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Research Article

Morphological, teratogenic and behavioral evaluations of *Gelidium spinosum* methanol extract on zebrafish embryos

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Abstract

Gelidium spinosum is an edible red seaweed from the family Gelidiaceae with possibility to be developed. The potential medical benefits of *G. spinosum* have been established, yet adequate empirical research on its toxicity is still lacking. Hence, the present work was aimed to examine the toxicity of *G. spinosum* methanol extract (GsME) on zebrafish (*Danio rerio*) embryos and to identify the phytoconstituents using gas chromatography-mass spectrometry (GC-MS) analysis. Results of this study showed that GsME induced morphological defects in zebrafish embryos, including reduction in eye size and body length. Moreover, yolk sac size and mortality were increased in zebrafish embryos exposed to GsME in a dose-dependent manner. GsME at high concentrations triggered teratogenic effects in zebrafish embryos such as decrease in heartbeat/minute, lack of pigmentation, lack of somite, structural deformity, pericardial oedema, and yolk oedema. The LC_{50} and EC_{50} of GsME were < 100 mg/L, which classified as a harmful category. The teratogenic index of GsME was found to be > 1, indicating its teratogenic attribute. Additionally, GsME exerted behavioral effects i.e. significantly lower total distance of movement and slower swimming speed of zebrafish embryos. GC-MS analysis of GsME was confirmed the presence of amino acid, phenolics, carboxylic acids, reducing sugars, saturated fatty acids and brominated saturated fatty acid in the extract. It suggesting that compounds of 13-bromotetradecanoic acid, palmitic acid, and stearic acid containing in GsME were contributed to the toxic effects.

Keywords

Behavior, *Gelidium spinosum*, GC-MS, Red seaweed, Toxicity

Introduction

Gelidium sp. is frequently recognized as red seaweed from division Rhodophyta (Schneider et al. 2020). The red seaweeds are widely distributed in the coastal area of Hainan and Xisha Islands in the South China sea, India, Indonesia, Korean Peninsula, Japan, Russia, Taiwan and Vietnam. Primary and secondary metabolites from this genus are recognized for use in the treatment of a variety of disorders. The plant is widely used in traditional medicine system for the treatment various of diseases, including ascariasis, bronchitis, crewels, constipation, diarrhea, enteritis, phlegm cough, goitre, nephropyelitis and tumor (Fu et al. 2016). *G. spinosum* is an edible red seaweed from the family Gelidiaceae. Several pharmacological activities of *Gelidium spinosum* have been investigated such as antidiabetic (Poulose et al. 2021) and antioxidant (Schneider et al. 2020; Warsi et al. 2023). However, studies on its toxicity are currently lacking despite wide availability of the extract for using in numerous beneficial purposes.

The zebrafish (*Danio rerio*) model is widely used in preliminary toxicology evaluations to verify teratogenic potential of both pharmaceutical products (Yamashita et al. 2014; Mersereau et al. 2016; Basnet et al. 2017) and drug candidates (Wibowo et al. 2018; Xavier and Kripasana 2020). In recent decades, the zebrafish (*D. rerio*) has evolved into an important model for ecological environmental monitoring of numerous pollutants (Dai et al. 2014), developmental hepatotoxicity (He et al. 2013), development of drugs for skin cancer (Bootorabi et al. 2017), and neurotoxicity in normal vertebrate development (Park et al. 2021). The zebrafish model behaves similarly to vertebrate models in assessments of developmental toxicity. In evaluating compounds for developmental toxicity, zebrafish and mammalian models accord 55%–100% of all studies (Horzmann and Freeman 2018). The zebrafish genome is also highly homologous to the human genome (Dai et al. 2014). Morever, the zebrafish growth is rapid, whole development of organs is manifested by 5 days post fertilization (dpf), and transparency makes it simple to see the extent of phenotypic changes (Zoupa and Machera 2017). Due to the advantages highlighted above, the zebrafish embryo is a rapid and effective preclinic model for evaluating a compound's toxicity.

The purpose of the current study was to assess the acute toxicity of *G. spinosum* methanol extract (GsME), by focusing on the morphological, teratogenic and behavioral effects on zebrafish embryos. Besides, this work also aimed to identify the phytoconstituents in GsME using GC-MS analysis.

Materials and methods

Sample material

Red seaweed (*G. spinosum*) was collected in January 2020 from Drini beach, Gunungkidul, Special Region of Yogyakarta, Indonesia. The sample was authenticated by Dr. Abdul Razaq Chasani (Ph.D.), Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia (document number 014893/S.Tb./IX/2020).

Extraction of red seaweed

Red seaweed was initially cleaned with distilled water and then dried at 28 ± 1 °C. The dried red seaweed was ground using a simplicia grinder. The resultant dried powder (25.0 g) was extracted with 150 mL of methanol (1:6 w/v) and stirred for 8 h at 800 rpm and then macerated at room temperature for 1 day. The crude extract was filtered using Whatman filter paper no. 1. Residue was again macerated with methanol (1:6 w/v) then filtered. The supernatant was concentrated using an evaporator at 65 ± 5 °C and then freeze-dried using a freeze dryer to dry the extract. The freeze dried red seaweed extract was maintained at -78 ± 2 °C for further experiments (Nurjanah et al. 2017).

Zebrafish toxicity assay

Zebrafish maintenance and spawning

Albino adult zebrafish were cared for at The Central Research and Animal Facility of International Islamic University Malaysia, Kuantan, Malaysia (authorized ethical registration number #IIUM/504/14/2/IACUC). The zebrafish were kept in an aquarium with a 12–16 h photoperiod and a 1 L water loading capacity per fish. Artemia dry granules of food were given three times each day. All the zebrafish were not given any medication for two months prior to spawning. The method used for spawning zebrafish corresponded with guidelines of standard literature (Busquet et al. 2013). Adult zebrafish aged 4–18 months, male and female (2:1) were kept apart and only permitted together reproduction when the light was first turned on. A few hours before dark, 30 zebrafish were kept in recirculated tanks of 10 fish/L in 100 liters of reverse osmosis water. Zebrafish were trained with a 14 h light and 10 h dark cycle at 26 ± 1 °C. Embryos were removed from the breeding tanks after thirty minutes, then mixed and selected at random for testing. The embryos then were rinsed with E3 medium (0.243 g of CaCl 0.33 mM, 0.064 g of KCl 0.17 mM, 0.407 g of MgSO4 0.33 mM and 1.461 g of NaCl 5.0 mM in 5 L of filtered water and added with 500 µL of 1% methylene blue) (Paatero et al. 2018).

Treatment of zebrafish embryos

Zebrafish embryos were processed with the sample in accordance with standard procedures (Busquet et al. 2014). At 5 hours post fertilization (hpf), the healthy spawned embryos were placed using a sterile pipette to a 96-well plate containing 125 µL of E3 medium. Each well contained a single embryo. Each GsME concentration (125 µL) was added to the 96-well. The final dosages of extract in dimethyl sulfoxide (0.1% v/v DMSO in E3 medium) were 0, 5, 15, 30, 62.5, 125 and 250 µg/mL (mg/L). The test was

performed with 20 embryos for the E3 medium (negative control), 20 embryos for each extract solution, 20 embryos for the drug control (3,4-dichloroaniline 4 mg/L in E3 medium), and 8 embryos for the internal plate control for each well. All the wells were incubated in the incubator at a temperature of 26 ± 1 °C.

Morphological observations

The morphological observations of zebrafish embryos were conducted at 1, 2, 3 and 4 dpf. Lethal concentration (LC_{50}) was determined using parameter endpoints of mortality (Equation 1), while effective concentration (EC_{50}) was calculated using endpoints of mortality and malformation (Equation 2) in accordance with The Organisation for Economic Co-operation and Development (OECD) guideline (Busquet et al. 2013). Meanwhile, morphological effect parameters that included eye size, rate of hatching, somite formation, movement defect and yolk size were observed using a microscope (TS 100, Nikon Corporation, Tokyo, Japan), while body length was captured using a microscope (Leica EZ4) equipped with DinoCapture software (2.0). Beats per minute was recorded as video and analyzed using DanioScope-1 software (Noldus Information Technology, The Netherlands). Teratogenicity parameters were also evaluated, such as less pigmentation, tail malformation, pericardial oedema, unhatched egg, and yolk oedema. For each of the dpf, teratogenic index (TI) was determined as the LC_{50}/EC_{50} ratio (Khan et al. 2020).

$$\text{Rate of mortality (\%)} = \frac{\text{Dead embryos number}}{\text{Embryos initial number}} \text{X}\,100 \quad (1)$$

$$\text{Rate of malformation (\%)} = \frac{\text{Morphological change embryos number}}{\text{Embryos initial number}} \text{X}\,100 \quad (2)$$

Behavioral assessment

Behavioral analysis was carried out following procedures previously reported by some researchers (Tzima et al. 2017; Achenbach et al. 2020). Briefly, ten zebrafish embryos in each sample concentration, solvent control and negative control were removed from the incubator and observed at 5 dpf using a Daniovision Behavioral Tracking instrument (Noldus Information Technology, The Netherlands). Each embryo was observed for 30 min under light and 30 min in the dark. Temperature was set at 27 °C. The total swimming distance and velocity of each embryo was recorded.

Phytoconstituents analysis

Phytoconstituents of *G. spinosum* methanol extract (GsME) were identified based on Gas Chromatography-Mass Spectrometry (GC-MS) profile by following a technique reported by Javadi et al. (2014) with few modifications. The red seaweed extract (25 mg) was dissolved in 50 µL of pyridine. The sample' solution was derivatized by adding 100 µL of methoxamine HCl (20 mg/mL in pyridine) and incubated at 60 °C for 2 h. 300 µL of *n*-methyl-*n*-(trimethylsilyl) trifluoroacetamide (MSTFA) was added to the reaction mixture and allowed to incubate for an additional 30 min at 60 °C. The sample was filtered and allowed to stand for the duration of the next day at 25 ± 2 °C.

The samples were analyzed by the gas chromatography mass spectrometry-electron ionization (GCMS-EI) system which was designed up of an Agilent 6890 GC-MS (Agilent Technologies, Santa Clara, CA, USA) and a HP 5973 selective mass detector (Agilent Technologies). A DB-5MS 5% phenyl methyl siloxane column with diameter of 250 µm, a film thickness of 0.25 µm and length of 30.0 m was chosen for the analysis. A 2 µL of the sample filtrate was injected into the GC-MS machine in splitless mode with helium supplied at a speed of 0.8 mL/min. The oven's first temperature was 85 °C. The starting temperature was consistently maintained to the intended temperature of 315 °C at a rate of 2 °C per minute, and then stabilized for 5 minutes. The temperatures of ion source and injector were established at 200 and 250 °C, respectively. The solvent barrier was set to 6 minute. Data were obtained in a mass scan ranging from 50 to 550 *m/z*, correlation between % abundance and retention time (minute). ACD/Spec Manager v. 12.00 (Advanced Chemistry Development, Inc., ACD/Labs Ontario, Toronto, ON, Canada) was used for converting the GC-MS raw data to the CDF file. The spectra were further analyzed using the Windows tool MZmine 2.53 (Institute of Organic Chemistry and Biochemistry, Prague, CZE). The National Institute of Standards and Technology's (NIST) 14 dataset library was used to compare the spectra for each peak of GC-MS, as well as compound name, m/z, retention time (RT), percentage of area, and similarity index (SI) of common primary and secondary metabolites. The SI for each of selected compounds was approximately ≥ 90.

Statistical analysis

All data were analyzed statistically using the IBM SPSS 23 (IBM Corporation, New Orchard Road Armonk, United States) statistics software package for Windows 10. Kolmogorov-Smirnov was used to verify normal distribution of the data, whereas Levene's test was used to confirm homogeneity of the data. The analysis then was followed by One-way ANOVA and Post-Hoc Tukey HSD tests to determine significant differences within the data groups. Body length, eye size, yolk size, total distance moved, and total swimming speed were parameters used to generate the data.

Results and discussion

Zebrafish toxicity evaluation

A toxicity examination is necessary to determine the potential risk from consumption of *G. spinosum* extract by human. The zebrafish as an animal model is assessed at different stages of its life: embryonic, larval and adult (Chahardehi et al. 2020). Zebrafish-based assay techniques have become more prevalent in the field of drug discovery over the last decade. It can be performed easily and

economically. Besides, the small size, fast growth, transparency, and genetic similarities to mammalian development make zebrafish an exceptionally excellent model for evaluating drug-induced teratogenicity (Yamashita et al. 2014).

Mortality rate in zebrafish embryos

Mortality rate (%) in zebrafish embryos exposed to GsME is displayed in Fig. 1. Data was acquired to determine the impact of dosage on mortality rate at 1, 2, 3, and 4 dpf. The data was collected at GsME doses of 0 (0.1% DMSO), 5, 15, 30, 62.5, 125, and 250 mg/L. Comparing GsME to solvent control (0.1% DMSO) groups revealed a dose-dependent increase in the mortality rate of zebrafish embryos. The outcome was consistent with an earlier investigation on the toxicity of *Curcuma longa* methanol extract on zebrafish embryos (Alafiatayo et al. 2019). The group that was administered 250 mg/L of GsME had the highest mortality. Each embryo at that concentration was killed between 1 and 4 dpf.

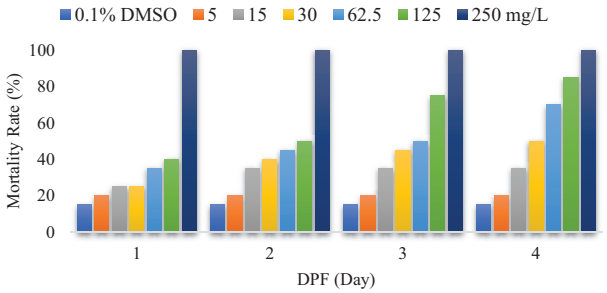

Figure 1. Effect of GsME on mortality rate (%) at 1, 2, 3 and 4 dpf, n = 20.

LC_{50}, EC_{50} and TI in zebrafish embryos

The dosage required to kill 50% or cause malformation and mortality in 50% of the investigated zebrafish embryos was used to determine the lethal concentration (LC_{50}) and effective concentration (EC_{50}), respectively. The linear regression curve relating log concentration and mortality rate (%) and relationship between log concentration and percentage malformation (%) were used to calculate the LC_{50} and EC_{50}) values, respectively (Fig. 2). Different concentrations of GsME caused developmental defects such as coagulation and body structural deformity in zebrafish embryos, as well as embryo death in a concentration-dependent fashion. Based on the OECD guideline, xenobiotics are classified as any substances that are harmful to zebrafish in the LC_{50} range of 10–100 mg/L, however concentration range at 1–10 mg/L is considered toxic and concentration range < 1 mg/L is considered highly toxic (Busquet et al. 2013).

The results of this research show that LC_{50} and EC_{50} values of GsME in zebrafish embryos were < 100 mg/L (Table 1). Hence, *G. spinosum* methanolic extract should be classified under the harmful category. Other earlier studies have reported that extracts with LC_{50} in the same harmful range i. e. *Polygonum multiflorum* methanol extract and *Allium flavum* 70% aqueous methanol extract with LC_{50} values of 48.8 mg/L and 50.3 mg/L, respectively (Yang et al. 2018; Aleksandar et al. 2019).

Zebrafish embryo LC_{50} and EC_{50} values were dosage and time-dependent manner. Longer exposures were associated with lower LC_{50} and EC_{50} values (Ali et al. 2012). Treatment 4 dpf had a strong effect on mortality and deformity in embry-

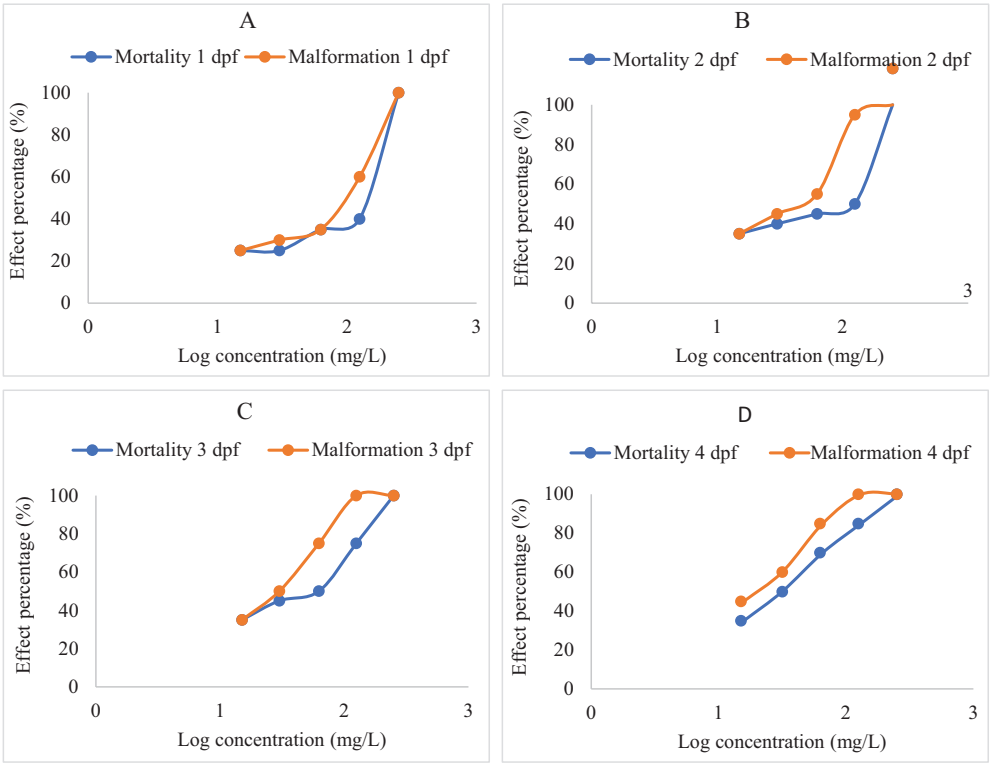

Figure 2. The LC_{50} and EC_{50} of GsME in zebrafish embryos: 1 dpf (**A**), 2 dpf (**B**), 3 dpf (**C**), 4 dpf (**D**).

Table 1. Values of LC_{50}, EC_{50} and TI of zebrafish embryos.

Timepoints (Day)	LC_{50} (mg/L)	EC_{50} (mg/L)	TI (LC_{50}/ EC_{50})
1 dpf	76.20	61.49	1.24
2 dpf	50.22	32.83	1.53
3 dpf	37.82	25.10	1.51
4 dpf	28.49	16.53	1.72

os. The teratogenic index (TI) value for each day of treatment was calculated using the LC_{50}/EC_{50} ratio. The teratogenic potential of toxic substance is determined according to its TI value. The higher the TI value, the more teratogenic potential the compound possesses (Alafiatayo et al. 2019). The TI of GsME at all observation days in this investigation had > 1 ratio. It was showed that GsME had teratogenic effects.

Effect on zebrafish heart rate

The heart rate of zebrafish embryo was recorded at 3 dpf (Fig. 3). Zebrafish embryos were administered with various dosages of GsME at 5, 15, 30 mg/L and negative control group to assess for effect on heart rate recorded as heartbeat per minute (BPM). At dose of 5 mg/L, zebrafish embryo's BPM was not significantly different from the control. GsME considerably lowered the heart rate of zebrafish embryos when compared to those of the negative control (p 0.00 < 0.05) at doses between 15 and 30 mg/L. The heartbeat per minute ranged from 86.9 to 124.0 on average. The greatest dose (30 mg/L) resulted in the lowest heart rate, whereas negative control embryos had the highest heartbeat per minute. This finding was contras to the earlier investigation showing that heartbeat per minute at high concentration of *Palmaria palmata* papain hydrolysate treatment (1000 mg/L) after 2 dpf was not significantly lower when compared to control. However, the study had reported that heartbeat per minute of zebrafish was significantly reduced at higher doses (5000 and 10,000 mg/L) when compared to the the control (Fitzgerald et al. 2013).

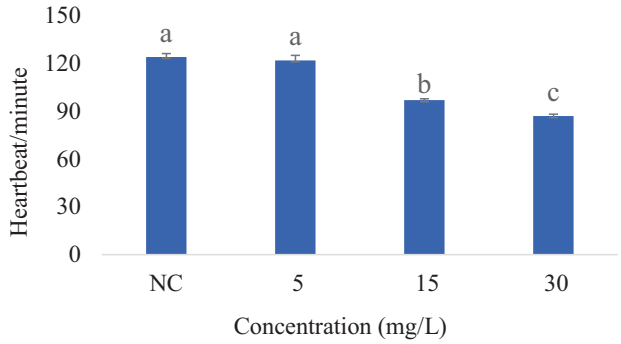

Figure 3. The effect of various dosages of GsME on zebrafish embryos heart rate

Effect on zebrafish body length

Body length is a morphological parameter that is used as an indicator to assess for malfunction in zebrafish embryos toxicity tests. Body length was used to determine the effect of GsME on the growth of the zebrafish embryos. It was measured at 5 dpf. The body length measurements of zebrafish embryos treated with GsME is presented in Table 2. Zebrafish embryos had significantly shorter bodies at GsME doses of 30 mg/L compared to concentrations between 5 and 15 mg/L, as well as the negative control and solvent control (0 mg/L) (p < 0.05). Zebrafish embryos administered with GsME at doses ranging from 0 to 30 mg/L produced a range of body lengths between 2.75 mm (maximum length) and 2.29 mm (minimum length). This result was supported by other investigation reporting dose-dependent reduction in the body length of zebrafish embryos when exposed to treatment (Zoupa and Machera 2017).

Table 2. Effects of GsME on morphology of zebrafish embryos.

Extract dosage (mg/L)	Hatching rate (%)	Eye size $\times 10^4$ (μm^2)	Body length (mm)	Yolk size $\times 10^5$ (μm^2)
250	0	NA	NA	NA
125	10	NA	NA	6.85 ± 0.03^a
62.5	25	NA	NA	6.03 ± 0.05^b
30	100	7.88 ± 0.06^a	2.29 ± 0.06^a	5.66 ± 0.03^c
15	100	9.15 ± 0.10^b	2.37 ± 0.05^b	NA
5	100	10.19 ± 0.13^c	2.42 ± 0.05^b	5.16 ± 0.02^d
SC	100	10.74 ± 0.06^d	2.75 ± 0.05^c	4.99 ± 0.03^e
NC	100	10.67 ± 0.05^d	2.76 ± 0.05^c	5.01 ± 0.02^e
PC	10	NA	NA	NA

NC: normal control, SC: solvent control, PC: positive control; NA: not assessed. The data were reported in average ± SD (n = 10); different letters: significantly different (p < 0.05).

Effect on zebrafish hatching rate

Zebrafish embryos have the capacity to hatch while they are still in the early stages of development between 2 and 3 dpf. Following exposure to GsME at concentrations between 5 and 30 mg/L, all zebrafish embryos were able to hatch. This was similar to the normal control and solvent control at 3 dpf. Meanwhile, a hatching defect was discovered in embryos treated with GsME at doses ranging from 62.6 to 125 mg/L. Because 100% of the embryos were coagulated at 1 dpf, the hatching rate of zebrafish embryos could not be measured at maximum concentration (250 mg/L).

Effect on zebrafish eye size

Another appropriate endpoint indicator for assessing a compound's toxicity is the size of the eyes in zebrafish embryos. This is due to the fact that zebrafish eyes and human eyes have several similarities, such as the predominance of cylinders in zebrafish vision and the way that zebrafish cells absorb light (Salbreux et al. 2012). Additionally, according to Ganzen et al. (2017), both zebrafish and humans have two neural layers, namely an interior and an exterior papillary membranes. GsME-treated zebrafish embryos exhibited significantly smaller eyes than untreated embryos (p < 0.05) in a dosage-dependent manner when compared against control groups. Previous investigations have also reported similar finding (Xia et al. 2020).

Effect on zebrafish yolk sac size

Yolk sac could be used to identify organ malfunction in zebrafish. The yolk sac provides the zebrafish embryo's main food supply for the first week of development. Zebrafish eggs have yolk sacs that are 70% neutral lipids, mainly liver-metabolized triacylglycerol and cholesterol ester. Thus, hepatic dysfunction causes a delay in the digestion and absorption of yolks as well as an increased fat deposition (He et al. 2013; Miyares et al. 2014). The data of yolk sac size was acquired at 1 dpf. When compared to the negative and solvent controls, GsME significantly increased the yolk size of zebrafish embryos in a dosage-dependent manner ($p < 0.05$). The outcomes of this study were corroborated by a number of earlier, related investigation that also found an increase in zebrafish embryo yolk size in a dosage-dependent manner (Park et al. 2019).

Teratogenic effects on zebrafish

The embryo tail-bud usually begins to detach at 1 dpf, and a non-detaching tail indicates the embryo is dead (Khan et al. 2020). All zebrafish embryos showed defects as described in Table 3 with GsME at concentrations of 62.5 and 125 mg/L, despite the fact that certain embryos exhibited a delayed hatching process. At the lowest extract concentration, no zebrafish embryos were affected. When compared to the control group (Fig. 4A), 100% of the eggs coagulated at 1 dpf (Fig. 4B), rendering it impossible to assess all teratogenicity parameters at a concentration of 250 mg/L. At 1 dpf, yolk oedema in zebrafish embryos exposed to GsME at dosages between 30 and 125 mg/L was examined (Fig. 4C). Embryos exhibited less somites at 5 dpf with GsME concentrations between 62.6 and 125 mg/L (Fig. 4F), when compared to normal control (Fig. 4E). Meanwhile, embryos expose to GsME at doses of 15 to 125 mg/L led to structural deformity. Structural abnormalities included non-development of the eyes, head, and tail as well as non-detachment of the tail at dose 125 mg/L (Fig. 4D); and non-linear extension of the tail at a dose of 62.5 mg/L at 3 dpf (Fig. 4H).

At 4 dpf, embryos exposed to GsME at concentrations of 62.5 and 125 mg/L had abnormal spines as well as reduced pigmentation (Fig. 4I). Embryos subjected to GsME at concentrations of 62.5 and 125 mg/L had pericardial oedema at 3 dpf (Fig. 4G).

Effect on zebrafish behavior

Several variables can be used in behavior analysis, such as spontaneous movement, which starts at 17 hours post fertilization (hpf), touch-evoked tail coiling which starts at 21 hpf, and swimming which starts at 27 hpf (McKeown et al. 2009). The spinal cord and nervous system's

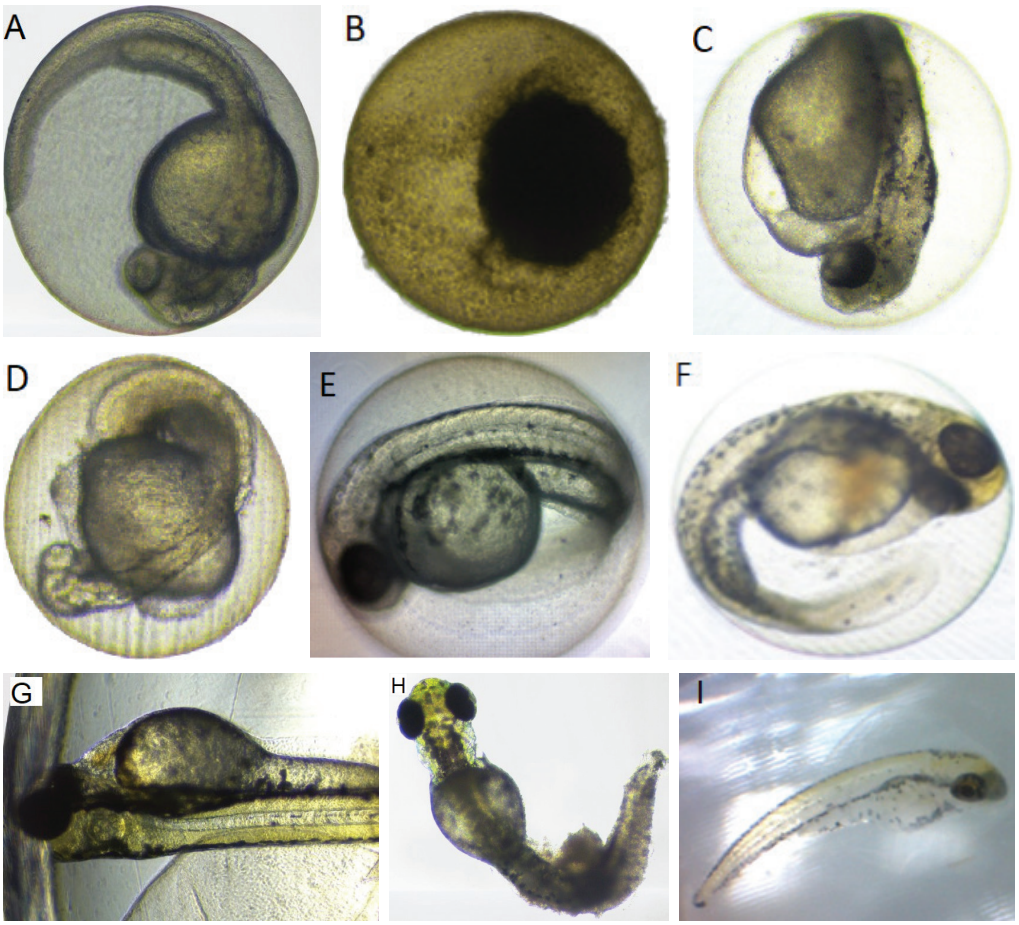

Figure 4. Teratogenic effects of various doses of GsME in zebrafish embryos. **A.** Normal embryo; **B.** 250 mg/L; **C.** 30 mg/L; **D.** 125 mg/L; **E.** Normal control; **F–I.** 62.5 mg/L.

Table 3. Teratogenicity of GsME on zebrafish embryos.

Extract dosage (mg/L)	Teratogenic parameters					
	LP	LS	SD	PO	UH	YO
250	NA	NA	NA	NA	NA	NA
125	+	+	+	+	+	+
62.5	+	+	+	+	+	+
30	-	-	+	+	-	+
15	-	-	+	-	-	-
5	-	-	-	-	-	-
NC	-	-	-	-	-	-
SC	-	-	-	-	-	-
PC	NA	NA	+	+	+	+

LP: less pigmentation; LS: less somite, SD: structural deformity, PO: pericardial oedema; UH: unhatched; YO: yolk oedema; NA: not assess, NC: normal control, SC: solvent control, PC: positive control; (-) not detected, (+) detected.

convolutional neural network, regulates motion in vertebrates, including walking and swimming. As a result, the swimming capacity of zebrafish can be investigated to determine function of the muscular system (Goody et al. 2012). The capacity of zebrafish embryo to move or swim at 5 dpf was observed during the behavior assay. Swimming was examined for 30 minutes under light and 30 minutes under darkness. The total distance traveled and swimming speed were recorded (Table 4). In comparison to solvent and negative control, application of GsME on zebrafish embryos resulted in a significantly lower total movement distance in zebrafish embryos ($p < 0.05$). Additionally, the swimming speed of zebrafish embryos treated with GsME displayed a significantly slower concentration-related trend in comparison to solvent and negative controls ($p < 0.05$). This finding was consistent with other studies on the toxicity of various water-soluble compounds (some compounds) that were considered to significantly reduce locomotor activity in a concentration-dependent manner (Ali et al. 2012). However, the results obtained further reveal that acute exposure to test extract resulted in an increase in dark-phase activity and decrease in light-phase activity.

Table 4. Effects of GsME in distance moved and swimming speed of zebrafish.

Extract doses (mg/L)	Total distance moved $\times 10^3$ (mm)		Total swimming speed $\times 10^4$ (mm/s)	
	Light	Dark	Light	Dark
30	2.37 + 0.05^a	2.81 + 0.06^a	7.92 + 0.07^a	8.19 + 0.13^a
15	2.65 + 0.05^b	3.22 + 0.06^b	9.41 + 0.09^b	9.86 + 0.09^b
5	3.09 + 0.05^c	3.45 + 0.05^c	10.27 + 0.11^c	10.52 + 0.10^c
2.5	3.39 + 0.05^d	4.00 + 0.03^d	10.83 + 0.09^d	11.16 + 0.13^d
SC	3.63 + 0.04^e	4.44 + 0.06^e	11.29 + 1.85^e	11.94 + 0.10^e
NC	3.62 + 0.04^e	4.45 + 0.05^e	11.21 + 1.41^e	11.90 + 0.14^e

mm/s: millimeter per second. The data are presented as mean + SD (n = 10); significant differences ($p < 0.05$) were stated in different letters.

Phytoconstituents of GsME

Identified metabolites in derivatized GsME of GC-MS analysis are presented in Fig. 5 and Table 5. Following derivatization, GC-MS analysis provided details regarding the chemical structure of the extract. Identification of the compounds was performed via comparison of each compound's fragment spectrum to the National Institute of Standards and Technology's (NIST) 14 spectrum database. The mathematical algorithm that were used including m/z, retention times (RT), area percentages (%), and similarity indexes (SI). The SI for each of the identified compound was approximately ≥ 90% (42.86% of compounds), whereas SI unidentified compound was ≤ 89% (57.14% of compounds). According to this discovery, GsME was contained amino acid (1-isoleucine), phenolics (catechin, protocatechuic acid), and carboxylic acids (succinic acid, citric acid, ribonic acid).

Table 5. The compounds identified in GsME by GC-MS.

Peak No	Compounds	m/z	RT (min)	Area (%)	SI
1	1-Isoleucine	86.05	5.06	0.77	90
2	Butanedioic acid (Succinic acid)	73.05	6.81	1.91	95
3	D-Arabinonic acid	73.05	8.68	1.33	91
4	Protocatechuic acid	193.00	11.11	0.53	91
5	1,2,3-Propanetricarboxylic acid (Citric acid)	73.05	11.39	8.63	90
6	D-(-)-Fructose	73.05	12.19	4.02	94
7	D-Psicose (D-Allulose, Pseudofructose)	73.05	12.32	3.32	93
8	D-(+)-Talose (D-Talopyranoside)	73.05	12.62	5.97	93
9	D-Galactose	73.05	12.91	1.53	91
10	Hexadecanoic acid (Palmitic acid)	117.05	14.57	7.84	94
11	Myo-inositol (Vitamin B_8)	73.05	15.78	1.04	93
12	Ribonic acid	73.05	17.40	1.17	94
13	Glyceryl glycoside	204.05	19.62	0.79	91
14	D-(+)-Galacturonic acid	73.05	20.60	0.86	91
15	O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (Sucrose)	73.05	22.48	3.14	90
16	Octadecanoic acid (Stearic acid)	73.05	23.02	1.12	94
17	13-Bromotetradecanoic acid	227.15	23.26	0.05	90
18	Catechin	73.05	23.97	0.70	90

GsME was contained mainly reducing sugars compounds, including D-arabinonic acid, D-fructose, D-(+)-galacturonic acid, D-galactose, glyceryl glycoside, myo-inositol, D-psicose, sucrose and D-(+)-talose. Based on evidence, it was reported that these sugars were not found to be toxic at low concentration and short time (Iida et al. 2013; Francisqueti et al. 2016). GsME was also contained saturated fatty acids, including palmitic acid and stearic acid. Recent finding reported that free fatty acid, such as palmitic acid was induced toxicity associated with metabolic system which triggers the production and release of reactive oxygen species (ROS) in the body (Osorio et al. 2020). Thus suggesting that the fatty acids compounds in GsME, including palmitic acid, and stearic acid were related to their toxicity. Additionally, GsME was also contained brominated saturated fatty acid such as 13-bromotetradecanoic acid. According to the latest study was mentioned that bromin-containing compounds can result in acute toxic effects by interfering with oxidative phosphorylation, as hormone antagonist, and induce both lethal and non-lethal deformities (Kammann et al. 2006: Dahlgren et al. 2015). Therefore, it appeared that 13-bromotetradecanoic acid in GsME contributed to the toxic effects.

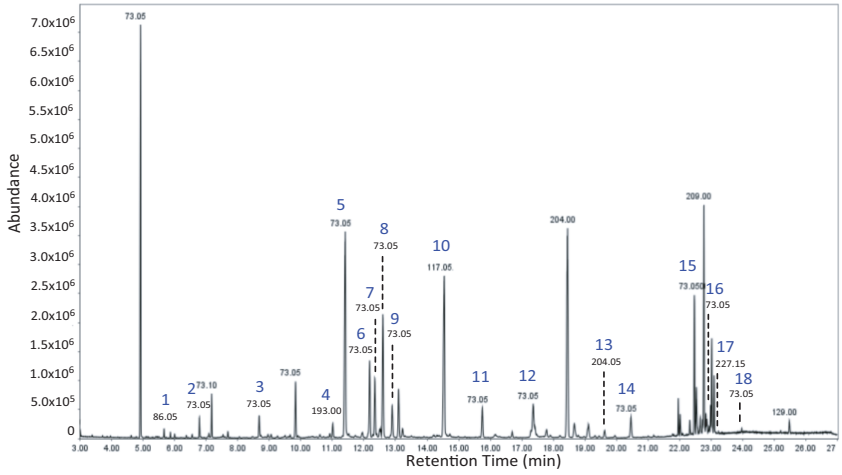

Figure 5. GC-MS analysis of phytoconstituents in GsME.

Conclusion

The findings of this toxicology investigation, show that *G. spinosum* methanol extract (GsME) had LC_{50} and EC_{50} of < 100 mg/L in a dose and time-dependent manner. A teratogenic index of > 1 suggested that GsME had teratogenic effects. GsME was significantly reduced eye size, body length, total distance of movement and swimming speed, meanwhile the amount of yolk in zebrafish embryos was increased in a dose-dependent manner. Low doses of GsME did not cause any developmental defects in zebrafish embryos, whereas high concentrations led to malformations including lack of pigmentation, lack of somite, structural deformities, pericardial oedema, and yolk oedema. GC-MS analysis of GsME was identified several important phytoconstituents belonging to different classes of biologically active compounds namely amino acid, phenolics, carboxylic acids, reducing sugars, saturated fatty acids and brominated saturated fatty acid.

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