (*S. tenerrimum*) in an adult zebrafish model.

Materials and Methods: The current preclinical toxicological interventional study was conducted at the Department of Pharmacology, Sri Ramachandra Medical College and Research Institute, SRIHER, Chennai, Tamil Nadu, India, in September 2022. The algae *S. tenerrimum* was extracted with methanol using Soxhlet extraction. In-vitro toxicity of *S. tenerrimum* was performed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay on the SH-SY5Y cell line. Acute toxicity was carried out for 96 hours according to Organisation for Economic Cooperation and Development (OECD) 203 guidelines, and subchronic toxicity was carried out for 14 days on the zebrafish model using the immersion method. Additionally, histopathological changes were observed after the acute toxicity study, and the Lethal Concentration 50 (LC50) value was analysed.

Results: The MTT assay of the methanolic extract of *S. tenerrimum* revealed an LC50 value of 140.014 µg/mL. An acute toxicity study conducted on zebrafish for 96 hours showed an LC50 value of 504.669 mg/L. Subchronic toxicity was done for 14 days, and on the 14th day of exposure, the LC50 value was found to be 404.196 mg/L. Histopathological changes were observed at higher concentrations (800 mg/L) of algal extract.

Conclusion: The present study revealed that the methanolic extract of *S. tenerrimum* showed a toxic effect at higher concentrations on the zebrafish model. However, at lower concentrations, *S. tenerrimum* was deemed safe for further exploration of pharmacological activities in a zebrafish model.

Keywords: Cytotoxicity, Lethal concentration 50 values, Macroalgae, Methanol, Soxhlet

INTRODUCTION

Macroalgae hold a significant importance in pharmaceutical research due to their rich repertoire of bioactive compounds and their potential therapeutic applications. Particularly, brown algae offer a vast array of natural compounds, including polysaccharides, proteins, polyphenols, pigments, fatty acids, and secondary metabolites which possess diverse biological activities [1]. Pharmaceutical researchers are chiefly interested in these compounds as they exhibit antioxidant, anti-inflammatory, antiviral, antibacterial, neurodegenerative diseases, and anticancer properties [2,3]. Algae provide a valuable source for drug discovery and development, serving as a starting point for identifying novel compounds with therapeutic potential. The importance of algae in pharmaceutical research lies in their vast potential for discovering new drugs, developing therapies, and addressing various medical challenges [4].

Sargassum tenerrimum, a brown alga, holds significant importance in pharmaceutical research and shows promising potential for future applications. It is commonly found on the coasts of various Asian nations like India, China, and Pakistan. In India, it is found along the shores of the states of Maharashtra (Bombay), Tamil Nadu (Tuticorin, Tirunelveli Cape Comorin, and Krusada Travancore), and Gujarat (Okha, Adatrareef, Cannanore, Dwarka, Saurashtra, Jalleshwar, Varaval, Gulf of Kutch) [5]. *S. tenerrimum*, a remarkable brown alga, has been the subject of a few scientific investigations that have shed light on its rich biochemical composition. These studies [6,7] have revealed a diverse array of secondary metabolites within this plant, underscoring its significance in the field of pharmacology and natural product research. Among the various secondary metabolites identified in *S. tenerrimum* [8], flavonoids stand out as a prominent group. Flavonoids are well known for their antioxidant properties and potential health benefits [9]. Alkaloids, another class of compounds found in this plant, often possess pharmacological activities, making them valuable for drug discovery and development. Alkaloids, saponins, carbohydrates, and anthraquinone glycosides are also notable constituents of *S. tenerrimum*. Saponins have been associated with various biological activities, including anti-inflammatory and anticancer properties [10,11]. In addition to these compounds, steroids, phenolic compounds, and tannins have been identified in *S. tenerrimum*. Steroids, such as phytosterols, can have cholesterol-lowering effects and are important in the synthesis of hormones [12]. Phenolic compounds have antioxidant and anti-inflammatory properties and are linked to potential health benefits [13]. Tannins, known for their astringent properties, have been used in traditional medicine for their ability to bind and precipitate proteins, making them relevant in various therapeutic applications [14]. In addition to their chemical diversity, these secondary metabolites have been investigated for their pharmacological properties, shedding light on the plant's potential medicinal applications.

Notably, *S. tenerrimum* has been studied for its antimicrobial properties, demonstrating efficacy against viruses and bacteria [15]. This suggests its potential use in the development of antiviral and antibacterial agents. Furthermore, the plant has exhibited anti-inflammatory and antiplasmodial activities [16]. Anti-inflammatory properties suggest its potential in alleviating inflammation-related disorders, while antiplasmodial activity highlights its relevance in combating malaria, which is caused by *Plasmodium* parasites. Moreover, *S. tenerrimum* has shown antioxidant properties, which are crucial for combating oxidative stress and preventing various

diseases associated with free radical damage. Additionally, its antitumor activity indicates a possible role in cancer therapy [17], while its antiallergic effects suggest potential use in managing allergic conditions [18]. The anticoagulant property of this algae is significant in the context of preventing excessive blood clotting, a condition associated with various cardiovascular diseases [19]. Its antidiabetic activity suggests a potential role in managing diabetes mellitus, a widespread metabolic disorder [20].

The scientific discipline of toxicology seeks to understand how certain substances may be hazardous to living things. The right dose differentiates between poison and treatment. The process of toxicological screening plays a crucial role in the advancement of new medications and the exploration of the therapeutic capabilities of compounds and crude extracts [21]. This provides a comprehensive explanation of the consequences of poison, including symptoms, biomechanism, treatment, and detection. In addition, the use of animal models is essential to comprehend the appropriate dose and concentration of fresh crude extract or by-product for human consumption. Long-term toxicity testing of brown algae *S. tenerrimum* has been limited. The novelty of this study was to explore the long-term toxicity of the brown algae *S. tenerrimum* in a zebrafish model. Adult zebrafish (*Danio rerio*) play a crucial role in toxicity testing and are of significant importance in pharmaceutical research [22]. One of the key advantages of using adult zebrafish in toxicity testing is their physiological maturity, which allows the study of the effects of substances on fully developed organisms. This is particularly important when evaluating chronic toxicity, long-term exposure effects, and the impact on adult organ systems. Adult zebrafish possess well-developed organ systems that closely resemble those of the human system, making them suitable for studying the potential toxic effects on various organs and tissues [23,24]. The primary goal of this research was to assess in-vitro toxicity in the neuroblastoma cell line SH-SY5Y and to investigate both acute and subchronic in-vivo toxicity in a zebrafish model. Additionally, the secondary aim of the study was to analyse the histopathological changes in zebrafish following a 96-hour exposure to acute toxicity.

MATERIALS AND METHODS

This preclinical interventional research was conducted at the Department of Pharmacology, Sri Ramachandra Medical College and Research Institute, SRIHER, Chennai, Tamil Nadu, India in September 2022. The study was approved by the Institutional Ethics Committee of Sri Ramachandra Institution of Higher Education and Research (Approval No. IAEC/62/SRIHER/721/2020).

Acute toxicity was carried out for 96 hours, and Subchronic toxicity was carried out for 14 days. Each group in the study contained seven fishes according to The Organisation for Economic Cooperation and Development (OECD) 203 guideline [23].

Study Procedure

Collection and verification of algae: The algae samples were collected from the Tuticorin region and verified by a former technical officer at the "Mandapam Regional Centre of Central Marine Fisheries Research Institute" located at Marine Post, Ramnad district, Tamil Nadu, India.

Preparation of methanolic extract: The seaweed *S. tenerrimum* was pulverised into a fine powder using a mixer grinder. The pulverised sample was extracted using a Soxhlet (Borosil) with methanol as the solvent. The porous cellulose thimble was loaded with 50 g of the powder sample after it was weighed. A total of 200 mL of methanol was poured into the round-bottom flask that was connected to an extractor and the condenser. The reservoir round-bottom flask was placed in a heating mantle and heated to 65°C for a period of 18 hours. The extraction process continued until the dark brown hue of the seaweed changed into a colourless state. The resulting solvent extract was then concentrated by passing it through a rotary evaporator at a lower temperature and in a vacuum condition. The concentrated extract was placed in a container for further investigation and cold storage under -4°C [4,25].

Cytotoxicity study of *S. tenerrimum* on SH-SY5Y cell line: 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay was used to assess the methanolic extract *S. tenerrimum* cytotoxicity in the neuroblast SH-SY5Y cell line (Mossman, 1983). The SH-SY5Y cell line is a widely used human neuroblastoma cell line. It was originally derived from a bone marrow biopsy of a neuroblastoma patient and has become a valuable tool in neurobiological and neuropharmacological research. SH-SY5Y cells are often used to study processes related to neurodevelopment, neuronal differentiation, neurotransmission, and neurodegenerative diseases, making them a valuable model for understanding various aspects of the nervous system. The cells were seeded into 96-well microplates at a density of 1×10^5 cells per well and incubated at 37°C for 48 hours in a 5% CO_2 incubator until they reached 70- 80% confluence.

Then, the medium was changed, and the cells were treated with five different concentrations of samples (6.25, 12.5, 25, 50, and 100 µg/mL) and incubated for 24 hours. The cells were then rinsed with phosphate-buffered saline (PBS, pH 7.4), and 20 µL of an MTT solution containing 5 mg/mL of MTT in PBS was added to each well. The plates were subsequently placed in a dark environment and maintained at a temperature of 37°C for a duration of two hours. The formazan crystals were dissolved in 100 µL of Dimethyl Sulfoxide (DMSO), and the absorbance was measured using spectrophotometry at a wavelength of 570 nm [26,27]. The digital inverted microscope (40x magnification) was used to observe and photograph the morphological changes of both untreated (control) and treated cells.

Cell viability (%)=(Absorbance of sample / Absorbance of control)×100 [28].

Acute toxicity testing of methanolic extract of *S. tenerrimum*

Experimental animal:

Inclusion criteria: Healthy, adult zebrafish (three months) of AB strain.

Exclusion criteria: Unhealthy or injured zebrafish, fishes less than three months.

A total of 102 Adult *Danio rerio* (Zebrafish) of AB strain of either sex were purchased from a certified vendor. Sixty fishes were used for acute toxicity. The fishes were divided into the seven groups with different concentrations of *S. tenerrimum* and along with one control group.

Fifty-six fishes were selected for the acute toxicity testing. Four fishes were excluded based on the exclusion criteria. For subchronic toxicity, another 42 fishes were used.

The zebrafish with an average weight of 0.3 g were acclimated for 10 days in a glass aquarium with a capacity of 50 liters and kept in well-aerated water with a temperature of 28°C. The light-dark cycle was about 14:10 hours. The zebrafish were given commercial food twice a day, in the morning they were given flakes, and in the evening, they were fed by live artemia [24,29]. The animal ethics committee approved the study.

Acute toxicity: According to the guidelines established by the OECD 203 (https://www.oecd-ilibrary.org/environment/test-no-203-fishacute-toxicity-test_9789264069961-en), a test to determine the acute toxicity of Methanolic Extract of *S. tenerrimum* (MEST) was carried out on adult zebrafish for 96 hours. Seven different concentrations of MEST (12.5, 25, 50, 100, 200, 400, and 800 mg/L) along with control were used in the study [30]. Seven zebrafish of either sex were exposed to each concentration in a 5L capacity rectangular tank for 96 hours.

After 24, 48, 72, and 96 hours of exposure, mortalities were reported, and the LC50 values were calculated. Fish were concluded dead when they showed no observable signs of movement and failed to react when their caudal peduncle was touched. After the dead fish were removed, the tank was carefully cleaned [31]. The fish were deprived of food either prior to the commencement or during the course of the experiment.

Histopathology of zebrafish based on acute toxicity: After the 96th hour of exposure, fish were euthanised using the hypothermia method. Euthanasia in zebrafish refers to the humane and ethical practice of intentionally causing the painless and swift death of the animal for scientific research or ethical reasons [32]. At the concentration of 400 mg/L, only one fish died, but at the concentration of 800 mg/L, all fishes died during the 96 hours of exposure. At the end of the experiment, a total of 48 fishes were alive and 8 fishes died for the histopathological study. The fish were placed in the cold temperature of 0-4°C for five minutes. The liver, brain, and heart were collected, and a smear with Haematoxylin and Eosin (H&E) was prepared for histopathological study [30].

Subchronic toxicity testing of methanolic extract of *S. tenerrimum*: Subchronic toxicity test was performed for 14 days. The concentration of the test compound used in this experiment was selected based on the LC50 value obtained from the acute toxicity test. For the subchronic study, another 42 fishes were selected. Fishes were divided into five different concentrations of algal extract and with one control. The fishes selected for the experiment were exposed to 31.25, 62.5, 125, 250, and 500 mg/L of *S. tenerrimum* extract for 14 days. The control group was treated with system water. Each group contained seven fish. Feeding was done once a day, and the extra food and excreted waste were eliminated one hour after feeding. The mortality of fish was tracked and recorded [33]. Dead fish were removed immediately from the test tank once mortalities were reported to avoid future infection.

STATISTICAL ANALYSIS

The LC50 values obtained from the MTT assay and the zebrafish toxicity model were determined through linear equation analysis and graphically represented in a bar chart using MS Excel.

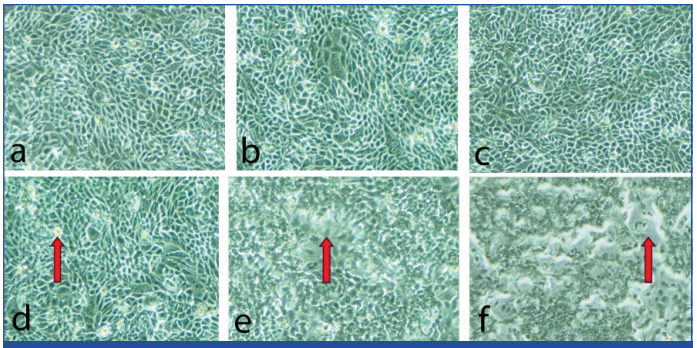
RESULTS

Cellular viability was assessed through an in-vitro MTT assay, providing critical insights into cell viability. The Optical Density (OD) served as the basis for determining the percentage of viable cells within the test sample [34]. [Table/Fig-1] succinctly summarises the percentages of viable cells and the extent of cell inhibition. The LC50 value of the MEST was meticulously determined via a dose-response curve analysis, yielding a precise value of 140.014 µg/mL. It is noteworthy that the negative impacts of MEST are primarily observed at higher concentrations, underlining its potential as a therapeutic agent at lower dosages.

S. no	Concentration (µg/mL)	Mean absorbance	Cell viability (%)	Inhibition (%)	LC50
1	6.25	0.916	99.457	0.543	140.014 µg/mL
2	12.5	0.915	99.348	0.652	
3	25	0.814	88.362	11.638	
4	50	0.702	76.221	23.779	
5	100	0.62	67.318	32.682	
6	Control	0.921	100	0	

[Table/Fig-1]: Cytotoxicity of methanolic extract of *S. tenerrimumon* SH-SY5Y cell line. Inhibition (%)= (Absorbance of control- Absorbance of sample / Absorbance of control)×100

At the concentrations of 6.25 and 12.5 µg/mL, no precipitated cell was observed, and at 25 µg/mL, precipitation of the cell was initiated. At concentrations of 50 µg/mL and 100 µg/mL, the precipitation of cells was increased and clearly visualised, indicated with the red arrow [Table/Fig-2].



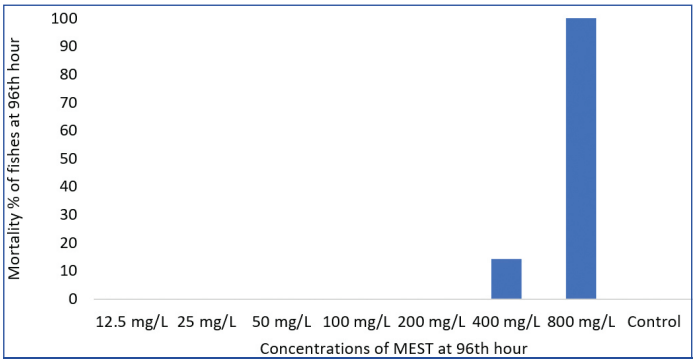
[Table/Fig-2]: Cytotoxicity of methanolic extract of *S. tenerrimum* on SH-SY5Y cell line: a) control; b) 6.25 µg/mL; c) 12.5 µg/ml; d) 25 µg/mL; e) 50 µg/ml; f) 100 µg/mL. (Red arrow: indicates the precipitation of the cells).

In screenings of natural products to assess their pharmacological potential, one of the essential preliminary steps is the evaluation of the toxic properties associated with medicinal products, including extracts, isolated compounds, and formulations. In this critical phase of assessment, the determination of the LD50 typically takes precedence. This measurement, which signifies the lethal dose at which 50% of the test subjects succumb, serves as the foundational point of reference. Moreover, the acute toxicity study plays a pivotal role by furnishing initial insights into the mechanism of toxic action exhibited by a given substance [35]. The present study findings indicate that the methanolic extract of *S. tenerrimum* exhibited a toxicity level resulting in a 100% fatality rate at the highest concentration of 800 mg/L [Table/Fig-3].

S. no.	Concentrations (mg/L)	24 hours	48 hours	72 hours	96 hours
1	12.5	0	0	0	0
2	25	0	0	0	0
3	50	0	0	0	0
4	100	0	0	0	0
5	200	0	0	0	0
6	400	1 (14.28%)	1 (14.28%)	1 (14.28%)	1 (14.28%)
7	800	1 (14.28%)	3 (42.86%)	5 (71.43%)	7 (100%)
8	Control	0	0	0	0

[Table/Fig-3]: Mortality percentage in acute toxicity at 24th, 48th, 72nd, and 96th hour exposure of methanolic extract of *S. tenerrimum*.

The LC50, or median lethal concentration, refers to the concentration of a tested compound that is estimated to cause mortality in 50% of the test organisms within the designated test duration. No mortality was detected within the concentration range of 12.5 mg/L to 200 mg/L. Fish mortality commenced at the 400 mg/L concentration, with an observed mortality rate of 14.28% after 24 hours of exposure to the algal extract [Table/Fig-3]. The maximum observed fish mortality occurred at the highest concentration tested, 800 mg/L, within just 24 hours of exposure, resulting in a 100% mortality rate at 96 hours of exposure MEST [Table/Fig-3,4]. Notably, as the exposure duration extended from 24 to 96 hours, there was a clear and dose-dependent increase in the mortality rate [Table/Fig-4].



[Table/Fig-4]: Mortality percentage in acute toxicity at 96th hour exposure of a methanolic extract of *S. tenerrimum*.

LC50 values at 24th, 48th, 72nd, and 96th hours is shown in [Table/Fig-5]. No mortality was observed in the control group.

S. no	Hour	LC50
1.	24	2352.824 mg/L
2.	48	990.3156 mg/L
3.	72	655.881 mg/L
4.	96	504.669 mg/L

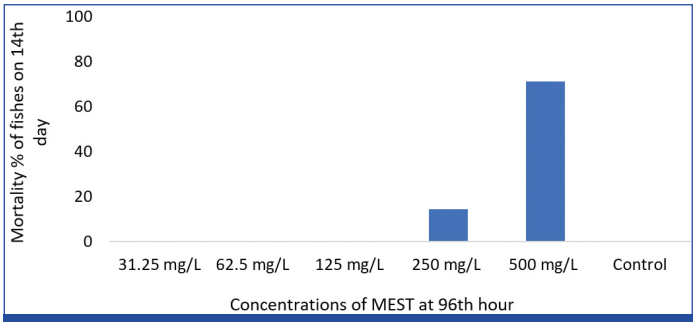
[Table/Fig-5]: LC50 of methanolic extract of *Sargassum tenerrimum* at different time intervals in acute toxicity.

Subchronic toxicity: No mortality was detected within the concentration range of 31.25 mg/L to 125 mg/L. Fish mortality commenced at the 250 mg/L concentration, with an observed mortality rate of 14.28% [Table/Fig-6].

Concentrations (mg/mL)	1st day	3rd day	5th day	7th day	9th day	11th day	13th day	14th day
31.25	0	0	0	0	0	0	0	0
62.5	0	0	0	0	0	0	0	0
125	0	0	0	0	0	0	0	0
250	0	0	0	1 (14.28%)	1 (14.28%)	1 (14.28%)	1 (14.28%)	1 (14.28%)
500	0	1 (14.28%)	1 (14.28%)	2 (28.57%)	4 (57.14%)	4 (57.14%)	5 (71.43%)	5 (71.43%)
Control	0	0	0	0	0	0	0	0

[Table/Fig-6]: Mortality percentage of methanolic extract of *S. tenerrimum* in subchronic toxicity at different time intervals.

The maximum observed fish mortality occurred at the highest concentration tested, 500 mg/L, resulting in a 71.43% mortality rate at the 14th day of exposure MEST [Table/Fig-6,7]. There was a clear and dose-dependent increase in the mortality rate [Table/Fig-7].



[Table/Fig-7]: Mortality percentage of methanolic extract of *S. tenerrimum* in subchronic toxicity on 14th day.

Subchronic toxicity: The LC50 values, which represent the lethal concentration at which 50% of a population is expected to perish, were determined at various time points during a toxicological study.

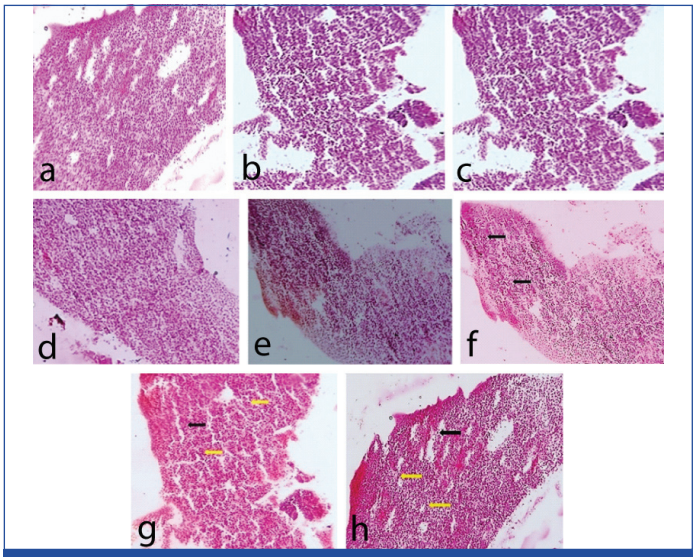
Specifically, these measurements were taken on the 3rd, 7th, 11th, and 14th days of the experiment. The results indicate a progressive decrease in the concentration required to cause a 50% mortality rate in the test subjects over time. On the third day of the study, the LC50 value was up to 1760.007 mg/L, signifying that a relatively large dosage of algal extract was required to induce mortality at this early stage [Table/Fig-8]. As the experiment progressed and continued over time, it became increasingly evident that the toxicity levels associated with MEST started to increase. By the seventh day, the LC50 had notably decreased to 824.455 mg/L, indicating a heightened level of toxicity in comparison to the initial measurement.

S. no.	Day	LC50
1.	3	1760.006 mg/L
2.	7	824.4550 mg/L
3.	11	477.1349 mg/L
4.	14	404.196 mg/L

[Table/Fig-8]: LC50 of methanolic extract of *S. tenerrimum* at different time intervals in subchronic toxicity.

This pattern persisted, with the LC50 values steadily declining to 477.135 mg/L on the 11th day and eventually reaching 404.196 mg/L by the fourteenth day. These findings highlight the dynamic nature of the substance's toxicity, with a notable increase in lethality over the course of the study period. There was no mortality observed in the control group.

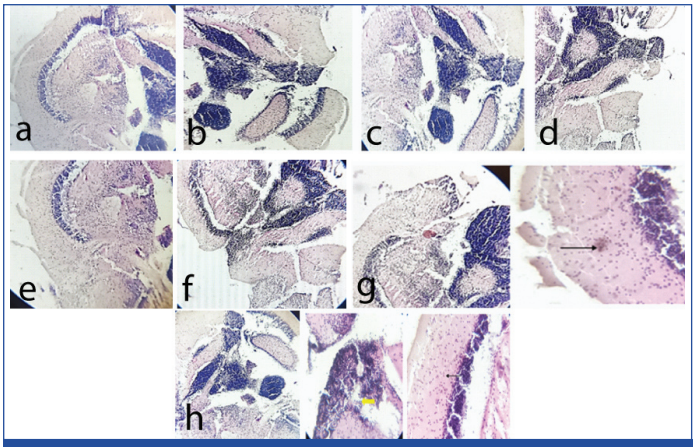
[Table/Fig-9] shows histopathological changes in the liver of zebrafish exposed to different concentrations of the methanolic extract of *S. tenerrimum*. [Table/Fig-10] shows the histopathological analysis of zebrafish brain exposed to different concentrations of the methanolic extract of *S. tenerrimum*. The heart of fish at 800 mg/L showed mild disintegration of muscle fibers (myomalacia) and oedema. The heart showed normal cardiac muscle bundle with myocytes in the group from 12.5 mg/L to 400 mg/L. No lesions or other pathological conditions were observed in the control group [Table/Fig-11].



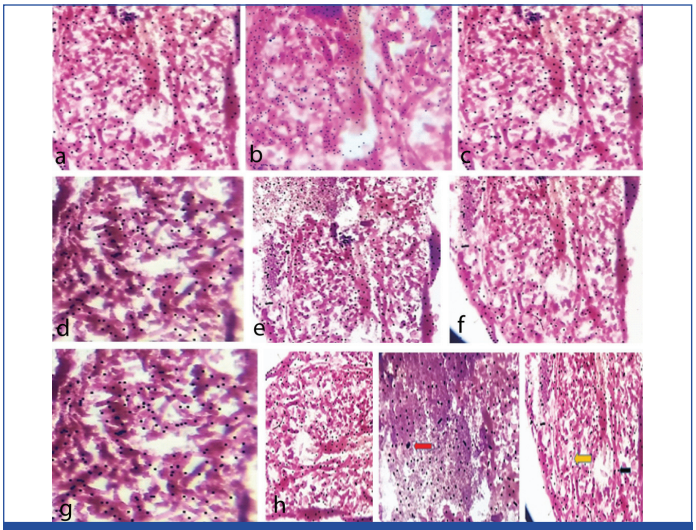
[Table/Fig-9]: Histopathological changes in liver of zebrafish exposed to different concentration of methanolic extract of *S. tenerrimum*. a) Control; b) 12.5 mg/L; c) 25 mg/L; d) 50 mg/L; e) 100 mg/L; f) 200 mg/L; g) 400 mg/L; h) 800 mg/L; In images the arrow mark represents the pathological changes in zebrafish liver- yellow indicates peripherally located nucleus; and black indicates vacuole formation and lipid accumulation in liver of zebrafish. The H&E staining analysis revealed that the cytoplasm of hepatocytes in the adult zebrafish control group was well-preserved, with a prominent nucleus and normal hepatic lobules. The liver of zebrafish exposed to *S. tenerrimum* (12.5 mg/L to 400 mg/L) did not exhibit any notable pathological alterations. In contrast, the higher dose of *S. tenerrimum* 800 mg/L exhibited; vacuole formation and a peripherally located nucleus. This was obviously due to the interference in the lipid metabolism of zebrafish. No necrosis or apoptosis was observed in the liver tissues treated with MEST. (Haematoxylin and Eosin stain 40x).

DISCUSSION

The study investigated the cytotoxicity of *S. tenerrimum*, specifically its impact on the SH-SY5Y cell line. Using an in-vitro MTT assay, cellular viability was assessed, and the MEST demonstrated as a potential therapeutic agent at lower dosages, with negative impacts observed at higher concentrations. The LC50 value was determined to be 140.014 µg/mL. The acute toxicity study reveals a 100% fatality rate at the highest concentration (800 mg/L) in fish, with a dose-dependent increase in mortality over 24 to 96 hours.



[Table/Fig-10]: Histopathological analysis of zebrafish brain exposed to different concentration of methanolic extract of *S. tenerrimum*. a) Control; b) 12.5 mg/L; c) 25 mg/; d) 50 mg/L; e) 100 mg/L; f) 200 mg/L; g) 400 mg/L (black arrows denotes thickening of Periventricular Gray Zone (PGZ) that was seen at 400 mg/L concentration of *S. tenerrimum*); h) 800 mg/L (black arrows in image H indicates that Inflammation with a decreased number of glomerules and yellow arrow indicates severe disrupted PGZ with congestion). In the brain, morphological changes were observed only at 800 mg/L. an inflammatory response was observed in the PGZ of the optic tectum in the zebrafish brain, characterised by a focal aggregation of lymphocytes and necrotic cells with a decrease number of glomerules (Haematoxylin and Eosin stain 40x).



[Table/Fig-11]: Histopathological analysis of zebrafish heart exposed to different concentrations of methanolic extract of *S. tenerrimum*. a) Control; b) 12.5 mg/L; c) 25 mg/L; d) 50 mg/L; e) 100 mg/L; f) 200 mg/L; g) 400 mg/L; h) 800 mg/L (black arrows in image H indicates myocardial fibres; yellow arrow indicates hyperplasia of myocardial cells; red indicates myocardial cell oedema may be due to raised capillary pressure) (Haematoxylin and Eosin stain 40x).

LC50 values were calculated at different time points. In subchronic toxicity, LC50 values progressively decreased over days, indicating an increase in toxicity during the study period. Histopathological examination after acute toxicity in zebrafish revealed morphological changes in the liver, brain, and heart at the highest concentration, with vacuole formation, inflammatory responses, and mild disintegration observed. Overall, the study suggested a dose-dependent cytotoxic and toxic effect of *S. tenerrimum*, emphasising the importance of careful dosage considerations in potential therapeutic applications.

Another study done by Purnomo Y et al., discovered that the toxicity level of *Urena lobata* leaf extract is notably higher on zebrafish embryos compared to juvenile and adult stages. This heightened sensitivity in embryos is attributed to their underdeveloped metabolic enzymes and organ systems, which increase their susceptibility to the extract's active constituents [36]. Xavier J and Kripasana K observed the toxic effects of *Enydra fluctuans*, noting that the plant extract proved to be 100% lethal within a mere 24 hours of exposure, with over 70% mortality at a dose of 200 mg/L in zebrafish. Interestingly, no toxicity was observed with ethanolic extract doses of ≤50 mg/L [30].

Moreover, Chinese *Momordica charantia* was found to induce scoliosis in zebrafish at concentrations ranging from 125 to 1000 µg/mL, while a concentration of 1000 µg/mL led to a gradual decrease in heartbeat. Severe necrotic liver damage was evident in fish treated with *Momordica charantia* extracts, primarily due to the presence of momordin, a key phytocompound in the plant known for inducing toxicity in animals and fishes [37]. Contrary to the preceding studies, the methanolic extract of *S. tenerrimum* was determined to be safe in adult zebrafish. However, additional long-term toxicological and histopathological data are required to further substantiate this finding.

Limitation(s)

Despite several advantages, some limitations emerge when attempting to study the zebrafish model, including the challenge of accurately measuring the compound intake by zebrafish. The process necessitates a skilled person to handle the zebrafish. Zebrafish are delicate creatures, and any mishandling can lead to inaccurate results or harm to the animals, making their care and experimentation a specialised task. Secondly, the dosages used in zebrafish experiments cannot be easily translated directly to rodent or human doses, and vice versa. This discrepancy arises from the significant physiological differences between these species. Zebrafish, being aquatic and considerably smaller than rodents or humans, have distinct metabolic rates, drug absorption rates, and sensitivities to certain compounds.

As a result, any findings from zebrafish studies must be carefully interpreted and cannot be directly applied to larger animals or humans without further adjustment and consideration of these interspecies variations [38]. These limitations underscore the importance of conducting additional research and validations when seeking to extrapolate zebrafish study results to broader applications, particularly in the context of drug development or toxicology studies.

CONCLUSION(S)

Currently, the zebrafish model has gained extensive recognition in the field of pharmacological and toxicological research owing to its diverse range of early morphology, husbandry, and size. In toxicology, the zebrafish model is used to assess human risk and screen drugs in the drug discovery process. From the results of acute and subchronic toxicity in this study, it was concluded that the methanolic extract of *S. tenerrimum* was found to be toxic to zebrafish in a dose-dependent manner. A clear correlation between concentration and mortality rate was observed, with an increase in concentration resulting in higher mortality rates. To ensure safety and avoid potential adverse effects, careful consideration of the extract's concentration is crucial.

At low concentrations, the methanolic extract of *S. tenerrimum* was found to be safe for zebrafish. However, when zebrafish were exposed to higher concentrations of 800 mg/L, severe histopathological changes were observed, leading to damage in vital organs. This finding highlights the importance of exercising caution and using appropriate dosages to prevent harm. To enhance safety data and gain more comprehensive insights, further long-term toxicity investigation in other higher species is recommended.

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