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Biological activities and chemical composition of solvent extracts of *Stoechospermum marginatum* (C. Agardh)

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The objective of this study was to evaluate the chemical composition CH3OH-CH2Cl2 (1:1) extract and biological activities of various extracts derived from the aerial parts of the brown marine alga *Stoechospermum marginatum* (C. Agardh). Gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS) were used to analyze the composition of the essential oil. Total phenolics assay demonstrated a high value in hexane extract (HE), with a lower value for chloroform extract (CE), and the lowest value for methanol extract (ME). DPPH (2,2-diphenyl-1-picrylhydrazyl) assay showed that extracts of *S. marginatum* possess radical scavenging activity (RSA). Tests of the antioxidant property of the extracts revealed both electron and hydrogen transfer mechanisms. The antibacterial activity of the ME, CE, and HE as well as an ethanol extract was estimated against seven Gram-positive and Gram-negative bacteria. The ethanol extract showed the highest antibacterial activity, and the HE showed the lowest.

Key words: *Stoechospermum marginatum*, antioxidant activity, antibacterial activity, extract, brown seaweed

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INTRODUCTION

Marine organisms are a rich source of structurally new and biologically active metabolites. In recent years, there have been many reports of macroalgae-derived compounds that posses a broad range of biological functions, such as antibiotic, antiviral, antioxidant, antifouling, anti-inflammatory, cytotoxic, and antimitotic activities (Vairappan *et al.,* 2001; Smit, 2004; Thirumaran *et al.,* 2006). Bioactive components have been revealed in many marine algae (Yang *et al.*, 2006; Venkateswarlu *et al.*, 2007; Oh *et al.,* 2008). Among the algal substances that have displayed this kind of activity, amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, fatty acids, and acrylic acid are of note (Yamamoto *et al.,*1984; Ely *et al.* 2004; Taskin *et al.,* 2007). Marine algal compounds are predominantly found with diverse functional groups and hence have different properties. They have been reported to possess antioxidant activity and thus have been widely investigated. The role of algae in human health has been demonstrated by studies confirming their antioxidant and antimicrobial activities, and as a result algal products are in demand by consumers. Marine algae are also well known as rich sources of bioactive natural products (Iliopoulere *et al.*, 2002; Metzger *et al.*, 2002). Additionally, marine algae have been shown to be effective in prevention of cancer as well as cardiovascular and degenerative diseases (Lordan *et al.,* 2011).

Oxidation is a natural process occurring in organisms in the production of energy to fuel biological cycles. However, on uncontrolled production of oxygen-derived free radicals is involved in the onset of such diseases as arthritis, atherosclerosis, and cancer as well as in many aging-related degenerative diseases (Halliwell & Gutteridge, 1984; Shahidi *et al.,* 1992). In recent years, numerous drug resistance mechanisms in human pathogenic microorganisms have developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. Therefore, novel compounds encompassing both antioxidant and antimicrobial activities would be of great commercial value to the pharmaceutical industries today. An important aspect of investigation into the antioxidant and antibacterial capabilities of substances includes determination of their radical scavenging activity (RSA) (Sohrabipour & Rabii, 1990).

The objective of this study was primarily to analyze the chemical composition of CH_3OH-CH_2Cl_2 (1:1) extract of *S. marginatum,* a brown seaweed collected from the waters of *Gwadar Bay*, Chah Bahar, and Sistan and Balochistan Province, *Iran*, using GC and GC-MS to determine its chemotypes. The secondary objective was to investigate the antibacterial and antioxidant activities of methanol, chloroform, hexane, and ethanol extracts of this promising alga.

MATERIALS AND METHODS

Plant material. *S. marginatum* (C. Agardh) was collected from the waters of Gwadar Bay, Chah Bahar, and Sistan and Balochistan Province, Iran, at a depth of 0.51 m in November, 2011. Voucher specimens were deposited at the Research Institute of Forests and Rangelands Herbarium, Tehran, Iran (Esmaeili & Amiri, 2011). The harvested seaweed was rinsed repeatedly in tap water to clean sand, dirt, and other particles from it. Using a procedure reported in similar studies the washed alga was then dried in the sun for 5 days (Gupta *et al.,* 2001). The resulting dry biomass was milled to a particle size averaging 2–5 mm. The algal powder was divided into four 90-g portions and extracted with, respectivity, CH_3OH-CH_2Cl_2 (1:1), methanol, chloroform, ethanol, or hexane for 24 h. After 24 h, each solvent extract was filtered

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Abbreviations: CE, chloroform extract; GC, Gas chromatography; GC-MS, gas chromatography-mass spectroscopy; HE, hexane extract; ME, methanol extract; RIs, retention indices; RSA, radical scavenging activity.

through Whatman filter paper (No. 1) and then concentrated in a vacuum for 3 h at 40°C using a rotary evaporator (Buchi, Flawil, Switzerland) to obtain a semisolid extract, which was then weighed.

Gas chromatography analysis. GC analysis was carried out using a Shimadzu 15A gas chromatograph equipped with a split/splitless injector (250°C) and a flame ionization detector (250°C). The carrier gas used was nitrogen (1 ml/min), and DB-5 (50 m × 0.2 mm, 0.32 μm film thickness) was used for as capillary column. The column temperature was kept at 60°C for 3 min and then heated to 220°C at a rate of 5°C/min and kept content at 220°C for 5 min. The relative percentage of all compounds was calculated from peak areas using a Shimadzu CR4A Chromatopac without the use of correction factors.

Gas chromatography-mass spectroscopy analysis. GC-MS analysis was accomplished using a Hewlett Packard 5973 with an HP-5MS column (30 m × 0.25 mm, film thickness 0.25 μm). The column temperature was as above. The flow rate of helium as a carrier gas was 1 ml/min. Mass spectroscopy readings were taken at 70 eV. The MSD was operated under 70 eV, with a scan range of 41–300 amu. The MSD was tuned every day using perfluorotributylamine as a tuning standard.

Qualitative and quantitative analyses. GC identification of most constituents was made by comparing retention indices (RIs) with those found in the literature or with RIs of authentic compound samples in our facilities. Determination of the RIs was made in relation to a homologous series of *n*-alkanes (C_8-C_{28}) under identical conditions of operation. Comparison of the mass spectra of constituents on both columns with mass spectra stored in the NIST 02 and Wiley 275 libraries or with mass spectra recorded in the literature or in our own library served as an additional identification. Relative concentrations of the components were calculated based on GC peak areas with no correction factors invoked (Mass Spectrometry Data Centre, 1991; Joulain & König, 1998; Adams, 2007).

Antimicrobial assay

Microorganisms. Pure cultures of *Staphylococcus aureus* (PTCC1113), *Staphylococcus epidermidis* (PTCC1349), *Bacillus anthracis* (PTCC1036), *Escherichia coli* (PTCC1330), and *Pseudomonas aeruginosa* (PTCC1310) were used as test organisms and the bioassay was done using an agar plate diffusion test.

Preparation of inocula. After incubating the nutrient agar slant of each test organism for 24 h, a loop full of the microorganism was inoculated in a nutrient broth with pH 7.4 so as to activate the bacterial strains. The broths were incubated at 37°C for 24 h to allow the microorganisms to grow. A separate uninoculated nutrient broth was maintained as a control.

Antibacterial activity test. Antibacterial activity was assayed using the agar well diffusion test technique. Müller Hinton agar (MHA) was prepared at pH 7.4 and then sterilized by autoclaving at 121°C for 15 min, after which 20 ml of the sterilized medium was poured into a sterilized Petri dish and allowed to solidify at room temperature. A sterile cotton swab was used for spreading each test microorganism from the inoculated broth evenly onto an MHA plate. The plates were allowed to sit for a few minutes to allow complete absorption of the inocula. In each plate a 5-mm diameter well was made at the center using an appropriately sized sterilized cork borer. One hundred microliter of each extract (HE, ME, and CE) was added to the wells on the MHA plates and allowed to diffuse at room temperature for 30 min. No extract was added to the control MHA plate, which was used for comparing the obtained results to detect any contamination. The plates were incubated at 37°C for 24 h. After incubation a clear zone was observed around the well, which was evidence of the presence of antibacterially active compounds in the extracts. The diameter of the zone of inhibition was measured in millimeters.

For comparing the antibacterial activity of the isolated extracts with the therapeutic action of a number of known, broad spectrum antibiotics. The zone exhibited by extracts was compared to the inhibition zone produced by the standard antibiotics (Seenivasan et al., 2010; Esmaeili & Amiri, 2011; Esmaeili & Khodadadi, 2012).

Antioxidant activity

Chemicals. Butylated hydroxytoluene (BHT) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (Sigma, Aldrich GmbH, Sternheim, Germany). Folin-Ciocalteau's phenol reagent, sodium bicarbonate, ethanol, chloroform, methanol, and the other chemicals and reagents employed in the study were purchased from Merck (Darmstadt, Germany). All other unlabeled chemicals and reagents used were of analytical grade.

DPPH assay. Kinetic data suggest that an electron transfer mechanism rather than the more commonly assumed hydrogen atom transfer reaction may be active in the DPPH assay. A published DPPH RSA assay method with minor modifications was employed to evaluate the RSA of the ME, HE, and CE of *S. marginatum*. In this assay, 10 mg/ml stock solutions were prepared by dissolving the extracts and the synthetic antioxidant BHT in methanol. The solutions were diluted to concentrations ranging from 1 until 5×10^{-6} mg/ml; 2 ml of each diluted solution was then mixed with 2 ml of 80 mg/ml DPPH methanol, chloroform, or hexane solution and left in the dark at room temperature for 30 min. Then absorbance of each solution was recorded on a spectrometer at 517 nm using a blank containing the same concentration of BHT or extract without DPPH. Percentage of inhibition of free radicals by DPPH was calculated as follows:

$$I\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100 \quad (1)$$

where $I\%$ is the inhibition percentage, A_{blank} is the absorbance of the control reaction, and A_{sample} is the absorbance of the test compound. Inhibition percentages were plotted against the sample concentrations to calculate the concentration providing 50% inhibition (IC_{50}). Each test was performed 3 times; the IC_{50} value was represented as means ± standard deviation.

Determination of total phenolic compounds. Total phenolic constituents in the methanol extract of *S. marginatum* were determined using a published method employing Folin-Ciocalteau's reagent, with gallic acid as the standard (Esmaeili & Khodadadi, 2012). Extract solutions (ME, HE, and CE) containing 0.1 ml of each extract were placed in volumetric flasks; 46 ml of distilled water and 1 ml Folin-Ciocalteu's were added, and the flasks were shaken thoroughly. After 3 min, 3 ml of a solution of 7% Na_2CO_3 was added to each flask, and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 765 nm. The same procedure was repeated for all standard gallic acid

solutions (0–1000 mg/0.1 ml), and a standard curve was obtained using the following equation:

$$\text{absorbance} = 0.0012 \times \text{gallic acid (mg)} + 0.0033 \quad (2)$$

Scavenging of hydrogen peroxide. The ability of the extracts to scavenge hydrogen peroxide was determined using the method described in a recently published paper (Esmaeili *et al.,* 2009). A solution of hydrogen peroxide (40 mM) was prepared in a phosphate buffer solution (pH 6.6). CE, HE, and ME extracts in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes and compared against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds were calculated as follows:

$$\%\ \text{scavenged}\ H_2O_2 = \frac{A_0 - A_1}{A_0} \times 100 \quad (3)$$

where A_o is the absorbance of the control and A_1 is the absorbance in the presence of the extract or standard (Esmaeili *et al.,* 2009).

RESULTS

Chemical components identified

The chemical composition of the CH_3OH-CH_2Cl_2 (1:1) extract of *S. marginatum* is given in Table 1. Twelve of the compounds identified represented 98.0% of the total composition of the oil. The major compounds identified were 4-epi-abietol (42.1%) and palustrol (14.5%). Another notable constituent was octane (13.2%). Diterpenes comprised the most abundant components of the oil (50.2%). Two sesquiterpene hydrocarbons (16.6%) and hydrocarbon aliphatic compounds (including acidic compounds and nonacidic compounds) (24.4%) were also found.

Amount of DPPH

The antioxidant activity of the CE, HE, and ME of *S. marginatum* was investigated using DPPH assay. The antioxidant activity of the extract compounds was measured in terms of hydrogen donating or RSA, using the stable radical DPPH. ME, CE, and HE were able to reduce DPPH to yellow-colored diphenylpicrylhydrazine with IC_{50} values of 0.064±0.0012 mg/ml for ME, 0.15±0.002 mg/ml for HE, and 0.21±0.06 mg/ml for CE. The concentration of the positive control BHT required to scavenge 50% of the free radical (IC_{50}) was 0.13±0.06 mg/ml.

Table 1. Chemical components of the essential oil of *Stoechospermum marginatum*

No.	Compound	R.I	%
1	Octane	800	13.2
2	Nonanone	1091	1.0
3	Hexadecanoic acide	1970	1.8
4	Sclarene	1967	1.2
5	Accidol acetate	1970	2.1
6	Manoyl oxide	1989	5.1
7	Manool	2056	1.8
8	Ethyl octadecanoate	2194	1.5
9	Docosene	2195	6.9
10	2-Keto-Manoyl oxide	2208	6.8
11	Palustrol	2314	14.5
12	4-epi-abietol	2341	42.1
Total			98.0

Total phenol content

The total phenolic content of the *S. marginatum* extracts was measured using Folin-Ciocalteau's reagent. The results were expressed as gallic acid. Our investigation found that the values of *S. marginatum* extracts (ME, HE, and CE) were 0.041±0.001 mg/ml, 0.018±0.005 mg/ml, and 0.02±0.003 mg/ml, respectively. The IC_{50} value for gallic acid was 0.13±0.065 mg/ml. The highest total phenolic content was found in the ME and the lowest in the HE.

Hydrogen peroxide scavenging

Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 from food systems is important (Ebrahimzadeh *et al*., 2009). Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water (Esmaeili & Khodadadi, 2012). The ability of the extracts to effectively scavenge hydrogen peroxide was determined according to the method used by Ruch, in which the extracts are compared with quercetin as the standard (Ebrahimzadeh *et al*., 2009). The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner and demonstrated moderately good scavenging activity. Our investigation found that the IC_{50} values for the *S. marginatum* extracts (ME, HE, and CE) were 4.21±0.14 mg/ml, 0.27±0.0.01 mg/ml, and 0.29±0.11, respectively. The IC_{50} value for ascorbic acid was 4.45±0.05 mg/ml. The effect was significant in some tests but further investigation of individual compounds and their antioxidant activities *in vivo* and in different antioxidant mechanisms is needed.

Antibacterial activity

The antibacterial tests were carried out at the Department of Biological Science, North Tehran Branch, Islamic Azad University, Tehran, Iran, using the following microorganisms: S*taphylococcus aureus* PTCC 1113 and S*taphylococcus epidermidis* PTCC 1349, (Gram-positive bacteria), and *Bacillus anthracis* PTCC 1036, E*scherichia coli* PTCC 1330, and *Pseudomonas aeruginosa* PTCC 1310 (Gram-negative bacteria), identified by the Iranian Research Organization for Science & Technology (Table 2).

In the study, we investigate ethanol extract only in microorganisms test. The microorganisms were obtained from enrichment culture in 1 ml of Müller-Hinton agar medium. Figure 1 shows the results of the investigation of the antibacterial action of the ethanol extract, ME, HE, and CE from the aerial parts of *S. marginatum* using the agar well diffusion test technique, with comparative growth inhibitory zones.

DISCUSSION

In this study, 12 compounds were identified in the CH_3OH-CH_2Cl_2 extract of *S. marginatum,* with diterpenes comprising over 50% of the compounds found. In

Table 2. Antibacterial activity of different extracts of *Stoechospermum marginatum*[a]

Bacterial Species	Gram +/–	ME[b]	HE[c]	CE[d]	Ethanol extract	Ciprofloxacin	Erythromycin
Staphylococcus aureus (PTCC 1113)	+	–	–	15	20		28
Staphylococcus epidermidis (PTCC 1349)	+	10	18	17	15		25
Bacillus anthracis (PTCC 1036)	+	15	–	–	13		20
Escherichia coli (PTCC 1330)	–	–	–	17	12	34	
Pseudomonas aeruginosa (PTCC 1310)	–	14	10	17	19	35	

[a]Agar disc diffusion method: Diameter of inhibition zone including disk diameter of 100 mm; [b]ME = methanol extract; [c]HE = hexane extract; [d]CE = chloroform extract

on other study, Iranian researchers isolated two spatane diterpenes, 5(*R*),16(*S*)-diacetoxyspata-13,17-diene and 5(*R*),16(*S*)-dihydroxyspata-13,17-diene from *S. marginatum* (Rosa *et al.,* 1991). A comparison of the analysis of the extracts of *S. marginatum* in that study with our result does shows many similar compounds. In previous studies, the essential oils of various brown algae have been investigated, isolating 8 compounds from *Dictyota dichotoma*, 12 compounds from *D. dichotoma*, 4 from *Petalonia fascia*, 4 from *Scytosiphon lomentaria*, and 14 compounds for *Colpomenia sinuosa*, accounting for 58.41%, 83.53%, 91.71%, 87.89%, and 74.17% of the total composition of the essential oils, respectively (Ozdemir *et al.*, 2006). The aliphatic hydrocarbons heptadecane and hexadecane have also been reported as common major volatile components in seaweeds (Stirk *et al.,* 2007).

ME, HE, and CE reduced the stable violet DPPH radical to the yellow diphenylpicrylhydrazine. The concentrations of ME, HE, and CE needed to scavenge 50% of the free radicals were 0.064±0.0012 mg/ml, 0.15±0.002 mg/ml, and 0.21±0.06 mg/ml, respectively. Phenolic compounds may affect the growth and metabolism of bacteria. They could have an activating or inhibiting effect on microbial growth according to their constitution and concentration (Ebrahimzadeh *et al.*, 2008; Esmaeili *et al.*, in press). Folin-Ciocalteau's reagent was employed to assay total phenolic compounds present in the extracts. The highest total phenolic content was found in the HE (0.018±0.005 mg/ml) and the lowest value in the ME (0.041±0.01 mg/ml). The phenolic content was high in the polar extracts. The importance of phenolic compounds as scavengers of free radicals is emphasized in a number of studies (Madsen *et al.,* 2000). It should be noted that the volatile components still present in the nonpolar ME, CE, and HE could also be responsible for the antioxidant activity.

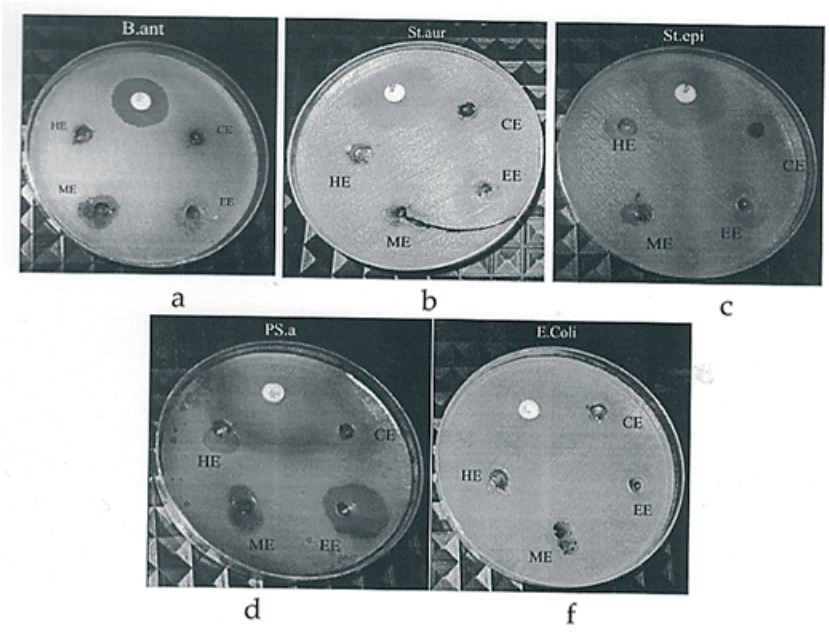

Figure 1. Diffusion disk antibacterial test of EE, ME, CE, and HE extracts grown in *S. marginatum* against five tested strains of bacteria
(a) *Bacillus anthracis*; **(b)** *Staphylococcus aureus*; **(c)** *Staphylococcus epidermidis*; **(d)** *Pseudomonas aeruginosa*; **(f)** *Escherichia coli*

The scavenging of H_2O_2 by the extracts may be attributed to their phenolics, which can donate electrons to H_2O_2 and neutralize it to H_2O. The ability of the extracts to effectively scavenge hydrogen peroxide was determined according to the Ruch's method (Ebrahimzadeh *et al.,* 2009), with quercetin employed as the standard. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. The HE extract showed moderately good scavenging activity, with an IC_{50} value of 0.27±0.0.01 mg/ml, while the IC_{50} value for ascorbic acid was 4.45±0.05 mg/ml.

When we investigated the antibacterial activity of the ethanol extract, ME, CE, and HE, we found that the CE and EE were the most effective in inhibiting bacterial activity. In an agar plate diffusion test, the ethanol extract, ME, CE, and HE of *S. marginatum* showed the highest antibacterial activity of *S. marginatum* (15,

10, 17, and 18 mm, respectively) and *P. aeruginosa* (19, 14, 17, and 10 mm, respectively). The EE of *S. marginatum* strongly inhibited the growth of *S. aureus* and *P. aeruginosa*. The CE showed the highest antibacterial activity against *E. coli* and was second to the EE in effectiveness against *P. aeruginosa*. The HE and CE showed the highest activity against *S. epidermidis*. Among all the test microorganisms, the lowest results were exhibited on *B. anthracis,* with the ME being the most effective (Table 2). These results indicate that the extracts contained different antibacterial components and may reflect the variety of secondary metabolites in the extracts (Ozdemir *et al*., 2004). Antimicrobial activities of essential oils could be attributed to camphor and it derivatives such as borneol. The synergistic effects of these chemicals with each other and the minor constituents of the essential oil should be taken into consideration. Although the mechanism of the antibacterial activity of the terpenoids making up essential oils is not fully understood, it is thought to involve membrane disruption by the lipophilic compounds (Cowan *et al*., 1999).

Inhibited formation of hydroperoxide is related to the presence of phenolic nuclei in essential oils and their extracts (Farag *et al.,* 1989). Phenolic compounds account for the antioxidative effectiveness of natural sources. Among the extracts, the ME exhibited the strongest and broadest antioxidant activity.

CONCLUSION

In our work we used GC and GC-MS to study the chemical composition of the CH_3OH-CH_2Cl_2 (1:1) extract derived from the aerial parts of *S. marginatum* (C. Agardh). We identified 12 constituents, of which 4-epi-abietol (42.1%) and palustrol (14.5%) were the major components. The constituents identified represented 98.0% of the total oil. Diterpenes comprised more than half of the oil's components (50.2%). Antibacterial effectiveness of various extracts of *S. marginatum* was also investigated. Antioxidant activity was investigated by assaying total phenolic content, scavenging activity of H_2O_2, and DPPH. In these tests the extracts of *S. marginatum* demonstrated antioxidant activity and potential usefulness in this arena. The antibacterial activity of the ME, CE, EE, and HE against Gram-positive and Gram-negative bacteria was also investigated. The strongest antibacterial activity was observed in the EE and CE of *S. marginatum*.

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