ABSTRACT

*Gelidium spinosum* belongs to red seaweed that is used as a food ingredient to heal a variety of diseases. Awareness about the potential medicinal value and actual research data on this plant's toxic effect is currently insufficient. Hence, the current study aimed to determine phytoconstituents using Gas Chromatography-Mass Spectrometry (GC-MS), and investigated the toxic effect of *Gelidium spinosum* Water Extract (GsWE) using zebrafish (*Danio rerio*) embryos model. Results of the identification confirmed some known phytoconstituents including alkaloid, cinnamic acid ester, heterocyclic aromatic, organic fatty acids, fatty alcohol, and sugars. Results of a toxicity study of GsWE showed a concentration-dependent increase in mortality and yolk size, meanwhile a decrease in eye size, body length, and heartbeat rate on zebrafish embryos. The median lethal concentration (*LC*$_{50}$) of GsWE was obtained at 707.38 mg/L. It was considered in the safe category. GsWE did not affect to zebrafish embryo development at low concentrations. In high concentrations, zebrafish embryos showed abnormalities, such as loss of pigmentation, pericardial oedema, and yolk oedema. The results of this investigation will contribute to reinforcing the safety of *G. spinosum*-related food supplement manufacturing.

**Keywords:** Zebrafish, *Danio rerio*, *Gelidium spinosum*, Toxicity, Water Extract, Seaweed.

INTRODUCTION

Red seaweed belongs to the *Rhodophyta* division. Red seaweed presents exciting prospects for the production of food supplements. *Gelidium spinosum* is an edible red seaweed from the *Gelidiales* order and *Gelidiaceae* family. The genus *Gelidium* is known as a source of primary and secondary metabolites that have been reported up to now including carotenoid, cyclosporin-like amino acids, R-phycoerythrin, polyphenols, agar, galactan, and sterols. This genus has a diverse range of biological activities, such as antibacterial, anticholinesterase, anticoagulant, anti-diabetic, anti-inflammatory, antiproliferative and Apoptosis-Inducing. Based on the research findings on the same genus, *G. spinosum* is a plant that has potential as a therapeutic agent as well. *G. spinosum* has been known to possess antioxidative and antidiabetic effects. Apart from numerous beneficial side effects, several phytoconstituents of the medicinal plants have the potential to be toxic effects, and have reportedly been shown to improve kidney function. Therefore, the plant that is traditionally used to treat various ailments needs to be thoroughly evaluated for its effectiveness and toxicity to confirm its safe nature before its prescription. In this regard,
zebrafish embryos are widely used to evaluate the toxicological effect of drug candidates. Zebrafish embryos as a model of toxicity assay have numerous advantages, including high fecundity, optical transparency, rapid development process, genomes, and physiology possessing a high similarity to humans. The current work aims to assess the preliminary phytoconstituents of Gelidium spinosum Water Extract (GsWE) using GC-MS. Besides, this work also aims to examine the acute toxic effects of GsWE using the zebrafish (Danio rerio) embryos model. This is the first study of GsWE to evaluate its acute toxicity in zebrafish embryos.

**EXPERIMENTAL**

**Collection and Identification of Sample**
The sample of Gelidium spinosum was obtained from the coastal area of Drini, Gunungkidul, in the Special Region of Yogyakarta, Indonesia. The authenticity of the sample was confirmed by the taxonomist, Dr. Abdul Razaq Chasani, Ph.D., from the Faculty of Biology at Universitas Gadjah Mada, Yogyakarta, Indonesia.

**Preparation of Plant Extract**
Initially, Gelidium spinosum was rinsed with water and allowed to dry at room temperature. The dried red seaweed was then pulverized and mashed using a mesh size of 40. The plant’s dry powder (25 g) was extracted with water (250 mL) in a ratio of 1:10 w/v for 8 hours with mechanical stirring at 800 rpm. The crude extract was filtered using filter paper of Whatman number 1 in a Büchner vacuum. The supernatant was then condensed using a rotating vacuum evaporator at 65-70°C and freeze-dried. The homogenized dried red seaweed extract that was obtained (6.14 ± 0.16 g) was kept at -80°C for further analysis.

**Assessment of Gelidium spinosum Water Extract Using GC-MS**
Metabolites of Gelidium spinosum Water Extract (GsWE) were characterized using GC-MS in compliance with a protocol that was released by researchers with minor modifications. The water extract was dissolved in pyridine, then derivatized using methoxamine HCl and n-methyl-n-(trimethylsilyl) tryfluoroacetamide. The mixed solution was pumped into the GC-MS machine with helium supplied at a speed of 0.8 mL/min. Data was collected in full scan mode from 50 to 550 m/z, and the correlation between % abundance and fragmentation (m/z) was recorded. Data was collected. The spectrum was then smoothed out using MZmine 2.18.3 Windows software.

**Toxicity Assay**

**Zebrafish Embryos Treatment**
Zebrafish embryos were procured from the Central Research and Animal Facility International Islamic University Malaysia, Kuantan, Malaysia. The ethical clearance of the zebrafish embryo was authorized by the Institutional Animal and Use Committee of the International Islamic University Malaysia under number IIUM/504/14/2/IACUC. E3 medium containing a combination of four different salts, including 0.097 g of CaCl 0.33 mM, 0.025 g of KCl 0.17 mM, 0.163 g of MgSO4 0.33 mM, and 0.584 g of NaCl 5.0 mM in 2 L of filtered water, was used to clean the embryos. The E3 medium was added with 200 µL of methylene blue to suppress the formation of fungal colonies. Embryos that were malformed were evacuated with a dropper that had a wider hole. The healthy eggs were transferred to a culture plate containing E3. The embryonic progress was observed using a microscope with a minimum magnification of 30X at 3 hours post fertilization (hpf). The zebrafish embryos were used for research only if the batch of eggs tested showed an overall healthy condition of ≥ 70%.

**Treatment of Embryos with The Sample**
Embryos of zebrafish were treated with samples according to the procedure of the standard. At 4 hpf, the viable spawned embryos were placed using a sterile pipette to a 96-well plate containing 150 µL E3 medium. Each well comprised a single embryo. A 150 µL of each red seaweed concentration was inserted into the 96-well. The final concentrations for extract were 0 (control of solvent), 50, 100, 200, 375, 500, 750, and 1000 mg/L in dimethyl sulfoxide (DMSO) at a concentration of 0.1 % v/v in E3 medium. Several embryos that were used in this test included, twenty embryos in each group, and eight embryos served as an inside plate control for each well in this test. 3,4-dichloroaniline (4 mg/L in E3 medium) was used as a control drug. All wells were incubated at 26 ± 1°C.
Microscopic Observations
The toxicity test (microscopic observation) of the sample on embryos of zebrafish was evaluated at 24, 48, 72, and 96 hpf. Parameter endpoints were conducted according to The Organisation for Economic Co-operation and Development (OECD) guidelines, comprising coagulation, and mortality to calculate the lethal concentration (LC50). The other abnormalities were observed using a Nikon light microscope (TS 100, Nikon Corporation, Tokyo, Japan), such as eye size, and yolk size. Teratogenicity parameters were also observed which included less pigmentation, pericardial oedema, and unhatched, and yolk oedema. Meanwhile, body length was captured using a microscope (Leica Ez4) that was equipped with DinoCapture software (2.0). Heartbeat per minute (BPM) was video recorded and analyzed using the software of DanioScope-1 (Noldus Information Technology, The Netherlands). Lethal concentration (LC50) was calculated based on the amount of mortality. The rates of mortality and hatching were estimated by following Formula 1, and 2, respectively.

\[
\text{Mortality Rate} \ (\%) = \frac{\text{Number of dead embryos}}{\text{Initial number of embryos}} \times 100
\]
\[
\text{Hatching Rate} \ (\%) = \frac{\text{Number of hatched embryos}}{\text{Initial number of embryos}} \times 100
\]

Statistical Analysis
Data of BPM, body length, eye size, and yolk size were expressed in average ± SD (n=10). IBM SPSS 23 statistics application for Windows 2021 was used to analyze all raw data. The raw data of each parameter was carried out using Kolmogorov-Smirnov for normality of variances, whereas homogeneity of variances was examined using a test of Levene. The difference between each group was determined by one-way ANOVA and Tukey tests. Each group was stated as significantly different at a p-value less than 0.05.

RESULTS AND DISCUSSION
Phytoconstituents of Gelidium spinosum Water Extract (GsWE)
Potential phytoconstituents in GsWE were determined based on GC-MS spectra (Fig.-1). The compounds were analyzed according to spectrum profile with the GC-MS catalog datasets of the National Institute of Standards and Technology (NIST) 11. Several phytoconstituents belonging to various groups were identified. Data of phytoconstituents' names, empirical formula, integration, retention time (RT), and similarity index (SI) were employed in Table-1.

The identified compounds included alkaloid (benzothiazole); cinnamic acid ester (ethyl p-methoxy cinnamate); heterocyclic aromatic (2-acetyl-3-methylbenzo[b]thiophene; organic fatty acids (acids of propanoic, lauric, myristic, and palmitic); fatty alcohol (octadec-9Z-enol); and sugars (D-pinitol, D-psicose, D-(−)-fructose, D-(+)-talose, d-Mannose). All of these compounds had a similarity index (SI) of ≥ 90%.

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Table-1: Potential Compounds Observed in GsWE

<table>
<thead>
<tr>
<th>No</th>
<th>Phytoconstituents name</th>
<th>Integration (%)</th>
<th>RT (min)</th>
<th>Empirical formula</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Propanoic acid</td>
<td>0.69</td>
<td>3.66</td>
<td>C₃H₆O₂</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>Benzothiazole</td>
<td>4.35</td>
<td>3.99</td>
<td>C₇H₅NS</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
<td>2-Acetyl-3-methylbenzo[b]thiophene</td>
<td>1.94</td>
<td>5.22</td>
<td>C₁₁H₁₀OS</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>Lauric acid</td>
<td>2.89</td>
<td>8.99</td>
<td>C₁₂H₂₄O₂</td>
<td>92</td>
</tr>
<tr>
<td>5</td>
<td>Ethyl p-methoxycinnamate</td>
<td>1.40</td>
<td>10.25</td>
<td>C₁₂H₁₄O₃</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>Myristic acid</td>
<td>1.46</td>
<td>10.38</td>
<td>C₁₄H₂₈O₂</td>
<td>93</td>
</tr>
<tr>
<td>7</td>
<td>D-Pinitol</td>
<td>3.79</td>
<td>11.04</td>
<td>C₆H₁₂O₆</td>
<td>91</td>
</tr>
<tr>
<td>8</td>
<td>D-Psicose</td>
<td>3.79</td>
<td>11.20</td>
<td>C₆H₁₂O₆</td>
<td>94</td>
</tr>
<tr>
<td>9</td>
<td>D(-)-Fructose</td>
<td>1.94</td>
<td>11.36</td>
<td>C₆H₁₂O₆</td>
<td>94</td>
</tr>
<tr>
<td>10</td>
<td>D(+)-Talose</td>
<td>6.24</td>
<td>11.52</td>
<td>C₆H₁₂O₆</td>
<td>93</td>
</tr>
<tr>
<td>11</td>
<td>D-Mannose</td>
<td>2.06</td>
<td>11.91</td>
<td>C₆H₁₂O₆</td>
<td>91</td>
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<tr>
<td>12</td>
<td>Palmitic acid</td>
<td>4.75</td>
<td>14.61</td>
<td>C₁₈H₃₆O₂</td>
<td>94</td>
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<tr>
<td>13</td>
<td>Octadec-9Z-enol</td>
<td>0.36</td>
<td>15.83</td>
<td>C₁₈H₃₈O₂</td>
<td>92</td>
</tr>
</tbody>
</table>

Toxicity Study of GsWE

Lethal Concentration and Mortality Rate

The lethal concentration (LC₅₀) was determined by calculating the dosage needed to cause mortality in 50% of the marine organisms tested. The LC₅₀ value for GsWE was calculated based on the curve of log concentration versus probit value (% mortality) as described in Fig.-2A. Different concentrations of GsWE caused embryo mortality in embryos of zebrafish in a dosage-relation pattern. The 96-hour LC₅₀ of GsWE was obtained at 707.38 mg/L. Compounds are classified as destructive to zebrafish if the LC₅₀ is between 10 and 100 mg/L, lethal: between 1 and 10 mg/L) and really lethal: < 1 mg/L according to the OECD guideline. Gelidium spinosum water extract’s LC₅₀ (707.38 mg/L) value for zebrafish embryos indicates that GsWE can be considered in a safe category. This research obtained LC₅₀ > 500 mg/L which was in contrast to the previous report on the zebrafish embryos' toxicity of water extracts of Piper sarmentosum in which at a dosage of 60 mg/L all embryos were killed. Another research study reported that LC₅₀ of Psychotria malayana Jack leaf water extract on zebrafish embryos was obtained as 252.45 mg/L. The mortality rate (%) in zebrafish embryos exposed to GsWE is shown in Fig.-2B. The data was observed at 24, 48, 72, and 96 hpf to know the effect of concentration on the mortality rate. This data was obtained in quantity at 200, 500, 750, and 1000 mg/L. GsWE markedly expanded the mortality rate on zebrafish embryos in a dose-dependent mode as compared to the control groups. This finding was found to be in line with the previous finding on zebrafish embryo toxicity of an ethanol extract of Clerodendrum cyrtophyllum. The 1000 mg/L of GsWE groups had the highest mortality rate in zebrafish embryos. All of the embryos at that concentration died between 24 and 96 hpf. Meanwhile, the control group and GsWE at 200 mg/L had the lowest mortality rate.

$$y = 2.6406x - 2.5248$$

$$R^2 = 0.9586$$
Hatching Rate

In the first growth phase, zebrafish embryos can hatch. It generally occurs between 48 hpf and 72 hpf. GsWE did not affect the hatching rate, like the normal control and solvent control groups (100% hatched), except at the highest concentration (750 mg/L). Some of the embryos (45%) at a dose of 750 mg/L showed a defect in hatching, and some died (55%). The hatching rate could not be detected at a concentration of 1000 mg/L due to 100% coagulation at 24 hpf. This finding was found to be similar to previous research in which a hatching defect was manifested on zebrafish embryos after exposure to *Hystrix Brachyura* water extract at a concentration of 750 mg/L.** Hatching rate on zebrafish embryos administered with GeWE at 72 hpf is displayed in Table-2.

Heart Rate

Heart rate constitutes a parameter to evaluate the cardiotoxicity in zebrafish model assay. The assay was performed when the heartbeat of zebrafish embryos was in stable condition.** Heartbeats of zebrafish embryos were video-recorded and analyzed using DanioScope software. The video was then converted to MP4 using any video converter software to calculate the heartbeat per minute (BPM).

The Zebrafish embryo's heartbeat was measured at 72 hpf, as shown in Fig.-3. In this research, the heartbeat of zebrafish embryos which were treated with four different dosages of GsWE was evaluated at doses of 50, 100, 200, and normal control (0 mg/L). The BPM of the zebrafish embryo was not significantly different at a dose of 50 mg/L from a dose of 0 mg/L. Meanwhile, BPM was decreased significantly with increasing dosage between 100 and 200 mg/L after treatment with GsWE in comparison to the dose of 0 mg/L (p <
The heartbeat’s mean was between 89.0 and 127.0 beats per minute. The highest BPM was achieved by the normal embryos (0 mg/L) and dose of 50 mg/L, meanwhile, the lowest BPM was obtained at the highest dose (200 mg/L). This result was consistent with an earlier finding on zebrafish embryo exposure with Clinacanthus nutans n-hexane fraction. This research reported that the BPM of zebrafish embryos was reduced proportionally with increasing doses of plant extract.\(^{30}\)

**Body Length**

Morphological abnormality, such as body length, constitutes a parameter that is usually used to assess the defects in a toxicity test with zebrafish embryos. In this assay, body length was used to determine the influence on the growth of zebrafish embryos and observed at 96 hpf. Body length was measured using a microscope that equipped with DinoCapture software. All data then was calculated using Adobe Photoshop CC 2019, a package for Windows 2021. The zebrafish embryos’ body length after treatment with GsWE was reduced significantly at a dosage between 375 and 500 mg/L compared to the concentration at a range of 50-200 mg/L as well as the normal control and control of solvent (\(p < 0.05\)). The shortest measured body length was 2.13 mm after treatment with 500 mg/L, whereas the longest measured body length was 2.40 mm after being subjected to 50 mg/L plant extract. This result was identical to previous research on zebrafish embryos after the treatment with fluoxastrobin in which the body length of zebrafish embryos was reduced at a high dose.\(^{31}\)

<table>
<thead>
<tr>
<th>Extract Dosage (mg/L)</th>
<th>BL (mm)</th>
<th>Eye size (\times 10^4) (µm(^2))</th>
<th>HR (%)</th>
<th>Yolk size (\times 10^5) (µm(^2))</th>
<th>Teratogenicity parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>NA</td>
<td></td>
<td>0</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>500</td>
<td>2.13 ± 0.09(^a)</td>
<td>6.82 ± 0.10(^a)</td>
<td>100</td>
<td>5.79 ± 0.02(^a)</td>
<td>+</td>
</tr>
<tr>
<td>375</td>
<td>2.18 ± 0.07(^b)</td>
<td>7.49 ± 0.09(^b)</td>
<td>100</td>
<td>5.51 ± 0.01(^b)</td>
<td>+</td>
</tr>
<tr>
<td>200</td>
<td>2.36 ± 0.08(^c)</td>
<td>8.70 ± 0.09(^c)</td>
<td>100</td>
<td>5.44 ± 0.01(^c)</td>
<td>+</td>
</tr>
<tr>
<td>100</td>
<td>2.34 ± 0.07(^d)</td>
<td>9.55 ± 0.15(^d)</td>
<td>100</td>
<td>5.32 ± 0.01(^d)</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>2.40 ± 0.07(^b)</td>
<td>9.64 ± 0.06(^d)</td>
<td>100</td>
<td>5.29 ± 0.02(^e)</td>
<td>-</td>
</tr>
<tr>
<td>NC</td>
<td>2.71 ± 0.06(^c)</td>
<td>10.54 ± 0.05(^e)</td>
<td>100</td>
<td>5.02 ± 0.03(^f)</td>
<td>-</td>
</tr>
<tr>
<td>CS</td>
<td>2.70 ± 0.06(^c)</td>
<td>10.46 ± 0.08(^e)</td>
<td>100</td>
<td>5.03 ± 0.01(^f)</td>
<td>-</td>
</tr>
<tr>
<td>DC</td>
<td>NA</td>
<td>NA</td>
<td>10</td>
<td>NA</td>
<td>+</td>
</tr>
</tbody>
</table>

BL: body length; HR: hatching rate; LP: less pigmentation; PO: pericardial oedema; UH: unhatched; YO: yolk oedema; NC: normal control, CS: control of solvent, DC: drug control; NA: not assessed. The data were reported in average ± SD (n = 10); different letters: significantly different (\(p < 0.05\)); (-) not detected, (+) detected

**Eye Size**

The eyes of zebrafish embryos have similarities with human eyes, including dominance in terms of vision which mediated cones, absorption of lighting by comparable cellular functions like in humans, and presence of two neural layers, i.e., an interior and exterior pupillary membrane. Both zebrafish and human eyes contain retinal pigment epithelium.\(^{32}\) Therefore, the eye size of zebrafish embryos could also be used as an endpoint parameter in a toxicity study. Alteration of eye size that was detected in zebrafish embryos exposed to GsWE was performed at 96 hpf. The eye size of zebrafish embryos after treatment with GsWE was lowered significantly in comparison to normal embryos (\(p < 0.05\)). The eye size of the zebrafish embryo was decreased significantly in a dose-dependent manner about the normal control. This study showed that the treatment of GsWE resulted in a reduction in the eye size of zebrafish embryos. It is indicating that the seaweed extract had an impact on eye growth or ocular hypoplasia. This finding was by previous investigation on the toxicity of Hystrix brachyura Bezoar extracts in which the eye size of zebrafish embryos was reduced at all concentrations.\(^{28}\)

**Yolk Sac**

The Yolk sac can also be used as an indicator of organ dysfunction in zebrafish. The yolk sac serves as the main source of nutrition for the zebrafish embryo for the first week of growth. The Yolk sac of zebrafish contains 70% of neutral lipid which is metabolized primarily in the liver. Therefore, liver dysfunction leads to latency in yolk degradation and absorption. This caused increasing lipid storage.\(^{33}\) Embryo with a dosage
of 375 mg/L revealed yolk oedema at 24 hpf (Fig.-4C). The Yolk sac of a zebrafish embryo administered with GsWE extract at 24 hpf, is shown in Table-2. The red seaweed extract has a considerably enhanced effect in a dosage pattern ($p < 0.05$) on yolk size when compared to the normal and control of solvent. The largest yolk size was found within the maximum dosage (500 mg/L) amount of $5.79 \times 10^5$ ($\mu$m²). This result was similar to the previous finding in the cytotoxicity study of *Lignosus rhinoceros* aqueous extract in zebrafish embryos in which edema of the yolk was found at higher dosages (0.3 and 0.5 mg/mL).\(^{34}\)

**Teratogenicity**

Teratogenic deformities in *Danio rerio* embryos exposed to various dosages of GsWE are presented in Table-2. These deformities were collected between 24 and 96 hpf. The highest extract concentrations (750, 500, and 375 mg/L) had the highest incidence of teratogenic deformities in *Danio rerio*. Meanwhile, the lowest extract concentrations (100 and 50 mg/L) had no defects except for a lack of pigmentation (i.e., 200 mg/L) and no deficiencies in unhatched embryos (unless at 750 mg/L). *Danio rerio* embryos exposed to GsWE at a dosage of 750 mg/L exhibited the most coagulation at 24 hpf (Fig.-4B), compared to a normal embryo at 6 hpf (Fig.-4A). Embryo exposure to GsWE at a high concentration (500 mg/L) demonstrated pericardial oedema at 72 hpf (Fig.-4E), but the embryo could still hatch and was distinguishable from the drug control (Fig.-4D). Embryo exposure to GsWE at a dosage of 200 mg/L resulted in less pigmentation at 96 hpf (Fig.-4F). Similar to the present study, it has earlier been suggested that xenobiotics can trigger teratogenicity in marine species at high dosages.\(^{35}\) Polysaccharides i.e., D-pinitol, D-psicose, D(-)-fructose, D(+)talose, and d-Mannose are the main compounds that found in the GsWE. According to the literature,\(^{36}\) D-psicose is nontoxic to animals when administered at low concentrations that parallel the current toxicity test.

![Fig.-4: Morphological Deformations in *Danio rerio* Embryos effect of GsWE (A: Normal Embryo; B: 750 mg/L; C: 375 mg/L, D: Drug Control and E: 500 mg/L; F: 200 mg/L)](image)

**CONCLUSION**

Analysis of GsWE using GC-MS confirmed some phytoconstituents that included benzothiazole, ethyl $p$-methoxyccinnamate, 2-acetyl-3-methylbenzo[b]thiophene, acids of propanoic, lauric, myristic, and palmitic, octadec-9Z-enol, D-pinitol, D-psicose, D(-)-fructose, D(+)talose, and d-Mannose. In a dosage mode, GsWE significantly increased yolk size and decreased the size of the eye, length of the body, and heartbeat rate, in zebrafish embryos when compared to with control group. GsWE did not induce any malformation in zebrafish embryos at low concentrations, but at high concentrations caused defects, such as less pigmentation, pericardial oedema, and yolk oedema. The findings of this study will help to improve the safety of food supplements based on *G. spinosum*.

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CONFLICT OF INTERESTS

The authors declare that have no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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