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Comparative Study: Extraction Conditions and Antioxidant and Antibacterial activities of *Gracilaria fisheri*

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Abstract

Gracilaria fisheri (G. fisheri), sourced from the southern region of Thailand and recognized for its sulfated polysaccharides with diverse health benefits, underwent extraction under varying conditions by adjusting the ratio of water to ethanol and the duration of maceration. Six extraction conditions were systematically investigated, and their yields of extraction, sulfate content, antioxidant activity, and antimicrobial activity were compared. Among the conditions examined, the extraction utilizing solely water for a duration of 2 hrs yielded the highest quantity (7.20±0.99 g) and highest sulfate content (215.86±2.67 µg/10 mg of crude extract). Furthermore, this extraction exhibited superior antioxidant activity as evidenced by the lowest IC_{50} values in DPPH (4.75±0.05 µg/mL) and ABTS (31.75±0.22 µg/mL) assays. Additionally, the extracts (10 mg/mL) manifested significant efficacy in inhibiting the growth of both *Staphylococcus aureus* (16.28±4.75 mm) and *Staphylococcus epidermidis* (15.33±3.27 mm) in comparison with the standard antibiotic, ciprofloxacin (5 µg), which exhibited inhibition zone diameters of 20.47±0.70 mm (*Staphylococcus aureus*) and 30.91±0.80 mm (*Staphylococcus epidermidis*). In conclusion, the most effective method for extracting *G. fisheri* is to solely utilize water for a duration of 2 hrs.

Keywords**:** *Sulfated polysaccharides; Gracilaria fisheri; antioxidant; antibacterial*

1. Introduction

G. fisheri is a red algae commonly found along the coast of the southern part of Thailand and Southeast Asia. Red algae are recognized for their ability to produce sulfated polysaccharides (SPs). SPs consist of repeating units of agarobiose, which alternate between 3-linked-β-D-galactopyranose (Gal) and 4-linked-3,6-anhydro-α-L-galactopyranose (Rudtanatip et al., 2022). SPs can be extracted from various types of seaweeds, such as green, brown, and red algae, each exhibiting distinct chemical structures due to the natural diversity of these seaweed species (Costa et al., 2010; Imjongjairak et al., 2015; Nagahawatta et al., 2023).

SPs derived from red algae demonstrate potent antioxidant properties, emphasizing their potential in combating and mitigating oxidative damage caused by reactive oxygen species in living organisms (Wang et al., 2009). The antioxidant efficacy of SPs depends on factors such as sulfate content, the distribution of sulfates along the polysaccharide chain, sugar composition, stereochemistry, and the presence of phenolic components within the polysaccharide structure (Devi et al., 2008; Ghosh et al., 2004). Additionally, the antioxidant properties are found to

be closely associated with the sulfate content (Qi et al., 2005; Imjongjairak et al., 2015). This emphasizes the significance of sulfate content in influencing the efficacy of SPs as antioxidants, providing valuable insights into their potential applications in mitigating oxidative stress-related conditions.

Additionally, SPs exhibit inhibitory properties against various types of bacteria (Jun et al., 2018). Therefore, the exploration and extraction of SPs as an alternative and novel approach for treating diseases caused by bacterial infections is of particular interest.

SPs can be extracted using various methods, including chemical processes, physical techniques, or enzymatic treatments. Traditional solvent extraction methods, such as methanol and acetone, have been widely used. However, these methods have limitations, including low separation efficiency and the use of large quantities of organic solvents, which can pose environmental risks. Moreover, certain solvents, particularly methanol, are restricted for use in the food industry. To address these challenges, a more environmentally friendly approach for extracting SPs from seaweed is water-based extraction (Chattopadhyay et al., 2008). However, it is crucial to consider that factors such as temperature during the extraction process have been reported to influence the sulfate content and the effectiveness of the polysaccharides in combating viruses and bacteria (Chattopadhyay et al., 2008). The investigation into solvent and duration is also a critical step in the extraction of bioactive compounds, as it profoundly impacts the efficiency and quality of the extraction process (Wassie et al., 2021).

The objective of this study, therefore, is to extract *G. fisheri* by various solvents and extraction durations. Furthermore, the study aims to investigate their antioxidant properties through DPPH and ABTS assays and their antibacterial characteristics. The main goal of this research is to explore the relationship between the extraction methods and chemical properties, such as phenolic compounds, sulfate content, bioactivity attributes, to provide valuable data for future research and development.

2. Objectives

The primary goals of this study are to explore appropriate solvents and extraction parameters for obtaining the extract from dried *G. fisheri* that exhibits the best antioxidant and antibacterial properties.

3. Materials and methods

3.1 Sample preparation

The *G. fisheri* was obtained from Koh Yor Subdistrict, located in the Mueang Songkhla District, Songkhla Province, Thailand. The *G. fisheri* sample preparation method was adapted from previous research (Mahae et al., 2010). The process involved taking 100 g of dried *G. fisheri*, soaking it in water for 3 hrs and filtering it through filter cloth. For the acid method an additional 300 mL of glacial acetic acid was introduced, and the sample was soaked for 30 minutes before filtering the mixture through filter cloth.

3.2 Solvent extraction method

G. fisheri samples were extracted using various solvents: 20% v/v ethanol, 80% v/v ethanol, and water. For the extraction process, 500 mL of the respective solvent was added to the sample from 3.1. Samples were extracted at 50ºC for 1 and 2 hrs. The supernatant was separated using filter paper No1. Afterward, the supernatant was collected and evaporated until dryness using a rotary evaporator. The obtained extract will be utilized in all further experiments. The yield percentage was calculated using the following equation (Kuda, & Ikemori, 2009).

$$\text{Percent yield} = \left[\frac{\text{dry weight of extract (g)}}{\text{dry weight of } \textit{G. fisheri} \text{ material (g)}}\right] \text{x} 100$$

3.3 Sulfate turbidity assay

The determination of sulfate content in the samples was conducted employing the turbidity assay method as outlined by Dodgson, & Price (1962), with appropriate modifications. Initially, diverse concentrations of *G. fisheri* extracts were prepared alongside a series of standard potassium sulfate (K_2SO_4) solutions spanning from 20 to 500 µg/mL. These solutions were dissolved in 5 mL of 4 M hydrochloric acid and subjected to boiling for 2 hrs. Following this, 15 mL of distilled water and 5 mL of sulfate turbidity conditioning reagent were introduced. The resultant solution was thoroughly mixed prior to the addition of 5 mL of 6% v/v barium chloride ($BaCl_2$), followed by a 10-minute mixing period. Turbidity measurements for both the sample and standard substances were performed at a wavelength of 420 nm utilizing a UV-Visible spectrophotometer (Thermo Fisher, G10s UV-Vis spectrophotometer, China). Subsequently, the sulfate concentration in the sample was determined using the linear equation derived from the standard curve of the potassium sulfate solution.

3.4 Total phenolic content

The total phenolic content was determined using the modified Folin-Ciocalteu method (Wojdyło et al., 2007). Various concentrations of standard gallic acid in methanol solutions were prepared at concentrations of 20, 40, 60, 80, and 100 µg/mL. For the assay, 2 mL of standard gallic acid solution and samples were mixed with 2 mL of Folin-Ciocalteu solution. After 10 minutes, 1 mL of 20% sodium carbonate (Na_2CO_3) was added and incubated in darkness for 30 minutes. The absorbance was measured at a wavelength of 765 nm using a UV-Vis spectrophotometer. The total phenolic content in the extract was calculated using a gallic acid curve and expressed as milligrams of gallic acid equivalent per gram of crude extract.

3.5 Antioxidant activity

3.5.1 DPPH assay

The antioxidant activity of the samples was determined using the modified 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (Baluchamy, & Subramanian, 2023; Adli et al., 2024). Various concentrations of standard ascorbic acid and the sample in methanol were prepared. Subsequently, 1.6 mL of the ascorbic acid or sample solution was mixed with 2.4 mL of DPPH solution and incubated in darkness for 30 minutes. The absorbance was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer. The % Inhibition of DPPH radicals was calculated using the formula:

$$\% \text{ Inhibition } \frac{(Abs_{control}\text{-}Abs_{sample})x100}{Abs_{control}}$$

The % radical scavenging activity values were utilized to generate a graph for the determination of the IC_{50} value.

3.5.2 ABTS activity

The evaluation of antioxidant activity was conducted using 2,2-azobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging method, (Dudonne et al., 2009). A 7 mM ABTS solution was prepared and mixed with 2.45 mM potassium persulfate (KSP) in a 1:0.5 ratio. The mixture was incubated at room temperature for 12 hrs and then it was diluted to an absorbance of 0.700±0.02 at a wavelength of 734 nm.

Standard ascorbic acid and sample solutions were dissolved in ethanol to desired concentrations. Subsequently, 20 µL of the standard or sample solution were mixed with 2 µL of the $ABTS^{\bullet+}$ solution. The mixture was incubated at room temperature in the dark for 6 minutes. The absorbance was measured at a wavelength of 734 nm using a UV-Vis spectrophotometer, The % inhibition of ABTS radicals was calculated using the formula:

$$\% \text{ Inhibition } \frac{(Abs_{control}\text{-}Abs_{sample})x100}{Abs_{control}}$$

The % radical scavenging activity values were used to generate a graph for the determination of the IC_{50} value.

3.6 Antibacterial activity

In this assay, the antibacterial activity was evaluated using the agar well diffusion method. Two strains of gram-positive bacteria, namely *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228), as well as two strains of gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), were employed.

The bacterial strains were cultured in tryptic soy broth (TSB) liquid medium and incubated at 30ºC for 24 hrs. After incubation, the turbidity of all four bacterial strains was adjusted to match the turbidity of a 0.5 McFarland standard solution.

The bacterial cultures were then evenly spread onto tryptic soy agar (TSA) culture plates. Wells were created in the agar using a sterile cork borer. Subsequently, 10 µL of crude extract, diluted with water to a concentration of 10 mg/mL, or 1 of ciprofloxacin disc (5 µg) as a positive control were placed into the wells. The plates were incubated at 37ºC for 24 hrs, and the results were recorded by measuring the diameter of the clear zones where bacterial growth was inhibited (Baluchamy, & Subramanian, 2023).

4. Results and discussion

4.1 Extraction

Two extraction methods for *G. fisheri* were employed in this study: the color removal method involving acetic acid (acid method) and the non-color removal method without acetic acid. Since the effect of solvent was identified as a crucial factor for effective extraction (Kuda, & Ikemori, 2009), different solvents (water and ethanol) were employed for extraction. Additionally, two different durations of extraction (1 and 2 hrs) were performed to assess the impact on the extraction efficacy. The extraction process was controlled at a temperature of 50ºC, as this temperature is conductive to efficient extraction

without causing thermal degradation of the polysaccharide (Imjongjairak et al., 2015). The *G. fisheri* -to-solvent ratio of 1:5 w/v was employed to maintain consistency in the experimental setup (Khongthong et al., 2021).

The extracts obtained from the acetic acid extraction (acid method) were found to have an unpleasant odor and limitations due to its corrosive nature, rendering it unsuitable for further research or practical application. Consequently, the acid method was not further performed. In contrast, the extraction method without acetic acid, utilizing water as the solvent, can overcome the problems associated with the acid method. It was found that a 2 hrs water extraction method resulted in the highest yield of crude extract (7.20±0.99% of the dry weight), as shown in Table 1. The aqueous extraction method yields a higher quantity of extract compared to ethanol extraction. This is due to the fact that the free sugars present in *G. fisheri* can dissolve more effectively in water than in ethanol. The extract obtained using 20% ethanol demonstrated a higher yield compared to that obtained using 80% ethanol. This phenomenon is attributed to 20% ethanol superior capacity to dissolve the free sugars inherent in *G. fisheri*. This observation aligns with the findings of the previous experiments (Mahae et al., 2010).

4.2 Sulfate content

In the analysis of sulfate quantities in the extracts using the sulfate turbidity assay, the extract obtained by water extraction over a 2-hour period exhibited the highest sulfate content of 215.86±2.67 µg/10 mg, followed by 20% (2 and 1 hr.) and 80% ethanol (2 and 1 hrs) extraction, respectively, as presented in Table 1. This suggests that enhancing the polarity of solvent and duration increases sulfate content. Water and ethanol are solvents with distinct properties regarding the dissolution of sulfate content in extraction. Water exhibits a higher capability to dissolve sulfate content, since the nature of sulfate negative ions favors interaction with water molecules. On the contrary, when ethanol is used as the solvent, the ability to dissolve sulfate diminishes. The hydrophobic nature of ethanol reduces solubility (Imjongjairak et al., 2015). In this study, it was found that there is a correlation between sulfate content and the percentage yield of extracts.

Table 1 Percentage yields and sulfate content of extracts under different condition of extraction (n=3)

Solvent	Extraction time (hrs)	% yield	Sulfate content (µg/10 mg)
80% ethanol	1	2.32 ± 0.10^{a}	155.86 ± 0.34^{a}
80% ethanol	2	$2.97\pm0.14^{a,b}$	167.43 ± 4.17^{b}
20% ethanol	1	3.67 ± 0.59^{c}	181.94 ± 2.56^{c}
20% ethanol	2	$4.21\pm0.59^{a,c}$	196.06 ± 1.02^{d}
H_2O	1	5.19 ± 0.51^{b}	203.31 ± 7.83^{e}
H_2O	2	7.20 ± 0.99^{d}	215.86 ± 2.67^{f}

The different lowercase letter (with in a column) indicates significant difference in data expressed as mean ± SD at $p<0.05$, according to Tukey test

Table 2 Total phenolic content and antioxidant activities from the DPPH and ABTS assays of various extracts and standard ascorbic acid (n=3)

Compound	Extraction time	Total phenolic content (mg GA/g crude extract)	Results of antioxidant activity	
			DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
80% ethanol	1	1.57 ± 0.03^{a}	16.09 ± 0.05^{a}	61.32 ± 0.82^{a}
80% ethanol	2	1.82 ± 0.05^{b}	15.56 ± 0.32^{b}	59.24 ± 0.34^{a}
20% ethanol	1	1.85 ± 0.04^{b}	15.52 ± 0.15^{b}	56.43 ± 3.07^{b}
20% ethanol	2	2.00 ± 0.05^{c}	14.96 ± 0.10^{c}	54.78 ± 2.55^{b}
Water extract	1	2.38 ± 0.14^{d}	8.23 ± 0.20^{d}	46.05 ± 0.69^{c}
Water extract	2	2.55 ± 0.08^{e}	8.06 ± 0.10^{e}	44.49 ± 0.07^{c}
Ascorbic Acid	-	NA	4.75 ± 0.05^{f}	31.75 ± 0.22^{d}

The same lowercase letter (with in a column) indicates no significant difference in data expressed as mean ± SD at $p<0.05$, according to Tukey test

4.3 Total phenolic content analysis

The total phenolic content of the extracts obtained through six different extraction conditions was determined. The results were calculated based on the linear regression equation of the standard graph of gallic acid as follows: $y = 0.06x + 0.1109$. Statistical analysis showed significant differences ($p<0.05$) in the total phenolic content among six extracts, as presented in Table 2. Among these extracts, the extract obtained from water extraction for 2 hrs exhibited the highest total phenolic content (2.55±0.08 mg GA/g crude extract). The second-highest total phenolic content was observed from extract obtained from water extraction for 1 hrs (2.38±0.14 mg GA/g crude extract) followed by 20% (2 and 1 hrs) and 80% ethanol (2 and 1 hrs) extracts, respectively. The significant variations observed in the total phenolic content among these extracts highlight the critical role played by the choice of solvent.

4.4 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant properties of extracts were assessed using the DPPH radical scavenging activity test. DPPH is a synthetic and stable purple-colored radical that absorbs light at 517 nm. When DPPH reacts with antioxidants, it gradually loses its purple color. Testing the six different extracts revealed significant variations ($p<0.05$) in their antioxidant properties. The extract obtained from water extraction for 2 hrs exhibited the highest antioxidant activity, with an IC_{50} value of 8.06±0.10 µg/mL. The second-highest antioxidant activity was observed in the extract obtained from water extraction for 1 hrs, with an IC_{50} value of 8.23±0.20 µg/mL) followed by 20% (2 and 1 hrs) and 80% ethanol (2 and 1 hrs) extracts, respectively.

4.5 2,2-azobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity

The evaluation of the antioxidant properties of extracts was conducted using the ABTS radical scavenging activity test. ABTS is a synthetic compound with a green-blue color that can absorb light at a wavelength of 734 nm. When tested with the six different extracts, it was observed that the efficacy in scavenging radicals with the ABTS method correlated with the results of the DPPH antioxidant activity test. The extract obtained from water extraction for 2 hrs exhibited the highest antioxidant activity, with an IC_{50} value of 44.49±0.07 µg/mL. The second-highest antioxidant activity was observed in the extract obtained from water extraction for 1 hrs, with an IC_{50} value of 46.05±0.69 µg/mL, followed by 20% (2 and 1 hrs) and 80% ethanol (2 and 1 hrs) extracts, respectively.

The extract obtained from water over a 2 hrs extraction period exhibits the highest yield, sulfate content, and antioxidant activities from all assays, followed by the extract obtained from water for 1 hrs, 20% ethanol extraction for 2 and 1 hrs, and 80% ethanol for 2 and 1 hrs, respectively. These findings align with previous research indicating a correlation between the concentration of SPs and their antioxidant efficacy (Costa et al., 2010; Nagahawatta et al., 2023). Due to the ability of sulfate groups to activate the hydrogen atoms of carbon, resulting in hydrogen atom donation (Wang et al., 2009). However, other components such as the molecular weight of sulfated polysaccharides, pigments, and proteins found in *G. fisheri* may also affect antioxidant activity (Devi et al., 2008). Additionally, the sulfate groups play a significant role in shaping the chemical architecture of SPs with sulfates, promoting structural stability and heightened resilience against degradation, thereby preserving the antioxidant properties of these compounds (Qiu et al., 2022).

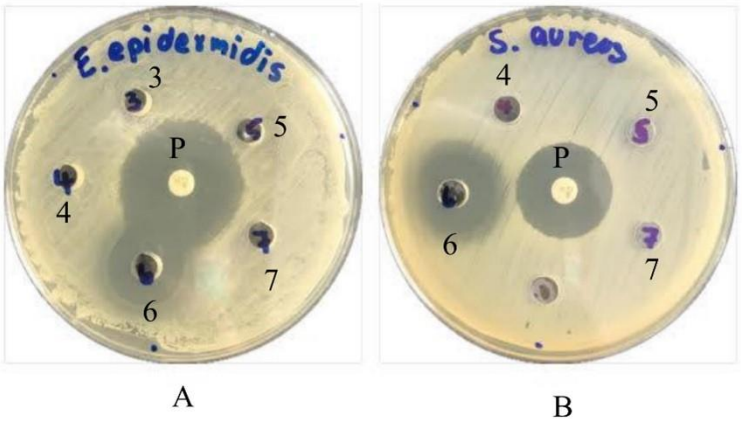

Figure 1 Inhibition of (A) *S. epidermidis*, (B) *S. aureus* using agar well diffusion method: (3) 80% ethanol 1 hrs (4) 80% ethanol 2 hrs (5) water 1 hrs (6) water 2 hrs (7) negative control (P) positive control of ciprofloxacin

4.6 Inhibition of pathogenic bacteria

The evaluation of the antibacterial properties of six extracts (10 mg/mL), employing the agar well diffusion method, revealed that the extract obtained with water over a 2-hour period effectively inhibited gram-positive bacterial strains under examination, specifically *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228), with inhibition zone diameters of 16.28±4.75 mm and 15.33±3.27 mm, respectively. In comparison, the standard antibiotic ciprofloxacin (5 µg) exhibited inhibition zone diameters of 20.47±0.70 mm *(S. aureus)* and 30.91±0.80 mm (*S. epidermidis)*. The extract of *G. fisheri* can inhibit the bacteria via sulfate groups that disrupts bacterial growth processes and the cell wall structure of bacteria, leading to the loss of integrity of the bacterial envelope and subsequently reducing the viability of *S. aureus and S. epidermidis* (Huang et al., 2019). Meanwhile, *G. fisheri* extracts are ineffective in inhibiting the growth of gram-negative bacteria, *E. coli* and *P. aeruginosa* which could be due to an outer lipid layer that obstructs the access of substances to the cells (Huang et al., 2019). The findings are in line with the research conducted by Kim et al., (2007), where they examined crude extracts from the red seaweed (*Ulva lactuca*) and reported about its antibacterial properties against *Bacillus subtilis*, *Listeria monocytogenes*, and *S. aureus.*

5. Conclusion

The most effective condition for extraction of *G. fisheri* was water extraction for 2 hrs duration, resulting in the highest percentage yield of extraction, sulfate and phenolic content, antioxidant activity, and antibacterial properties against *S. aureus* and *S.epidermidis*. Consequently, *G. fisheri* extract offers promising potential for future health-related applications.

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