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Antioxidant and Antimicrobial Potential of *Sargassum* spp. Extract from Gunungkidul Regency, Indonesia Using Triphasic Extraction Method

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ABSTRACT

Sargassum is the most abundant and diverse macroalgae in Gunungkidul Coast, Indonesia. The use of *Sargassum* as a cosmetic ingredient in Gunungkidul is limited, despite its antioxidant and antibacterial properties. This study aimed to identify *Sargassum* based on DNA barcoding and to explore its antioxidant and antimicrobial properties. Three types of *Sargassum* were collected from the Gunungkidul coast and were identified through DNA barcoding targeting the nuclear ribosomal Internal Transcribed Spacer 2 (ITS2) sequences. Triphasic method of n-heptane/EtOAc/acetonitrile/butane-1-ol/water solvents was employed for extraction. The extract bioactivity was evaluated by testing it against the minimum inhibitory concentration (MIC) of four bacterial species and its IC_{50} capacity. DNA barcoding identified three confirmed species: *S. oligocystum*, closely related to *S. aquifolium*; *S. ilicifolium*, closely related to *S. yinggehaiense*; and *S. aquifolium*, closely related to both *S. oligocystum* and *S. megalocystum*. The three phases of triphasic extract were separated into phases: 1 (top), 2 (middle), and 3 (bottom). MIC test against four microbes showed a range of activity from no inhibition to minimal activity. The antioxidant capacity examination of *S. ilicifolium* and *S. aquifolium* extracts using IC_{50} yielded values ranging from strong to very strong. These findings were further supported by the LC-MS dereplication, which revealed the presence of sargachromanols A and cystodione I molecules.

INTRODUCTION

Sargassum is a macroalgae species characterized by its rapid growth and adaptability to the environment. *Sargassum* typically thrives in intertidal to subtidal

regions by living attached to open rock substrates **(Kadi, 2005; Low *et al*., 2019; Fidai *et al*., 2020; Yip *et al*., 2020)**. The genus *Sargassum* has high phenotypic variation among the Phaeophyceae class **(Yip, 2020; Guiry & Guiry, 2022)**. In Indonesia, 57-62 species have been found, spread across Yogyakarta (17 species/30.35%), Sulawesi (14 species/25%), West Java (12 species/21.42%), Seribu Islands and Sunda Strait (10 species/7.85%) and Sumatra and eastern Indonesia (4-9 species/7.14%-16.07%). The high phenotypic variation results in frequent species confirmation, re-taxonomy, and the discovery of different species but shows closeness in their phylogenetics **(Yip *et al*., 2018; Soliman & Tawfik, 2020; Sargazi, 2021; Prasetyaningsih, *et al*., 2024)**. Utilizing fragments and morphological observations of anatomy to identify diversity is still inadequate in providing a precise representation of variation **(Rindi *et al*., 2012)**. Hence, additional supporting evidence derived from molecular analysis is necessary to validate the findings.

The compounds contained in *Sargassum* are ingredients in the health, food, feed, and cosmetics industries **(Hu *et al*., 2016; Gazali *et al*., 2018; Prasetyaningsih *et al*., 2018; Jayadi *et al*., 2019; Handayani *et al*., 2021; Kusmita *et al*., 2024; Meiyasa *et al*., 2024; Nurhidajah *et al*., 2024)**. The bioactivity of *Sargassum* as an antioxidant and its molecular structure are the most widely studied. According to **Yangthong (2009)**, the *Sargassum* extract concentration required to inhibit 50% DPPH (IC_{50}) is 1.08 ± 0.83g/ mL, which is higher than the IC_{50} values of algae from the Rhodophyta (*Gracilaria* sp.) and Chlorophyta (*Caulerpa racemosa*, and *Ulva lactuca*) groups, which are approximately 15.05 ± 0.61, 103.73 ± 0.59, and 24.22 ± 0.87g/ mL, respectively. Using ethanol extract as a solvent increased the IC_{50} value to 57.05g/ mL **(Nurjanah *et al*., 2018)**. Secondary metabolites present in *Sargassum* have been identified as promising antibacterial agents with a wide range of activity **(Prasetyaningsih *et al*., 2018; Jatmiko *et al*., 2019; Prasedya *et al*., 2019; Puspita *et al*., 2020; Susanto *et al*., 2021)**. *S. crassifolium* extract is reported to have the potential as an antibacterial for Gram-positive and negative *Escherichia coli* and *Staphylococcus aureus* **(Huang *et al*., 2018)**. However, *Sargassum* extract is more effective as a bacteriocidal against Gram-positive bacteria than Gram-negative due to differences in susceptibility to bacterial cell wall structure and composition **(Taskin *et al*., 2007)**. Scientific data searches from 2011 to 2022 found more than 30 species of *Sargassum* that have been studied for their potential as antimicrobials. Among these species, 26 have been documented to possess antioxidant properties against 21 bacterial types and 4 fungal types that specifically target the mouth, skin, and vagina.

Extensive research has been undertaken in the past decade to investigate *Sargassum*'s potential. Metabolomic studies have found various potential compounds in *Sargassum*, including polyphenols, terpenoids, fucoxanthin, fatty acids and their derivatives, vitamins, minerals, proteins, steroid polysaccharides, and crude fiber **(Joob & Wiwanitkti, 2016; Pakidi & Suwoyo, 2017)**. Fucoxanthin is the primary carotenoid pigment synthesized solely by brown algae **(Pereira *et al*., 2021)**. As the primary

constituent of *Sargassum*, this pigment plays a crucial role in photosynthesis and providing photoprotection. This compound also provides color to the *Sargassum* thallus, a trait exclusive to brown algae and absent in red or green algae **(Liu *et al*., 2012; Mikami & Hosokawa, 2013; Perfeito *et al*., 2018; Pereira *et al*., 2021)**. Additional chemicals exclusive to Sargassaceae include the phenolic meroditerpenoid group (plastoquinone, chromanols, and chromenes), which exhibit bioactivity in the pharmaceutical industry of fisheries products. Aside from enhancing fish health, these chemicals are considered oceanic flavors that provide a unique flavor to seaweed and fish **(Kim & Kong, 2010; Kellogg *et al*., 2014; Murray *et al*., 2018)**. Phlorotannins are distinctive polyketides identified exclusively in this group of algae, not in terrestrial plants **(Meslet-Cladière *et al*., 2013)**. The phlorotannin content in *Sargassum* ranges from 17.10 to 884.80mg/ g, or around 1 to 14% of dry weight. This content is influenced by salinity, light, and available nutrients **(Farvin *et al*., 2019)**.

In natural product research, extracting chemical molecules from samples is a crucial first step in obtaining sample extracts. Conventionally, different extraction techniques are employed based on their intended application, such as maceration, soxhletation, digestion, decoction, infusion, percolation, Soxhlet extraction, superficial extraction, ultrasound-assisted, and microwave-assisted extractions **(Abubakar & Haque, 2020)**. One of the extraction methods that are developed is triphasic, utilizing five solvents consisting of n-heptane, acetonitrile, butane-1-ol (p.a), ethyl acetate (technical), and water, which are classified into three polarity gradients, namely polar, semi-polar and non-polar. The solvent is prepared by combining five components, n-heptane, EtOAc, acetonitrile, butan-1-ol, and water, in a proportion of 22:14:29:8:27 **(Gori *et al*., 2021)**. This mixture is expected to enhance the efficiency of both time and solvent usage, thereby maximizing the achievable outcomes.

Gunungkidul Regency is a regency in the Daerah Istimewa of Yogyakarta Province, Indonesia. It is situated between 07°16’30” - 07°19’30” S and 110°19’30” - 110°25’30” E. It covers an area of 1,485km^2 and has a coastline of 60.83% (Vertical Data from the Indonesian Navy Base at http://bappeda.jogjaprov.go.id/dataku). The presence of rocky and sandy beaches promotes the proliferation of *Sargassum*. Scientific data searches revealed that 14 types of *Sargassum* (out of 57-62 types of *Sargassum* in Indonesia) thrive on the rocky beaches of Gunungkidul, making them abundant during the harvest season. However, research on molecular-based diversity and the potential of *Sargassum* in Gunungkidul is still limited, especially in terms of its role in the health and cosmetic sectors. Therefore, this study aimed to molecularly identify the three most commonly found *Sargassum* species in Gunungkidul and to evaluate their antimicrobial and antioxidant activities.

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MATERIALS AND METHODS

Samples collection

Fig. (1) displays three macroalgae samples from 30 beaches in Gunungkidul Regency, Yogyakarta, chosen for further examination.

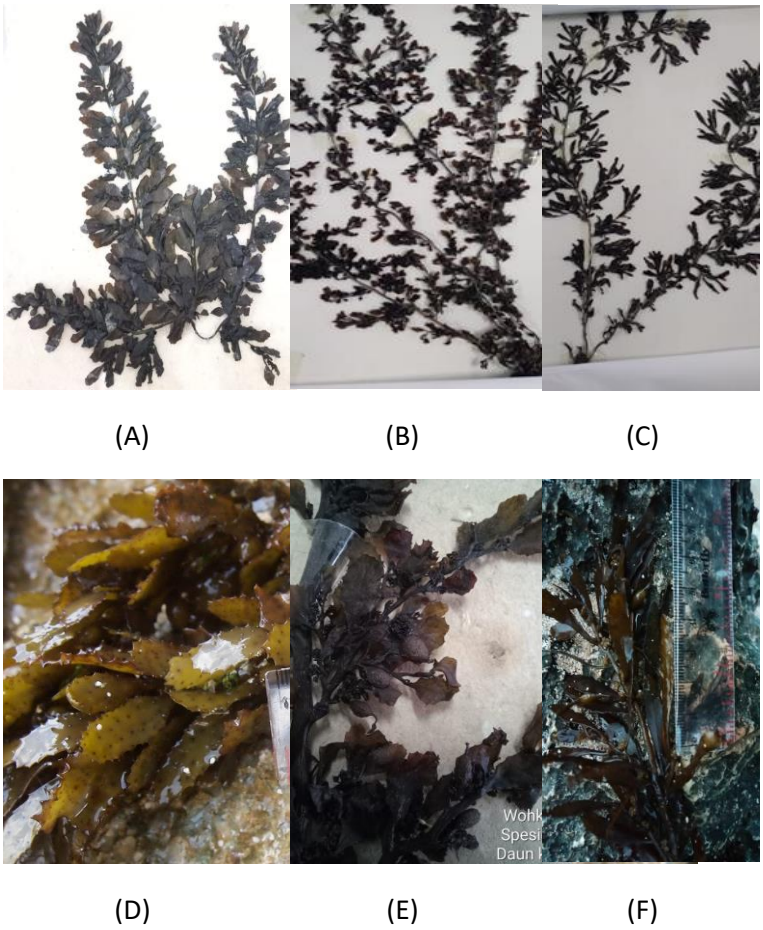


Fig. 1. Sample 2954-2 (**A.** herbarium, **D.** fresh), Sample 2939-2 (**B.** herbarium, **E.** fresh) and Sample 2939-1 (**C.** herbarium, **F.** fresh)

Samples with codes 2939-1 and 2939-2 were collected from Wohkudu Beach (8°05'56"S 110°26'26" E). A sample with code 2954-2 was collected from Ngunggah Beach (8°05'20"S 110°25'18" E). The three samples were selected based on the breadth of *Sargassum* distribution on the 30 beaches (Fig. 2)

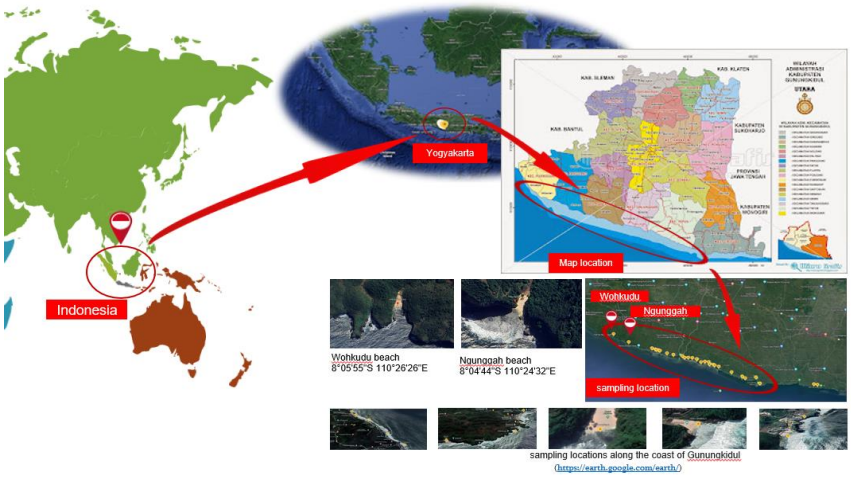


Fig. 2. Map of the sampling location along the coast of Gunungkidul-Yogyakarta

Molecular identification of *Sargassum* spp.

Genome extraction and DNA amplification

Molecular identification of *Sargassum* was conducted by PT. Genetika Science Indonesia. The samples used were from the herbarium with codes 2954-2, 2939-2, and 2939-1 (Fig. 3A, B, and C). The extraction of total genomic DNA was performed using the Quick-DNA Plant/seed Kit (Zymo Research, D6020), and the concentration of the collected DNA was determined using nucleic acid (Genomic DNA) quantification (Nanodrop). DNA amplification was performed using (2x) MyTaq HS Red Mix (Bioline, BIO-25048) and KOD FX Neo (Toyobo, KFX-201) kit. The primers used were nuclear ribosomal Internal Transcripted Spacer 2 (ITS2) sequences, namely 5.8S BF (5'CGATGAAGAACGCAGCGAAATGCGAT-3') and 25BR2 (5'-TCCTCCGCTTAG TATA TGCTTAA) **(Kantachumpoo *et al*., 2015)** with a target DNA amplification of 500-700 bp. Genomic DNA amplification was conducted using the protocol outlined in Tables (1, 2).

Table 1. PCR optimization of primer 5.8S BF and 25BR2 with samples 2939-1 and 2939-2 using (2x) MyTaq HS Red Mix (Bioline, BIO-25048)

Component	1x 50 µL (µL)	Final concentration
MyTaq HS Red Mix, 2x	25	1 x
Forward Primer*	2	0.4 µM
Reverse Primer**	2	0.4 µM
DNA template	X	50 ng
Water, nuclease-free	Up to 50	

*5.8S BF 5'- CGATGAAGAAGCAGCGAAATGCGAT -3'

**25BR2 5'- TCCTCCGCTTAGTATATGCTTAA -3'

Table 2. PCR optimization of primer 5.8S BF and 25BR2 with sample 2954-2 via optimization using KOD FX Neo (Toyobo, KFX-201)

Component	1x 50 µL (µL)	Final concentration
KOD FX Neo	1	1 x
2× PCR Buffer for KOD FX Neo	25	1 x
2mM ddNTP	10	0.4 mM
Forward Primer*	3	0.6 µM
Reverse Primer**	3	0.6 µM
DNA template	X	100 ng
Water, nuclease-free	Up to 50	

*5.8S BF 5'- CGATGAAGAAGCAGCGAAATGCGAT -3'

**25BR2 5'- TCCTCCGCTTAGTATATGCTTAA -3'

The amplification of internal transcribed spacer 2 of nuclear ribosomal DNA (ITS2) from *Sargassum* samples was performed according to the cycles outlined in Table (3).

Table 3. Cycles of the PCR amplification condition

Phase	Step	Temperature	Time
1	Denature	95°C	3 min
2	Denature	95°C	15 sec
3	Anneal	60°C	30 sec
4	Elongate	72°C	45 sec
Repeat steps 2-4 (35 times)			
Termination	Step	Temperature	Time
5	Elongate	72°C	5 mins
6	Hold	4°C	Until removed from the machine

*Thermal cycler: VeritiPro Thermal Cycler, 96 well (Applied Biosystems, A48141)

Top10 Hit BLAST Results Against NCBI Database, Excluding Uncultured Sample

DNA sequencing

PCR products as much as 1µL were checked using electrophoresis 1% Tris Buffer borate EDTA (TBE) agarose with a 100 bp DNA ladder marker (loaded 2.5µL). Band visualization was performed by observation under ultraviolet (UV) light. The DNA amplification product sequences were subsequently tracked and analyzed automatically using the Basic Local Alignment Search program (BLAST) database tracking program (ABI 3130XL, Applied Biosystem). BLAST compared sequences with the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed using the

Mega 10 version with neighbor-joining (NJ method) to reconstruct trees and estimate genetic distance.

Metabolite extraction

Three identical *Sargassum* samples were collected from 30 beaches in October - December 2022. The samples were sorted by species, cleaned with running fresh water, and dried with a fan for 24 hours. The samples were then dried in an oven at 40°C until the dry weight was constant. The dried samples were pulverized with a grinder and were sifted through a 400-mesh screen. The fine powder was stored in food-grade ziplock plastic.

Triphasic extraction method (Gori *et al*., 2021)

The solvents used in the triphasic method consisted of n-heptane, acetonitrile, butan-1-ol (p.a), ethyl acetate (technical), and distilled water with a ratio of 22:14:29:8:27 **(Gori *et al*., 2021)**. Prior to usage, the solvent was shaken in a separating funnel to induce a triple-layer separation according to the solvent's polarity. Five grams of *Sargassum* powder from each species was dissolved in 75mL of solvent with a 1:15 (w/v) ratio. After separation, the powder was added and shaken vigorously until mixed, then left for 24 hours with occasional shaking. After 24 hours, the results of each phase were collected and separated according to their layers, and the residues were macerated again for 1 x 24 hours with the same process. The extract was filtered twice using cloth and Whatman©42 cellulose filters with a diameter of 110mm. Evaporation (IKA®HB.10) was carried out for 2-3 hours at 40- 50°C at a speed of 50 rpm until it thickened. The remaining solvent was evaporated using an oven at 40°C until a dry extract was obtained. The extract powder was weighed and stored in a freezer (temperature 4°C). Each extraction result was coded Phase 1 for the top extract, Phase 2 for the middle extract, and Phase 3 for the bottom extract. The yield was calculated using the formula in Eq. 1 **(Dewatisari *et al*., 2017)**.

$$\%Yield = \frac{Weight\ of\ extract\ obtained}{Initial\ weight\ of\ sample} \times 100\% \quad (1)$$

Antioxidant activity (Aminina *et al.*, 2020)

An antioxidant test of each extract phase was carried out using the 1,1-diphenyl-2-picrylhydrazil (DPPH) Radical Scavenging Assay method using a UV-visible spectrophotometer at a wavelength of 517nm. The concentration of sample solutions in all phases was prepared by gradually diluting an initial stock of 10,000ppm in methanol to concentrations of 1000, 500, 250, 125, 62.5, 31.35, 15.62, and 57.812ppm. Furthermore, 120µL of the extract was combined with 80µL of 0.4mM DPPH (in methanol p.a). As a positive control of butylated hydroxyl-toluene (BHT) solution, ascorbic acid was made with concentrations of 1000, 500, 250, 125, 62.5, 31.35, 15.62,

57.812ppm (in methanol p.a). The samples were then incubated in the dark at 40°C for 30 minutes before reading on a spectrophotometer at a wavelength of 517nm. The percentage of sample inhibition was calculated based on the difference between the absorption of the DPPH solution and the absorption of the sample solution divided by the absorption of the DPPH solution and multiplied by 100%. The concentration of antioxidants that can inhibit free radicals by 50% is called the IC_{50} value. The IC_{50} of the sample was determined based on regression, where Y was 50, while X showed the IC_{50} value obtained from the dose-response curve.

Antibacterial assay (Hutomo *et al*., 2018)

Preparation of bacterial suspension

The microbiological suspension stock of *Streptococcus mutans* ATCC 25175, *S. aureus* ATCC 29213, and *Escherichia coli* ATCC 29213 was prepared according to the procedure described by **Hutomo *et al*. (2018)**. Bacteria were grown in the Brain Heart Infusion (BHI) medium, and fungi were grown in the Yeast Dextrose Agar (YDA) medium. Microbial cultures were centrifuged for 15 minutes at 3000rpm until pellets were obtained. The pellets were then mixed with liquid peptone media to obtain the same turbidity level as the McFarland 0.5 solution standard (1.5 x 108 CFU/mL). This bacterial suspension was utilized promptly, within a maximum time frame of 15 minutes.

***Bacterial activity test* (Hutomo *et al*., 2020)**

A minimum inhibitory concentration (MIC) test was conducted to assess the susceptibility of *Streptococcus mutans* ATCC 25175, *S. aureus* ATCC 29213, and *Escherichia coli* ATCC 25922 colonies to different phases of *Sargassum* extract. The test was performed using the broth microdilution method diluted in 1% DMSO. Microbial cultures of 1.5 x 108 CFU/mL as much as 10µL were inoculated into 100µL of BHI broth containing *Sargassum* extract with concentrations ranging from 100,000 to 1,563 ppm (µg/mL) in 96-well plates. These plates were then replicated twice following initial tests. Ciprofloxacin of 10µg/ mL was used as a positive antibacterial control. Negative controls were inoculated using test microbes without the inclusion of any extract. The microplate cultures were incubated at 37°C for 24 hours. The MIC value was determined by visually assessing the clarity of the culture in comparison to positive controls **(Hutomo *et al*., 2020)**. The minimum MIC value was grouped into the categories of highly active extracts (<100µg/ mL), significantly active (100 ≤ MIC ≤ 512µg/ mL), moderately active (512 < MIC ≤ 2048µg/ mL), low activity (MIC> 2048µg/ mL), not active (MIC> 10mg/ mL), weak, moderate, and strong antibacterial activity **(Tamokou *et al*., 2017)**. The ability to inhibit bacteria was measured at the bacterial absorbance value (OD) at λ 595nm using a microplate reader.

Candida albicans antifungal activity test (Brillianti *et al.*, 2022)

Preparation of fungal isolates

A clinical isolate of *Candida albicans* was obtained from a patient at Bethesda Hospital Yogyakarta and was identified using a chrom agar medium. *C. albicans* colonies on chrom agar exhibited a green color. *C. albicans* colonies were cultured on solid Yeast Peptone Dextrose (YPD) media, re-cultured on liquid YPD media, and incubated at 37^{o}C for 24 hours. After incubation, the culture was centrifuged for 15 minutes at 3000rpm. The supernatant was discarded, and the sediment was resuspended using glycerol:water until the turbidity was equivalent to the McFarland standard solution 0.5.

***Antifungal activity test* (Brillianti *et al.*, 2022)**

The antifungal activity of *Sargassum* extract was assessed using a multilevel microdilution method. The *Sargassum* phase concentrations used in the experiment were 150,000, 75,000, 37,500, 18,750, 9,375, and 4,688ppm (µg/mL) in a 96-well plate. Each dose was repeated twice following a preliminary assay. As a positive control, 2mg/ mL fluconazole (dissolved in aquadest) was used **(Brillianti *et al.*, 2022)**. Negative control was done by not adding the extract to the media inoculated with *C. albicans* isolates. Incubation was carried out for 24 hours at room temperature. The MIC value was established by visually assessing the clarity of the culture in comparison to the positive control **(Hutomo *et al.*, 2021)**. Bacterial inhibition capacity was quantified by measuring the OD at λ 595nm using a microplate reader.

Phytochemical profiling and dereplication

The chemical composition of all extract phases was analyzed using Liquid Chromatography coupled with Mass Spectrometry (LC-MS). The LC-MS test was conducted at the Forensic Laboratory in Sentul, Bogor. The LC-MS test method employed was the m/z [M+H+] method, which involved the addition of 1 proton. This implies that during the sample injection procedure, 1 hydrogen atom was emitted, resulting in the reduction of 1 hydrogen element in the chemical components for compound identification. The chromatogram results were a picture of the compounds identified during running of 22 minutes. The LC-MS test results were identified using the Masslynx 4.1 application. The peaks on the chromatogram indicate the abundance of an identified compound at a specific retention time. The peak identified by knowing the value of the dominant mass molecule when the peak occurs. The identification of the mass molecule value was then used as a reference to find references related to compounds that have been published or have not been found (dereplication) and dominate the sample. Dereplication of mass spectral data was determined based on compounds from the Comprehensive Marine Natural Products Database (CMPD) database based on species and genus.

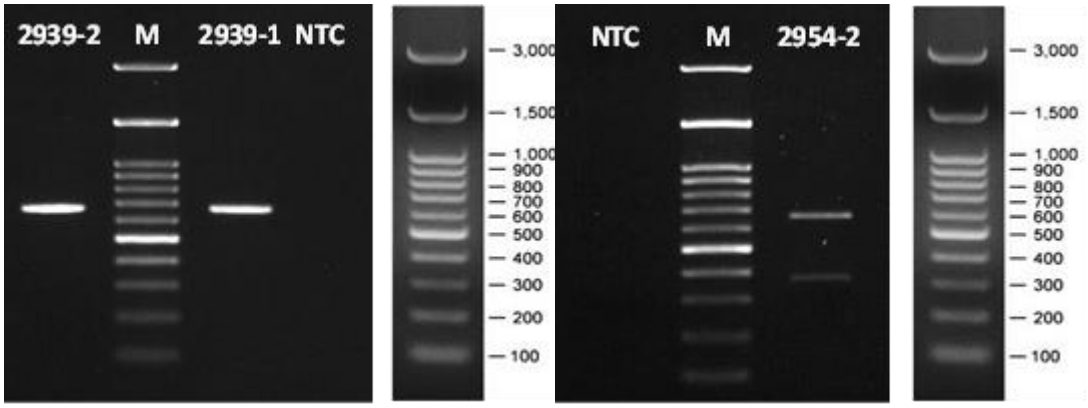
Statistical analysis

The MIC and inhibition percentage were expressed as mean ± standard deviation (SD). The relationship between independent variables (type of *Sargassum*, part of each phase/treatment, and concentration) and dependent variables (inhibition percentage value and IC_{50}) was analyzed using the multivariate dependence method. Test with significance level at 5% (P<0.05).

RESULTS

1. Molecular identification

Molecular identification (extraction, DNA amplification, and sequencing) was conducted at PT. Genetica Science, Jakarta, Indonesia. DNA amplification using nuclear ribosomal Internal Transcripted Spacer 2 (ITS2) sequences primers, namely 5.8S BF (5'CGATGAAGAACGCAGCGAAATGCGAT-3') and 25BR2 (5'-TCCTCCGCTTAGTATATGCTTAA) **(Kantachumpoo *et al.*, 2015)**. The amplification results were visualized on 1% TBE agarose gel electrophoresis (Fig. 3).



(A) (B)

Fig. 3. Electrophoresis visualization of primary amplification of the ITS2 gene (5.8S BF and 25BR2) from three *Sargassum* samples code (A) 2939-1 and 2939-2, (B) code 2954-2. M: 100 bp DNA ladder marker, NTC = Non-Template Control

This study utilized nuclear ribosomal DNA internal transcription spacer sequence primers ITS2 (5.8S BF and 25BR2). This choice was made after the RbCL 2 primer was previously tried but did not yield adequate results. The results of DNA amplification using the ITS2 primer produced amplicons measuring between 596-627bp. The amplicon from sample code 2939-1 produced a sequence of 609bp. The amplicon from sample code 2939-2 was 627bp sequence. Moreover, the amplicon from sample code 2954-1 was

596bp sequence. The sequencing data were processed using MEGA-X 10.1.8. This program did nucleotide sequence analysis by segmenting, matching the input sequence (query) with the database sequence (NCBI), computing the genetic distance, and determining the proportion of the size/length of the input sequence match in relation to the target DNA sequence. The sequence alignment and phylogenetic tree results are depicted in Fig. (4) **(Kumar *et al*., 2018)**.

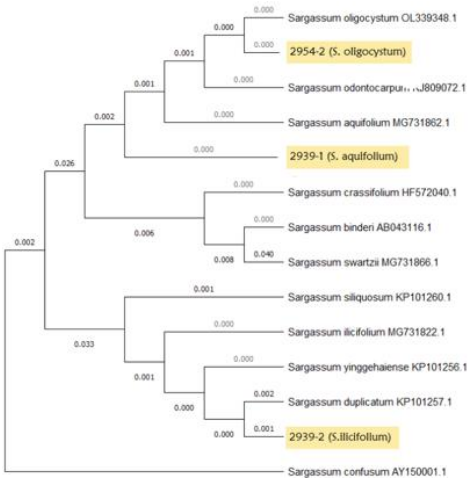


Fig. 4. The Phylogenetic tree of the Sargassum based on ITS 2 gene by Mega 10

2. Extraction

The solvent gradient employed in this work consisted of a mixture of n-heptane, EtOAc, acetonitrile, butan-1-ol, and water at a ratio of 22:14:29:8:27. The solvent exhibited a separation into an upper phase (25%) with apolar (Ap) properties, a middle phase (48%) with medium polarity (Int), and the lower phase (27%) with higher polarity (Pol). Below are the polarity characteristics of the five solvents employed in the maceration process. n-heptane is non-polar, Ethyl Acetate is polar, Acetonitrile is polar, Butanol-l is polar, and Aquades is polar. The presence of butan-1-ol, which is more polar than EtOAc and does not mix with water, makes the apolar and middle phases more polar. A distinct division between the solvent property groups, namely Phase 1 (top layer), Phase 2 (middle layer), and Phase 3 (bottom layer), was shown in the study results (Fig. 5).

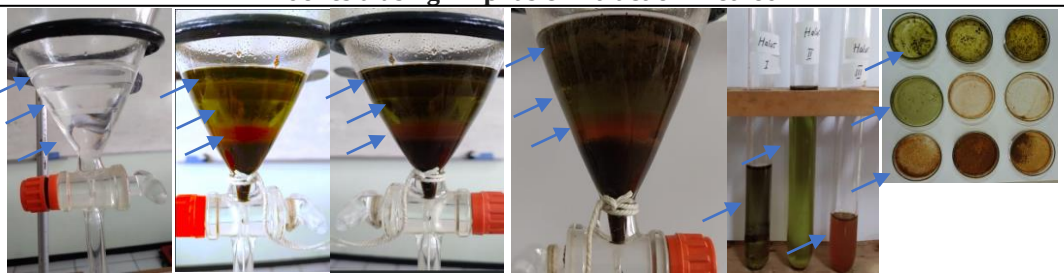


Fig. 5. The separation process occurs during the extraction of *Sargasum* using the triphasic method. (a) The agitated solvent, (b) the powder immersed in the solvent, (c) the powder immersed for 12 hours, (d) the powder immersed for 24 hours, (e) the obtained extract, and (f) the evaporated extract. The blue arrows indicate the separation of the extract in the separating funnel, Phase 1 (top), Phase 2 (middle), and Phase 3 (bottom)

The yields of crude extract varied in each phase among all species of *Sargassum* spp. The highest yield was obtained from *S. oligocystum* extract (44.18%), whereas *S. aquafolium* and *S. ilicifolium* exhibited nearly identical values, namely 25.13 and 24.57% (Table 4).

Table 4. Yield of *Sargassum* spp. extraction at each separation phase

Sample	Phase 1 (Apolar)	Phase 2 (Intermediate)	Phase 3 (Polar)	Total yield (%)
	rendemen (%)±sdv	rendemen (%)±sdv	rendemen (%)±sdv	
S. oligocystum	12.70±3.60	8.15±2.50	23.33±1.67	44.18
S. aquifolium	11.98±3.00	9.33±2.65	3.82±0.52	25.13
S. ilicifolium	10.90±3.10	8.00±2.90	5.67±2.67	24.57

These results differ from the research reported by **Puspita (2017)**. Among the three types of *Sargassum* spp. tested with six different solvent modifications, *S. aquifolium* exhibited the greatest yield in comparison to the other two species.

3. Antimicrobial assay

MIC in this study aimed to determine the lowest extract concentration that can inhibit the growth of test microbes based on observations of culture turbidity compared to positive controls. Three test microbes were used, namely colonies of *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922. *S. mutans* ATCC 25175 is the main Gram-positive bacterium responsible for dental caries (DC) and oral health issues **(Kováč *et al.*, 2022)**. *S. aureus* ATCC 29213 is a Gram-positive bacterium that is usually found on the skin **(Hidayah *et al.*, 2017)**. *E. coli*

ATCC 25922 is a Gram-negative bacterium that are commonly found on the skin. In addition to testing bacteria, the extract was also tested on eukaryotic cells of clinical isolate *Candida albicans* fungi. The results of the MIC showed that the three *Sargassum* spp. samples have a high minimum inhibitory power concentration of ≥100,000ppm. Thus, these samples were classified as part of the non-active group (MIC > 10mg/ mL) **(Tamokou *et al*., 2017)**. No inhibitory effect was observed in the test findings on *C. albicans*, even at concentrations up to 150,000ppm (Table 5).

4. DPPH free radical scavenging activity

DPPH radical scavenging assay is a standard method used to measure the total antioxidant capacity in natural extracts. The outcomes are reported as IC_{50} values, which represent the number of antioxidants needed to decrease 50% of the original DPPH concentration **(Deng *et al*., 2011; Granados-Guzman *et al*., 2017)**.

Table 5. The minimum MIC results of the test microbes and the average optical density at a wavelength of 595nm

Types of *Sargassum*	Test microbes	*S. mutans* ATCC 25175		*S. aureus ATCC 29213*		*E. coli ATCC 25922*		*C. albicans* clinical isolate	
	Extract phase	MIC (µg/ml)	Mean OD ± SD	MIC	Mean OD±SD	MIC (µg/ml)	Mean OD±SD	MIC (µg/ml)	Mean OD±SD
S. oligocystum	Phase 1	-	-	-	-	100,000	0.245 ± 0.006	-	-
	Phase 2	100,000	0.219± 0.000	-	-	-	-	-	-
	Phase 3	-	-	-	-	-	-	-	-
	Mix	-	-	-	-	-	-	-	-
	Ciprofloxacin	10	0.349 ± 0.140	10	0.206 ± 0.001	10	0.174 ± 0.046	-	-
S. ilicifolium	Phase 1	-	-	-	-	100,000	0.629 ± 0.388	-	-
	Phase 2	100,000	0.236 ± 0.012	100,000	0.629 ± 0.388	-	-	-	-
	Phase 3	-	-	-	-	-	-	-	-
	Mix (µg/ml)	100,000	0.188 ± 0.008	100,000	0.634 ± 0.411	-	-	-	-
	Ciprofloxacin	10	0.152 ± 0.011	10	0.641 ± 0.284	10	0.650 ± 0.478	-	-
S. aquifolium	Phase 1	-	-	-	-	100,000	0.632 ± 0.393	-	-
	Phase 2	-	-	-	-	-	-	-	-
	Phase 3	100,000	0.321 ± 0.020	100,000	0.650 ± 0.396	-	-	-	-
	Mix	100,000	0.428 ± 0.070	-	-	-	-	-	-
	Ciprofloxacin	10	0.308 ± 0.003	10	0.664 ± 0.425	10	0.182 ± 0.046	-	-
Fluconazole (mg/ml)								2	0.241 ± 0.003

The IC_{50} value category is very strong if the IC_{50} value is <50ppm, strong if the IC_{50} value is between 50 and 100ppm, moderate if the IC_{50} value is between 100 and 150ppm, weak if the IC_{50} value is between 150 and 200ppm and not active if the IC_{50} is above 200ppm **(Li *et al.*, 2018)**. The IC_{50} results of each phase indicate variations in antioxidant capacity. Phases 1 and 3 had an average of strong antioxidants compared to Phase 2. Extracts from *S. aquifolium* and *S. ilicifolium* had antioxidant abilities in the strong to very strong categories.

Results from multivariate testing indicated that the IC_{50} value was strongly influenced by the type of *Sargassum* and phase but not by the treatment concentration. This phenomenon was also observed in the combination variable, where the concentration and phase of the extract had a statistically significant impact on the IC_{50} at a 50% confidence level (Tables 6, 7).

Table 6. The antioxidant activity of *Sargassum* spp. extract using DPPH in each phase against the percentage inhibition and IC_{50} values

Types of *Sargassum*	Extract phase	% inhibition (µg/mL/ppm)	IC_{50} (µg/mL/ppm)	Category
S. oligocystum	Phase 1	33.984±0.00/250	446.045	Not active
	Phase 2	71.484±0.319/1000	288.131	Not active
	Phase 3	73.047±0.552/31.35	171.771	Moderate
	Mix	35.807±0.697/62.5	-41.354	Not active
S. ilicifolium	Phase 1	33.984±0.000/250	22.751	Very strong
	Phase 2	74.870±0.83/250	71.986	Strong
	Phase 3	70.443±0.436/500	145.649	Moderate
	Mix	69.141±0.390/31.35	65.571	Strong
S. aquifolium	Phase 1	65.775±0.610/62.5	52.831	Strong
	Phase 2	42.839±0.558/7.812	-635.946	Not active
	Phase 3	74.089±0.664/125	24.673	Very strong
	Mix	67.188±0.884/31.35	55.630	Strong
Asam ascorbat			91.766	Strong

Table 7. Results of the significance test of *Sargassum* types and extract phases on the percentage of inhibition and IC_{50}

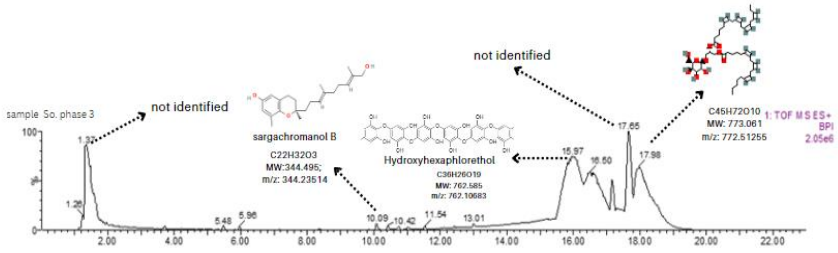
Variable	Influenced factors			
	% inhibition		IC_{50}	
	sig value	Conclusion	sig value	Conclusion
Types of Sargassum	0.437	not a statistically significant	0.000*	statistically significant
Extract phase	0.010*	statistically significant	0.000*	statistically significant
Extract concentration	0.000*	statistically significant	1.000	not a statistically significant
Concentration and Type of *Sargassum*	0.958	not a statistically significant	1.000	not a statistically significant
Sargassum types and extract phases	0.957	not a statistically significant	0.957	not a statistically significant
Concentration and extract phase	0.000	statistically significant	0.000*	statistically significant

analyzed using the multivariate dependence method. Test with significance level at 5% (P<0.05).

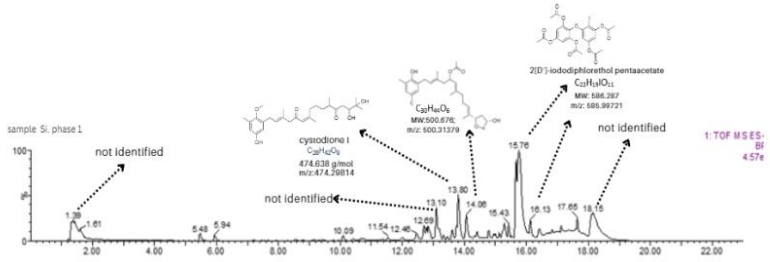
*Statistically significant with significance level at 5% (P<0.05).

5. Chemistry profiling

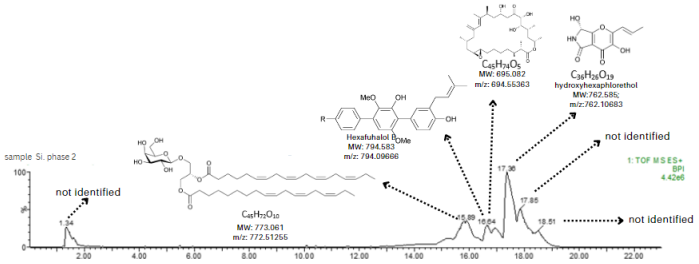
The LC-MS chromatogram of three phases of *Sargassum* spp. extracts revealed that Phase 1, the top layer or apolar phase, had the greatest number of peaks. Conversely, Phase 2, the intermediate layer, had the fewest peaks, except for *S. ilicifolium*. Phase 3, the polar phase or bottom layer, exhibited peak numbers that fell between Phase 1 and 2 (Fig. 6).



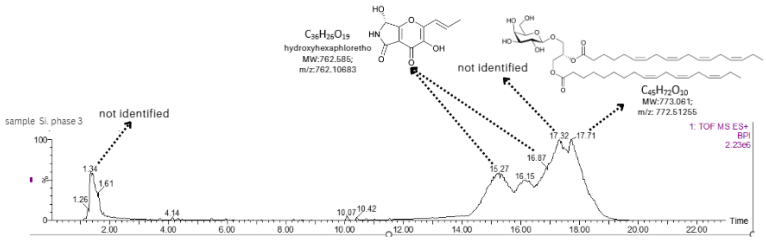
Extract Phase 3 of *S. aquifolium*



Extract Phase 1 of *S. ilicifolium*



Extract Phase 2 of *S. ilicifolium*



Extract Phase 3 of *S. ilicifolium*

Fig. 6. LC-MS of three-phase extract of *Sargassum* spp.

Not all peaks observed in the LC-MS Chromatogram of *Sargassum* spp. could be recognized as they were not present in the dereplication database derived from literature, compound databases, or other sources. Further observations were made on the eight dominant peaks appearing at retention times of 10.09, 12.65, 13.80, 14.06, 15.27, 16.64, 17.36 and 17.98 (Table 7).

Table 7. Results of compound dereplication from the CMPD database

Retention	Compound	Mol/MW formula	m/z	*Sargassum* spp.	Reference	Phase extract
10.09	Sargachromanol A	$C_{22}H_{30}O_3$ 342.479	342.479	*S. aquifolium*	(Blunt et al., 2007)	3
12.65	(E)-6,10,14-trimethylpentadec-5-ene-2,12-dione	$C_{18}H_{32}O_2$ 280.452	280.24023	*S. aquifolium*	CMPND	1
13.80	cystodione I	$C_{28}H_{42}O_6$ 474.638	474.3791	*S. oligocystum* *S. ilicifolium*	EMBL-ChBI,	3 1

					CMNPD	
14.06	[(2E,6E,10E)-11-[(2S,4S)-4-hydroxy-5,5-dimethyloxolan-2-yl]-1-(2-hydroxy-5-methoxy-3-methylphenyl)-3,7-dimethyldodeca-2,6,10-trien-5-yl] acetate	$C_{30}H_{44}O_6$ 500.676	500.31379	*S. ilicifolium* *S. aquifolium*	CMNPD	1 1
15.27	3''-isopropyl-3c-{3b-[(2-oxo-3,4-dihydro-2H-chromen-3-yl)methyl]butyl}-2''-butenyl-3'-hydroxy-2'-(2'b-methoxy-2'-oxoethyl)-3',4'-dihydro-2H-pyran-4'-carboxylate	$C_{30}H_{40}O_8$ 528.642	528.27232	*S. ilicifolium*	CMNPD	3
16.64	[3-[(2E,6E,10E,14R)-14,15-dihydroxy-3,7,11,15-tetramethylhexadeca-2,6,10-trienyl]-4-hydroxy-5-methylphenyl] (Z)-octadec-9-enoate	$C_{45}H_{74}O_5$ 695.082	694.55363	*S. ilicifolium*	(Blunt et al., 2007)	2
	Hexafuhalol B	$C_{36}H_{26}O_{21}$ 794.583	794.09666	*S. ilicifolium*	(Faulkner, 1993)	3
17.36	hydroxyhexaphlorethol	$C_{36}H_{26}O_{19}$ 762.585	762.10683	*S. ilicifolium*	(Faulkner, 1993)	2
17.98	[(2S)-2-[(6Z,9Z,12Z,15Z)-octadeca-6,9,12,15-tetraenoyl]oxy-3-[(2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxypropyl] (9Z,12Z,15Z)-octadeca-9,12,15-trienoate	$C_{45}H_{72}O_{10}$ 773.061	772.5944	*S. aquifolium*	https://pubchem.ncbi.nlm.nih.gov/#query=C45H72O10	3

DISCUSSION

The genus *Sargassum* is one of the genera with the highest phenotypic variation among the Phaeophyceae class. This high phenotypic variation results in difficulties in species-level taxonomic classification. Therefore, species confirmation, re-taxonomy, and the discovery of different species are often carried out while showing closeness in phylogenetics **(Soliman & Tawfik, 2020; Yip *et al*., 2020; Sargazi, 2021)**. The polymorphic character of *Sargassum* spp. explains the genetic variety seen. It is challenging to rely only on morphometric diversity for precise taxonomic identification **(Mattio & Payri, 2010; Rindi *et al*., 2012)**. The present work involved the identification of three predominant forms of *Sargassum* spp. within the waters of Gunungkidul (Fig. 1). The significance of DNA barcoding on *Sargassum* lies in its remarkable diversity, particularly in Gunungkidul. A literature study revealed that Gunungkidul contains 14 *Sargassum* out of the 57-62 species known in Indonesia. This level of diversity is the most extensive compared to other regions in Indonesia. Nevertheless, as of 2020-2022, the number of species documented in these waters was limited to 2-6 **(Sodiq & Arisandi, 2020; Dwi *et al*., 2021; Ningrum & Chasani, 2021)**.

Three samples with codes 2939-1, 2939-2, and 2954-1 were amplified by PCR method using the primer sequence of nuclear ribosomal DNA ITS 2 (5.8S BF and 25BR2) **(Kantachumpoo *et al*., 2015)**. ITS is a nuclear ribosomal DNA with extensive application. This ITS2 primer is classified as a micro barcode because of the relatively short length of the target base pair, approximately 700bp **(Arulandhu *et al*., 2019)**. However, this barcode has been successfully used in research as one of the molecular markers to analyze phylogenetic relationships between *Sargassum* species and populations **(Dixon *et al*., 2014; Kantachumpoo *et al*., 2015; González-Nieto *et al*., 2020; Sulistiyani *et al*., 2022)**.

The three samples exhibited amplification ranging from 596 to 627bp (Fig. 1). Upon comparing the sequencing of the amplification with NCBI's BLAST database, the following similarities were identified. The specimen code 2839-1 (Sn Wohkudu) was determined to be *Sargassum aquifolium* with a Query Cover of 100% and a similarity value of 99.67%. Nevertheless, the BLAST results on NCBI revealed a strong familial relationship between *S. aquifolium* and both *S. oligocystum* and *S. megalocystum*, as indicated by the same similarity score. Sample code 2939-2 (Sf Wohkudu) was identified as *Sargassum ilicifolium* with a Query cover value of 100% and a similarity of 99.84%. This result is the same as *S. yinggehaiense*; both show close kinship and are in the same group. Sample code 2954-2 (Si Ngunggah) was identified as *S. oligocystum* with Query cover of 100% and similarity of 100% (Fig. 4). In the top 10 Hit BLAST NCBI database, several other species were still found with the same similarity value. Therefore, efforts must be made to use other barcodes, such as Cox 3, to provide more accurate identification data.

The Soxhlet method and maceration with solvents of varying polarities are classic and simple techniques for isolating and characterizing compounds. However, these methods have limitations due to the restricted polarity of both the plant material and the solvent, and they are time-consuming. To address these issues, this study employed a triphasic extraction system that uses multiple solvents simultaneously. This approach is expected to increase yields, reduce reaction time, facilitate compound partitioning between phases, and minimize solvent consumption. The triphasic system was created by mixing five solvents (n-heptane, ethyl acetate, acetonitrile, butan-1-ol, and water) in different proportions to form a polarity gradient **(Gori *et al.*, 2021)**. The extraction resulted in three distinct layers (phases) in all *Sargassum* spp. species.

The highest yield was obtained from the *S. oligocystum* extract, which reached 44.18%, while *S. aquifolium* and *S. ilicifolium* showed almost the same results (25.13 and 24.57%) (Table 4). This outcome surpasses the reported results of several *Sargassum* extractions using a single solvent, namely ethanol extract (1.39%) **(Hidayati *et al*., 2017)**, ethanol extract (0.54 and 0.13%) **(Renhoran *et al*., 2017)**, ethyl acetate extract (0.420%) and n-hexane extract (0.265%) **(Gazali *et al*., 2018)**, and methanol extract (2.69%) **(Sedjati *et al*., 2018)**. The high number of extracts is possible because secondary and primary metabolites are bound in the solvent. As **Gori *et al*. (2021)** stated, polar properties might extract carbohydrates, and apolar ones might bind apolar ones, such as fats. Thus, this method cannot replace the extraction method used to target a particular group of molecules **(Gori *et al*., 2021)**. Extracts of *S. oligocystum* showed the highest yield compared to two other species. This result contrasts with the findings of **Puspita's (2017)** study, which isolated three species of *Sargassum* spp. using six different solvents. They found that *S. aquifolium* exhibited the greatest yield compared to the other two species **(Puspita, 2017)**.

In triphasic extraction, the metabolite extract from *Sargassum* spp. is divided into three parts according to its polarity (Fig. 4). Separation occurs due to different solvent polarities (polar, semi-polar, and apolar). The separation consists of three phases: Phase 1 (top layer) is apolar (Ap), Phase 2 is intermediate (Int), and Phase 3 is nonpolar (Pol). The results of this study did not show the same separation pattern between samples. This contradicts the findings of **Gori *et al*. (2021)**, who predicted a division into the upper phase (25%), medium phase (48%), and lower phase (27%). This phenomenon can be attributed to variations in metabolite compositions among species, metabolite characteristics (such as stability and sensitivity), and mixing proportions **(Seidel, 2012; Roopashree & Dhananjay, 2019; Gori *et al*., 2021)**.

Microorganisms *S. mutans* ATCC 25175, *S. aureus* ATCC 29213, and *E. coli* ATCC 25922 represent Gram-positive and Gram-negative bacteria commonly found in the mouth and skin. *C. albicans* is a pathogenic fungus found in the vagina and oral cavity **(Mmola *et al*., 2016)**. The effect of *Sargassum* spp. extract was tested against these microorganisms. The MIC test of all *Sargassum* spp. samples on the three test bacteria

revealed that a high concentration of extract (100,000ppm) was required to suppress bacterial growth. Therefore, it was classified in the inactive category (MIC >10mg/ mL) **(Tamokou *et al.*, 2017)**. The findings presented in this study are distinct from previous reports on the effects of *Sargassum* extract against *S. aureus* **(Jaswir *et al.*, 2014; Hidayah *et al.*, 2017; Menezes-Silva *et al.*, 2020; Prasedya *et al.*, 2020)**, *S. mutans* **(Amirsharifi *et al.*, 2020; Magesh *et al.*, 2020)**, and *E. coli* **(Sayin *et al.*, 2022)**. Furthermore, the presence of a phase mixture did not alter these results (Table 5). The MIC findings on *C. albicans* **(Sayin *et al.*, 2022)** did not demonstrate any inhibition up to 150,000ppm, so these findings are disregarded. The absence of activity in the extract may be attributed to the inability of the potential active chemicals present in *Sargassum* from Gunungkidul to suppress the tested microorganisms' development effectively.

It is estimated that extracts consisting of several compound molecules can influence each other, both primary and secondary metabolites. The mixture extract phase can produce multi-target mechanisms due to the presence of compounds that can suppress bacterial resistance mechanisms and pharmacokinetic or physicochemical effects that result in increased bioavailability, solubility, resorption rate, neutralization of side effects, and reduced toxicity. However, this investigation did not demonstrate such findings. The MIC in the mixed phase exhibited activity at a concentration of 100,000ppm (Non-active). This anomaly brings difficulties in investigating the mechanism of action and interactions with other compounds in each phase of the extract. It is necessary to prioritize the investigation of the mechanism of action, interactions with other substances, and pharmacokinetic and/or pharmacodynamic profiles of medicinal plant extracts in order to characterize them as potential antimicrobial agents **(Wagner & Ulrich-Merzenich, 2009; Almabruk *et al.*, 2018; Vaou *et al.*, 2021)**. Nevertheless, antimicrobial substances classified as mildly active can be recommended for application as antiseptics rather than antibacterials.

The DPPH antioxidant test showed an IC_{50} value categorized as very strong for the extract Phase 3 of *S. aquifolium* and the extract Phase 1 of *S. ilicifolium*. In both *Sargassum*, the IC_{50} results were categorized as strong in Phases 1 and 2 and the mixture of extracts (mix). The extract from *S. oligocystum* showed an IC_{50} value ranging from not active to moderate. The difference in IC_{50} capability of the three *Sargassum* spp. sample extracts are *S. ilicifolium* > *S. aquifolium* > *S. oligocystum*. Statistical analysis indicates that the inhibition percentage and IC_{50} are primarily influenced by the extraction phase and the type of *Sargassum* (Tables 6 and 7). Several studies of *Sargassum* spp. extraction from Indonesia with a single solvent reported varying IC_{50} values, ranging from 66.1554 (strong) for *S. aquifolium* methanol extract, 2048.0810 (weak) for *S. aquifolium* ethanol extract, 4065.6250 for *S. aquifolium* acetone extract **(Firdaus, 2013)**, and 57.050 (strong) for *Sargassum* sp. methanol **(Noorjanah *et al.*, 2019)**.

The combined extract of *S. oligocystum* exhibited the greatest percentage of inhibition against free radicals, measuring 73.047 ± 0.139% at a concentration of

15.25ppm. This finding is nearly equivalent to ascorbic acid, with an inhibitory power of 78.225 ± 0.187% (15.25ppm). This value is higher than *S. plagyophyllum* obtained from Pasauran Beach, Banten, West Java, Indonesia (% inhibition 37.58 ± 0.03-41.61 ± 0.02) **(Mansauda *et al*., 2018)**, *S. naozhouense* from Leizhou Peninsula of Guangdong Province, China (IC50 17 ± 0.35) **(Peng *et al*., 2018)**, and hot water and ethanol extracts of *S. macrocarpum* from Jeju Island, Republic of Korea **(Kim *et al*., 2022)**. The antioxidant capacity in this study generally showed high results. The observed effect arises due to the apolar to polar characteristics of the solvents employed. Butan-1-ol, being somewhat more polar than EtOAc and immiscible with water, enhances the polarity of both the apolar and intermediate phases. Apolar (non-polar) solvents will bind fat, intermediate (semi-polar) solvents will bind secondary metabolites, and polar solvents will bind carbohydrates **(Gori *et al*., 2021)**.

Metabolomics analysis is an important part of systems biology for product development. The LC-MS technique is widely employed for the multi-target quantification of polar metabolites, particularly in identifying plant-isolated compounds. The information generated on accurate mass molecular ions and characteristic fragmentation ions (production) in LC-MS can be used for compound identification. LC-MS is suitable for analyzing known and unknown metabolites **(Liu & Rochfort, 2014)**. This specification is very suitable for this study in exploring non-target compounds. Several dominant peaks between 20-26 of each species were found at different retention times and m/z. However, there is a constraint that around 35-50% of peaks are not found in the reference. The LC-MS chromatogram shows the results of dereplication; eight compound molecules have high peaks and are identified in the literature (Fig. 6 & Table 8).

Sargachromanols A is one of sixteen new meroterpenoids from the Chromene class in the sesquiterpenes group. Meroterpenoids are a group of secondary metabolites that partly originate from the terpenoid biosynthesis pathway. Meroterpenoids exhibit structural variability, varying lengths of terpenoid chains, and specific terpenoid components that undergo cyclization to form phenolic derivatives ranging from simple compounds to more intricate meroterpenoids. These compounds come from various natural sources, such as animals, fungi, marine organisms, and plants. Undoubtedly, fungi and aquatic organisms are the richest sources of meroterpenoids **(Matsuda & Abe, 2016; El-Demerdash *et al*., 2020)**.

Sargachromanols A was identified in the extract Phase 3 (polar) of *S. aquifolium* at a retention time of 10.09 with m/z 342.479. This compound gave an IC_{50} value of 24.63µg/ mL (ppm) and was categorized as very strong. These results are in line with several reports that sargachromanols A showed antioxidant activity from *S. siliquastrum* from Jaeju Island, Korea, with a radical scavenging activity value of 87-91% from 100µg/ mL **(Jang *et al*., 2005)**, *S. serratifolium* **(Lim *et al*., 2019)**, and the presence of reactive antioxidants from the genus Sargassum, namely sargahydroquinoic acid and

sargachromanol and several of its derivatives **(Farrokhnia, 2020)**. Research on sargachromanols A is very limited, most of which state that the main activity of this type of meroterpenoid is as an antioxidant.

Cystodione I compound molecules are included in the organic compound group of lipids, prenol lipids, and diterpenoids. This group of compounds is one of the most active and was first reported in the Sargassaceae family, *Cystoseria usneoides* species originating from the coast of Tarifa (Spain) **(De los Reyes *et al*., 2016)**. In this study, this compound was found in Phase 3 (polar) of *S. oligocystum* extract and Phase 1 (apolar) of *S. ilicifolium* extract. The antioxidant activity of Phase 3 showed moderate values and was very strong in Phase 1. This indicates that in Phase 1, there is a possibility of cystodione I bioactivity together with other compounds that provide an IC_{50} value of 22.751µg/ mL (very strong). According to **De los Reyes *et al*. (2016)**, cystodiones and cystone, 11-hydroxyamentadione, and amentadione exhibit radical-scavenging activity.

Hexafuhalol B and hydroxyhexaphlorethol are polar organic compounds belonging to the phenylpropanoid and polyketides group of tannins. These compounds are reported to be found in the *Carpophyllum maschalocarpum* (Turner) Greville species of the Sargassaceae family, distributed in New Zealand (CMNPD). **Petchidurai *et al*. (2023)** found that tannins in *S. wightii* and *S. polypodioides* seaweeds exhibit insecticidal properties by exerting their effects through different mechanisms. These tannins disrupt normal physiological metabolism, leading to detrimental alterations in several key body proteins. Consequently, they induce the death of insects *Amrasca devastans* that target cotton leaves **(Petchidurai *et al*., 2023)**. The results of molecular tracing of compounds using LC-MS showed the presence of hexafuhalol B compound, which belongs to the tannin group at a retention time of 17.64 with m/z 794.09666. However, in this study, toxicity tests were not carried out on insects, but it is possible to continue research as an insecticide.

CONCLUSION

Molecular identification confirmed that the three most abundant *Sargassum* species collected from Gunungkidul were *S. oligocystum*, closely related to *S. aquifolium*; *S. ilicifolium*, closely related to *S. yinggehaiense*; and *S. aquifolium*, closely related to *S. oligocystum* and *S. megalocystum*. Triphasic extraction produced three phases: extract, Phase 1 (apolar), Phase 2 (intermediate), and Phase 3 (polar). MIC tests against *S. mutans* ATCC 25175, *S. aureus* ATCC 29213, *E. coli* ATCC 25922, and *C. albicans* indicated that the extracts were classified as non-active. However, antioxidant activity, measured by IC50, showed strong to very strong results for *S. ilicifolium* and *S. aquifolium* extracts. Sargachromanols A and cystodione I are considered the primary compounds responsible for the observed antioxidant activity.

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