

Acute Toxicity Study and Antidiabetic Activity of Marine alga-*Halimeda gracilis* Chooranam (HGC) in Freshwater Zebrafish Model

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The study aimed to assess the acute toxicity and anti-diabetic activity of *Halimeda gracilis* (green marine alga). The *Halimeda gracilis* were collected from the coastal area of the Gulf of Mannar biosphere reserve and shade dried. Methanolic extract of *Halimeda gracilis* (MEHG) was prepared and it was screened for acute toxicity and anti-diabetic activity in the Zebrafish model. In the Acute toxicity study, the Zebrafishes were grouped into 6 groups and dosed with 6.25, 12.5, 25, 50, and 100mg/L of MEHG and observed at 0, 24, 48, 72, and 96 hours' intervals. For anti-diabetic activity analysis diabetes was induced using streptozotocin (STZ). The Zebrafish were divided into six groups- control group, positive control, diabetic Zebrafish with three doses of MEHG, and standard control (treated with metformin). Acute toxicity study showed no significant behavioral changes and LC₅₀ was determined as 100mg/L. In the diabetic study, test groups when compared to the control group showed: a significant reduction in both fasting and postprandial blood glucose levels and significant changes in the regeneration of pancreatic β -cells, and reduced vacuolization in the islets of Langerhans. Images of the regenerating caudal fins taken at 24, 48 and 72-hours post-amputation displayed significant limb regeneration in MEHG treated fish compared to the control group. These results prove that MEHG in STZ- induced diabetic Zebrafish possess potent anti-diabetic action by ameliorating blood glucose regulation, promoting pancreatic cell regeneration, minimizing long-term diabetic complications by preventing the emergence of metabolic memory but no behavioral changes.

Keywords: *Halimeda gracilis*, Streptozotocin, Acute toxicity study, Anti-diabetic activity, Zebrafish model.

Diabetes Mellitus is one of the heterogeneous metabolic disorder causing both microvascular & macro vascular complications

which is characterized by hyperglycaemia due to alterations in the storage & mobilization of metabolic compounds, including catabolism &

anabolism of carbohydrates, lipids & proteins, resulting from defects in insulin synthesis & secretion, resistance to action or both¹.

The long-term effects of diabetes lead to the development of specific complications like retinopathy, nephropathy, neuropathy, cardiovascular diseases, and co-morbidities². Hence, it is vital to diagnose the undiagnosed people with diabetes or pre-diabetic state and impart proper care for them as early as possible. Regular medications are utilized to treat diabetes by enhancing insulin sensitivity, increasing insulin production, and reducing blood glucose levels. There is always a drawback in drug treatment, in regulating the blood glucose levels due to certain adverse effects like GIT disturbance, tiredness, weight gain., etc.

In modern medicine, no satisfactory effective therapy is yet available. Marine algae (or) seaweed compounds have contributed to the global search for novel medicinal agents. In the current time, a noticeable number of novel metabolites with potent pharmacological properties have been distinguished from the marine organism which is one of the abundant source of structurally diverse natural products³⁻⁵. These algae are the most essential part of the diet of many eastern countries, and their use as food is well documented. Marine algae have a wide variety of compounds which have promising health benefits, and some have exhibited chelating property on heavy metals⁶. They are an abundant natural resource for many bioactive substances like polyunsaturated fatty acids, sterols, proteins, polysaccharides, antioxidants, colours, and trace elements in a focus a lot higher than in earthly plants⁷ and offer a wide range of secondary metabolites, which shows different pharmacological actions like anti-cancer, antimicrobial, antifungal, anti-inflammatory, antioxidants, anti-fouling, and anti-diabetic activity, etc⁸⁻⁹.

Hence, in this study, we have tested the acute toxicity and antidiabetic activity of *Halimeda gracilis* as various studies on other macro algae have potentially proven to have anti-diabetic action in animal models & patients and it's being used in alternate medicines to treat diabetes mellitus.

METHODS

Collection of *Halimeda gracilis*

The fresh marine green alga *Halimeda gracilis* was collected from Rameswaram coastal area, Tamil Nadu, India, and was carried to the lab in plastic bags with seawater. The alga was processed for a thorough wash with seawater & then tap water to remove epiphytes, salts, and other extraneous materials. The seaweeds were identified and authenticated as *Halimeda gracilis* (*Halimeda gracilis* Harvey ex. J Agarah 1887) by Dr. S. Bragdeeswaran, Associate Professor, Centre of Advanced Study (CAS) in Marine biology, Annamalai University, Parangipettai, Tamil Nadu, India & also prepared herbarium & museum specimens for the repository. The sample was then shade dried at 37°C and ground to a fine powder. The powder was then stored in the refrigerator for further use¹⁰.

Preparation of extracts

The dried *Halimeda gracilis* was made into a coarse powder in a mechanical grinder and it was subjected to maceration at 24-25°C in 95% methanol for 72 hours. The methanolic extract was derived after the process of distillation, evaporation, and drying under reduced pressure as per the standard procedure¹¹.

Acute toxicity study

Zebrafish were housed in a home tank at the population density of 2 fish per litre. The aquarium was filled with dechlorinated purified tap water and reverse osmosis water in the ratio of 1:2. The water was analysed and adjusted for the optimum pH, conductivity, salinity, and hardness. The aquarium was equipped with an aquarium filter and aerator units (aquarium aerator and pump). The fish was quarantined for a minimum period of 12 days in the laboratory test room before starting the experiment and the fish was fed with commercial fish food pellets two times per day. The temperature was maintained in the range of 27±2°C. The fish was provided with a photoperiod of 12 hours of artificial light and 12 hours of darkness throughout the experiment. They were acclimatized in the test tanks for 4 days before the study and the feeding was stopped 24 hours before the initiation of the drug dose.

Procedure

As per OECD guideline 203¹², the limit test at 100 mg/L of the test compound demonstrated that the LC₅₀ is greater than this concentration. The maximum concentration of the study was fixed as 100mg/L. The spacing factor of the concentration range was 2. The volume of the exposure medium was 2.5 litres per tank (not exceeding the maximum load of 1g of fish per litre). Exposure medium, maintained at the optimum pH, temperature, dissolved oxygen, and 12 hours of photoperiod was maintained throughout the study.

Grouping and dosing

As the test compound was sparingly soluble in water, the stock solution was prepared by

dissolving the 500 mg of the test compound in 1ml of methanol. The stock solution is aliquoted into different tubes based on the desired concentrations as mentioned (Table 1). The desired concentrations of exposure medium were prepared by adding the aliquoted quantity of the stock solution in the aquarium habitat water of known quantity. During the test period of 96 hours, all experimental fish were treated with MEHGC except normal control fish and observed for mortality, morbidity, and other behavioural changes in Zebrafish.

Anti-diabetic activity of MEHGC

Induction of diabetes in Zebrafish

All experimental fish received STZ (0.35 mg/g body weight, i.p.) single dose for 5 groups for

Table 1. Acute toxicity study groups and doses

Group	Total number of fish	Concentration	The volume of test sample per tank (μL)
1	8	100mg/L	500
2	8	50mg/L	250
3	8	25mg/L	125
4	8	12.5mg/L	62.5
5	8	6.25mg/L	31.3
Control	8	NULL	0



Fig. 1. Inducing STZ -intraperitoneal injection (50μL gas tight syringe)

19 days, except normal control based on its body weight (Figure 1). The fishes were kept on fasting for 12 hours on 20th day and on Day 21, blood samples (1.0 to 2.0 μl/fish) were collected through caudal fin and analysed for blood glucose level as a baseline. Diabetic animals were randomized into 6 groups based on an acceptable range of glucose level \pm 20% mean between the groups (Table 2). It was ensured to have a minimum of 8 animals with disease induction in each group at the start of drug treatment¹³.

Table 2. Dosing for diabetic induced Zebrafish

S. No	Group	Treatment	Total Fish
1	Normal Control	Control (Non-Diabetic Fishes)	10
2	Test Drug	STZ + MEHG low dose (200μg/g body weight)	10
3	Test Drug	STZ + MEHG mid-dose(300μg/g body weight)	10
4	Test Drug	STZ + MEHG high dose(500μg/g body weight)	10
5	Positive Control	Streptozotocin (STZ) of 0.35 mg/g body weight	10
6	Standard Drug	STZ + metformin of 0.001 mg/g body weight	10

Antidiabetic activity of MEHGC

In experiment groups, group 4 Zebrafish treated with a high dose of MEHG showed significant reduction [$p < 0.001$] in both the fasting and postprandial blood glucose levels compared to MEHG treated group 2, 3 & untreated group 5 – positive control [Table 8].

Fasting and postprandial glucose levels are expressed as mean \pm SEM, $P < 0.05$ when compared to control and group 6.

Histopathological examination

The pancreatic cells in group 1 fishes exhibited normal morphology, whereas, in group 2 and group 3 fishes, vacuolation in the exocrine

Table 5. Observation at 48th hours of dosing

Observations	Group 1	Group 2	Group 3	Group 4	Group 5	Control
No. of live fish	8	8	8	8	8	8
Mortality	Nil	Nil	Nil	Nil	Nil	Nil
pH	7.80	7.59	7.40	7.54	7.50	7.33
Temperature	26.43°C	26.43°C	26.43°C	26.43°C	26.43°C	26.43°C
Behavioral/visible abnormality	Normal	Normal	Normal	Normal	Normal	Normal

Table 6. Observation at 72nd hours of dosing

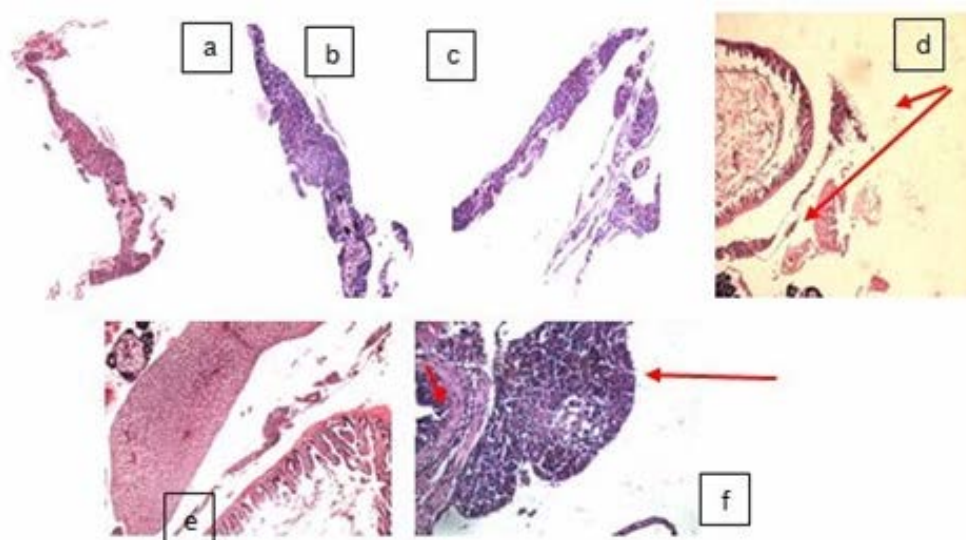
Observations	Group 1	Group 2	Group 3	Group 4	Group 5	Control
No. of live fish	8	8	8	8	8	8
Mortality	Nil	Nil	Nil	Nil	Nil	Nil
pH	7.80	7.61	7.50	7.17	7.38	7.18
Temperature	27.22°C	27.22°C	27.22°C	27.22°C	27.22°C	27.22°C
Behavioral/Visible abnormality	Normal	Normal	Normal	Normal	Normal	Normal

Table 7. Observation at 96th hours of dosing

Observations	Group 1	Group 2	Group 3	Group 4	Group 5	Control
No. of live fish	8	8	8	8	8	8
Mortality	Nil	Nil	Nil	Nil	Nil	Nil
pH	7.67	7.46	7.39	7.18	7.21	7.18
Temperature	27.46°C	27.46°C	27.46°C	27.46°C	27.46°C	27.46°C
Behavioral/Visible abnormality	Normal	Normal	Normal	Normal	Normal	Normal

Table 8. Antidiabetic activity of MEHG in Zebrafish

Groups	Fasting blood glucose level	Postprandial glucose level	P values
1 – Control	52.0 \pm 5.732	80.13 \pm 9.311	<0.001
2 - STZ + low dose	249.50 \pm 11.123	330.13 \pm 8.509	<0.001
3 - STZ + mid dose	186.38 \pm 15.892	290.50 \pm 6.162	<0.001
4 - STZ + high dose	99.13 \pm 8.459	140.13 \pm 5.194	<0.001
5 - Positive control	294.75 \pm 9.192	359.50 \pm 9.871	<0.001
6 - STZ + metformin	66.0 \pm 7.17	107.25 \pm 10.512	<0.001



A: Normal Control pancreas section showing normal morphology (10X)
 B: High dose of MEHGC showed normal pancreatic architecture (20X)
 C: Metformin showing pancreatic cells are arranged in normal cellular architecture (10X)
 D: Mild dose of MEHGC showing vacuolation in the exocrine pancreas (10X)
 E: High dose of MEHGC showing vacuolation in the exocrine pancreas (10X)
 F: Untreated group showed degenerated and vacuolation in islets of Langerhans of the pancreas (40X)
 Therefore, these results revealed that group 4 exhibited better efficacy compared to other treated and untreated groups.
 However, group 6 exhibited a good ameliorative effect compared to group 4.

Fig. 3. Histopathological examination of Zebrafish from experimental groups.

pancreas was observed. In group 4, most of the fishes exhibited regenerative morphology and exhibited normal pancreatic architecture. Few fishes exhibited vacuolation in the exocrine pancreas. In the untreated group 5, high pancreatic damages were observed like pancreatic degeneration and vacuolation in islets. Group 6 fishes showed normal pancreatic cellular architecture [Figure 3].

Hence from obtained histopathological examination analysis group 4 exhibited almost normal pancreatic cellular morphology compared to MEHG treated group 2, 3 & untreated group 5.

DISCUSSION

Seaweeds or marine algae are crude nonflowering plants without true root stem and leaves¹⁰. They are the sustainable living sources of food, fodder, and fertilizer in many parts of the world. Macro algae are found to have potentially highly active secondary metabolites with diverse activities in various researches reports which have been screened widely to isolate life-saving drugs

or biologically active substances worldwide. Marine algae are a rich source of ingredients such as polyunsaturated acids, β -carotene, and their pigment carotenoids, sulphated polysaccharide, and sterol¹⁵. They also contain secondary components that have the potential to be developed in various fields such as pharmaceuticals, cosmetics, and other industrial purposes such as biomass, biofuels, bio-oil, biodiesel, etc., and the waste can be used as fertilizer and fodder for animal (or) fish¹⁶.

In the present study, HGC used in acute toxicity study didn't show any abnormal behavioural changes, mortality, and morbidity in the Zebrafish even after 96 hours at maximum concentration 100mg/L.

The MEHGC showed quite significant antidiabetic activity at higher doses (500 μ g/g body weight) by reducing blood glucose levels ($p < 0.001$) showing better efficacy in comparison with other doses. It also maintained the normal morphology of pancreatic cells & regenerative property.

We would like to conclude that *Halimeda gracilis* has better efficacy as antidiabetic, by

maintaining the normal morphology of pancreatic cells & also has regenerative property without causing any mortality & morbidity in the acute toxicity test. It can be included in the diet of diabetic patients for the prevention and treatment of their condition.

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Ethical Approval

Not applicable (Ethical approval for Zebra fish study is not required as there are no ethical committees formed for the fish studies).

Competing interests

The authors have no competing interest to declare.

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Ethical clearance

Not applicable

Code availability

Not applicable

Author's contribution

SAJV- Initiated and conducted experiments. SRN- wrote manuscript. UNS- analysed the data. SV –is an animal biotechnologist - helped in animal studies. MK – Drafted the manuscript. SM- Conceived and designed the research. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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