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RESEARCH ARTICLE
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In vitro Evaluation of Antibacterial Activity of *Caulerpa Cylindracea* Crude Extract against *Morganella Morganii*

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ABSTRACT

Caulerpa (C.) cylindracea is a species of green seaweed known for its antibacterial bioactivity. Crude extracts of weeds are promising to way to check their antimirobiolal potential against various pathogens. Alongwith other factors, the bioactivity of weed extracts also greatly dependent upon method of its extraction. Consequently, this study aimed to identify the bioactive constituents and antibacterial bioactivity of crude extracts of *C. cylindracea* obtained through the *ultrasound-assisted extraction (UAE)* method against *Morganella morganii* bacteria. The *C. cylindracea* was extracted using UAE with different methanol and acetone ratio and various time. Best extract against *M. morganii* would be further identified its bioactives using LC-HMRS. A15-minute extraction time, a maximum temperature of 40°C, a frequency of 50 Hz, an amplitude of 50%, and a solvent ratio of methanol to acetone (2.5:7.5) in the stolon, showed best antibacterial activity. A total of 24 bioactive compounds is identified, among which three exhibit antibacterial activity: betaine, 5-Fluoro-2-hydroxybenzoic acid, and Isoamylamine. Their inhibitory mechanisms involve the disruption of bacterial membranes and metabolic processes, ultimately leading to cell death.

Keywords: Antibacterial, *Caulerpa cylindracea, , Morganella morganii*, Ultrasound-Assisted Extraction

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INTRODUCTION

Seaweed is an abundant bioactive chemical source due to its capacity to generate a variety of secondary metabolites (Val et al., 2001; Almeida et al., 2020). These metabolites exhibit a diverse range of biological activities, encompassing antibacterial, antiviral, antioxidant, anti-aging, anti-inflammatory, anticancer and antihypertensive properties (Tanna et al., 2018; Yap et al., 2019; Belkacemi et al., 2020; Permatasari et al., 2021; Avila-Romero et al., 2023; Nurkolis et al., 2023; Nurkolis et al., 2023). Seaweeds are categorized into three types according to their pigments: red *(Rhodophyceae)*, brown *(Phaeophyceae)* and green *(Chlorophyceae)* seaweeds (Hamid et al., 2019; Rajivgandhi et al., 2021). *C. cylindracea* is the most prevalent green seaweed; however, despite its potential as an antibacterial agent, its antibacterial biological activity has not been thoroughly investigated, in contrast to *C. racemosa* and *C. lentilifera* (Yap et al., 2019; Marraskuranto et al., 2021; Palaniyappan et al., 2023).

The efficacy of crude seaweed extracts is contingent upon the particular seaweed species, its growth environment, solvent concentration, polarity, and extraction process (Nawaz et al., 2020; Ruslan et al., 2021). Selecting the appropriate extraction method is essential for maintaining the desired quality of the target compounds (Gullón et al., 2020). The UAE is an eco-friendly extraction process that presents several benefits (Muhammad et al., 2023), including low disruption to the stability of bioactive substances and the potential to decrease reliance on harmful solvents. Moreover, the UAE enhances efficiency, lowers extraction duration and is economically viable.

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It improves the quality and yield of bioactive chemicals (Zhu et al., 2017; Cikos et al., 2018; Ummat et al., 2021; Putra et al., 2022). Moreover, UAE is an exceptionally effective technique that circumvents the necessity for elevated temperatures. It has been effectively utilized in the extraction of thermally labile compounds, as evidenced by research undertaken by Zhou et al. (2013) and Skenderidis et al. (2017).

Morganella (M.) morganii, an enteric bacterium within the *Scombroidae* fish group (Rodtong et al., 2005; Ferrario et al., 2012; Gopi et al., 2016), this bacterium possesses the enzyme *histidine decarboxylase*, which facilitates the conversion of histidine into histamine in fish (Tsai et al., 2006; Ruiz-Capillas & Herrero, 2019) The resultant histamine can lead to poisoning, manifesting symptoms such as rash, urticaria, nausea, vomiting, diarrhea, and erythema (Chen et al., 2010; Silva et al., 2011; Evangelista et al., 2016; Elik et al., 2019). The overuse of antibiotics in food production contributes to antimicrobial resistance, threatening global health and sustainability. Seaweed extracts offer a promising alternative, leveraging their natural bioactive compounds with antimicrobial properties to combat pathogens effectively in food systems (Callaway et al., 2021).

The utilization of seaweed crude extract as an antibacterial agent primarily targets a range of human pathogenic bacteria, including *Salmonella paratyphi* (Gram-negative), *Vibrio cholerae* (Gram-negative), *Escherichia coli* (Gram-negative), *Bacillus subtilis* (Gram-positive), and *Staphylococcus* (Gram-positive) (Avila-Romero et al., 2023; El-Gammal et al., 2024; Honey et al., 2024). Consequently, it is essential to examine the efficacy of *C. cylindracea* crude extract as an antibacterial agent against *M. morganii* and to assess the bioactive component of the seaweed utilizing the *UAE* approach.

MATERIALS & METHODS

Collection and Preparation of *Caulerpa Cylindracea*

C. cylindracea was obtained from Puntondo Beach in Takalar Regency, South Sulawesi Province, Indonesia. The sampling location's coordinates are 05035'33.87'' S 119029'00.92'' E. Fig. 1 illustrates the specimen of *C. cylindracea* and the precise location of its collection. Mud or any other clinging contaminants were removed using pure water. After that, the material was diminished by slicing it into segments, which are further chopped and ground to produce powdered seaweed. The samples were kept at 4°C until future usage.

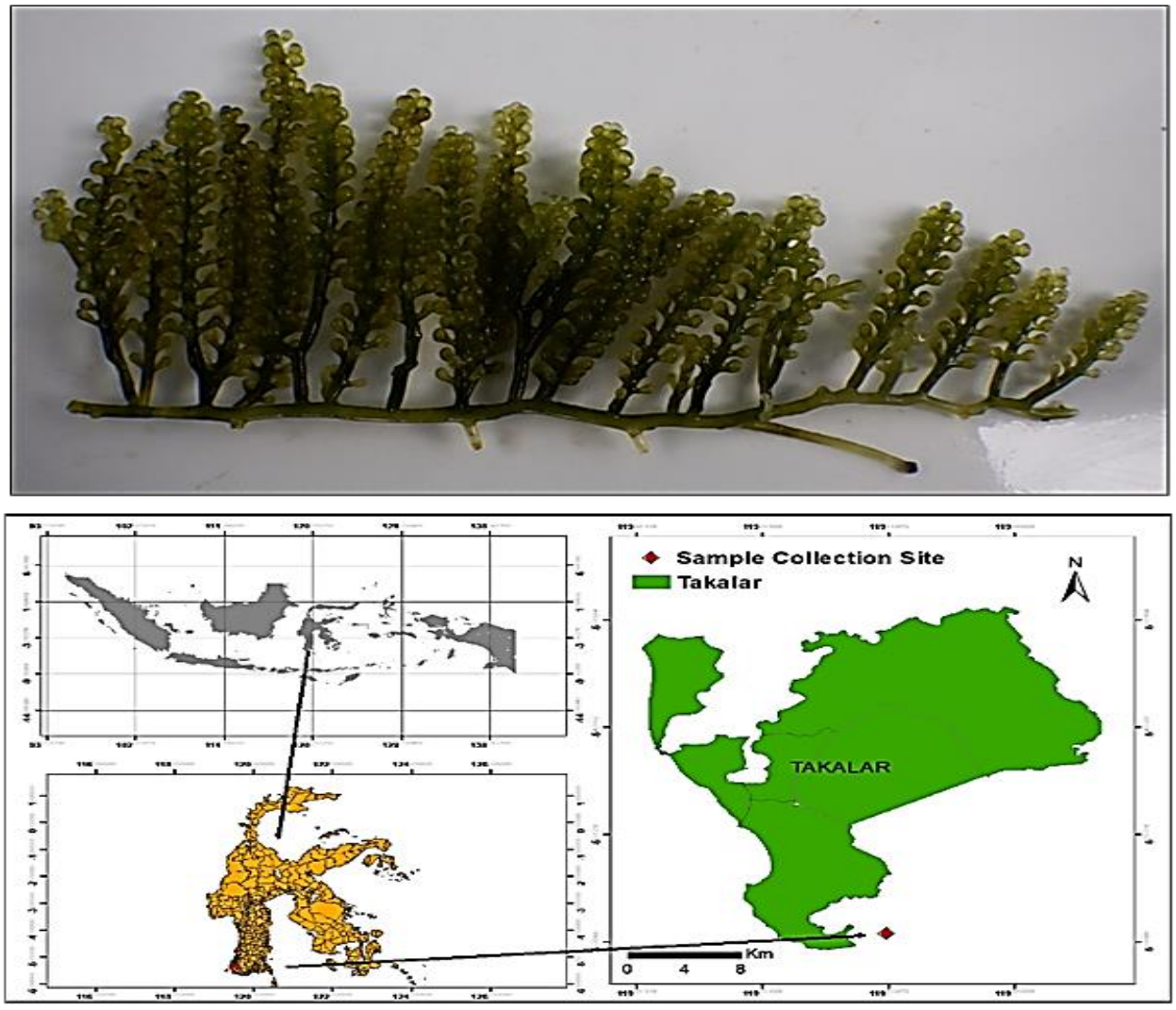

Fig. 1: Physical map indicating site of sample collection and *Caulerpa cylindracea*.

Ultrasound-Assisted Extraction* of *C. Cylindracea

The extraction process employs the *UAE* method utilizing the ultrasonicator model UCD-950 (Biobase) with a power supply of 1000W and a voltage of 220V at 50Hz. The extraction procedure is described by (Kadam et al., 2015) with modifications. Briefly, 10g of powdered simplisia from each seaweed component, specifically the stolon, ramuli, and complete organism (whole), were extracted using 100ml of solvent. The methanol and acetone were utilized in several ratios: 5:5 (A), 7.5:2.5 (B), 2.5:7.5 (C), pure methanol (D), and pure acetone (E). The extraction was conducted for 15, 25 and 35min at a maximum temperature of 40°C, with an amplitude of 50%. The collected materials were subsequently centrifuged at 6000rpm for 15min. The supernatants were evaporated using a vacuum evaporator at 40°C for 25min. The extracts were quantified and stored at -20°C. The yield value of each treatment extract of *C. cylindracea* was determined by weighing the extract and applying the subsequent formula:

$$\text{Yield} = \frac{\text{Amount of product}}{\text{Amount of sample}} \times 100\%$$

Antibacterial Activity of *C. Cylindracea* Crude Extract

M. morganii was obtained from the microbiology laboratory at the Faculty of Medicine, Brawijaya University. The analysis utilized the disc diffusion method, as described by Yusuf et al. (2020), with slight modifications. 20ml of sterile nutrient agar was dispensed into a petri dish and incubated at 37°C, followed by the inoculation of approximately 0.1ml of *M. morganii* bacterial culture. Dissolve the resultant extract in a 10% DMSO solution containing 0.5% Tween 80 at 500 (I) and 1000mg/ml (II), respectively then sterilise it with a 0.45µm filter membrane. Apply the solution onto blank paper discs and, using sterile tweezers, position them on a Sodium Agar plate within a petri dish. Subsequently, incubate at 37°C for 24hrs. The negative control employed a 10% DMSO solution. Antibacterial activity is classified as highly active when the zone exceeds 10mm, mildly active for 7-10mm, minimally active for 6-7mm, and inactive when the zone is less than 6mm (Chandra et al., 2011).

Identification of Bioactive Compounds by Liquid Chromatography High-Resolution Mass Spectrometry *(LC-HRMS)*

The LC-HRMS analysis of *C. cylindracea* crude extract was conducted at the integrated research laboratory of Brawijaya University. The extracted sample was diluted with ethanol solvent to achieve an appropriate density, neither overly concentrated nor excessively dilute, resulting in a final volume of 1500µL. The mixture was then vortexed at 2000 rpm for 2min and centrifuged at 6000 rpm for 2min. Obtain the supernatant, filter it through a 0.22µm syringe filter, and transfer it into a vial for insertion into the autosampler prior to injection into the LC-HRMS. The liquid chromatography system is an HPLC utilizing the thermo scientific dionex ultimate 3000 RSLCnano, equipped with a microflow meter and an analytical column, hypersil GOLD PFP, measuring 50 x 1mm with a particle size of 1.9µm. Solvents: 0.1% Formic acid in Water (A), 0.1% in Acetonitrile (B). Analytical flow rate of 40µL/min with a runtime of 30min in a column oven at 30°C. The HRMS equipment is the thermo scientific Q exactive, operating at a full scan resolution of 70,000 and data-dependent MS2 resolution of 17,500. The runtime is 30min, with positive polarity and data processing is conducted using Compound Discoverer software in conjunction with the mzCloud MS/MS Library.

Scanning Electron Microscopy (SEM) Observation

Cell observation of *M. morganii* pertains to the modification by Wang et al. (2022). Bacterial cells were produced by centrifugation at 8500 rpm for 5min, followed by three washes with 0.01M Phosphate Buffered Saline (PBS). The resultant cell pellets were dehydrated in graded ethanol solutions (30, 50, 70, 85, 90 and 100%, respectively) and washed twice with isopentyl acetate. The coating process was conducted using a sputter coater (Quorum type Q150R S Plus) with a sputter current of 20 mA and a duration of 35 seconds, utilizing gold material. Cell morphology was then observed with a scanning electron microscope (SEM) instrument, FEI Quanta FEG 650, equipped with an EDS detector: X-act Oxford Instrument and EDS software: AZtecOne. This analysis was conducted at the integrated research laboratory of Brawijaya University.

Statistical Analysis

Data were analyzed using ANOVA, and if a significant difference between the treatments was seen, the analysis proceeded with the Tukey HSD (High Significant Difference) test. Data analysis employed IBM SPSS Statistics 27 software.

RESULTS & DISCUSSION

Extract Yield of *C. Cylindracea* by UAE

The maximum extract yield on the stolon part was 10.82%, while the minimum was 2.48%. The maximum yield on ramuli parts was 11.55%, the minimum was 2.98%, the best yield was 11.18%, and the lowest was 2.61% (Table 1). The extract of the ramuli part of *C. cylindracea* produced the maximum extract yield (11.55%) when using pure methanol as a solvent for 35min, with statistical significance ($P<0.05$).

Table 1: Effect of solvent concentration, extraction time on extract yield of various parts of *C. cylindracea* by UAE

Solvent Concentrations	Extraction Time								
	Stolon			Ramuli			Whole		
	35' (%)	25' (%)	15' (%)	35' (%)	25' (%)	15' (%)	35' (%)	25' (%)	15' (%)
A	7.59±0.03f	7.10±0.02f	6.12±0.02e	8.66±0.025h	7.99±0.04f	6.87±0.02e	8.05±0.025h	7.54±0.02f	6.96±0.026e
B	8.61±0.02h	8.00±0.05g	7.08±0.02f	9.81±0.025i	8.83±0.025h	7.48±0.02f	9.10±0.025i	8.38±0.02h	7.19±0.026f
C	4.17±0.02d	3.76±0.025c	2.93±0.025b	4.83±0.02d	4.31±0.015d	3.30±0.025c	4.55±0.02d	4.07±0.02d	3.16±0.025c
D	10.82±0.025m	9.89±0.04i	8.43±0.02h	11.55±0.02o	10.42±0.02m	8.63±0.025h	11.18±0.025n	10.20±0.025m	8.69±0.02h
E	3.25±0.02c	2.98±0.025b	2.48±0.02a	4.22±0.03d	3.81±0.025c	2.98±0.02b	3.55±0.015c	3.24±0.02c	2.61±0.02b

Ratio of solvent concentrations between methanol and acetone: A (5:5), B (7.5:2.5), C (2.5:7.5), D (pure methanol), E (pure acetone). Different superscript letters within the same column or row signify statistically significant differences ($P<0.05$) among the groups

The yields from all treatments indicate that applying polar solvents (methanol) results in maximum yields. In contrast, reducing solvent ratio, specifically with the inclusion of semi-polar solvents (acetone), leads to a loss in yield. The polarity of components in seaweed influences the yield of the extract (Agregán et al., 2017). Consequently, the solvent's polarity and the extraction duration significantly affect the extraction process (Kumar et al., 2020) Applying polar solvents, such as methanol, yields higher results than semi-polar solvents like acetone (Airanthi et al., 2011; Higgins et al., 2019; Afrin et al., 2023). In addition, modern extraction techniques, such as microwave-assisted extraction and ultrasonic-assisted extraction, enhance seaweed extract yields by optimizing the interaction between the solvent and the seaweed matrix (Dulanlebit & Hernani, 2023). UAE has emerged as an effective technique for enhancing the yield of seaweed extracts. This method employs ultrasonic waves to generate cavitation bubbles in the solvent, which subsequently disrupt the cell walls of the seaweed, thereby facilitating the release of bioactive compounds into the solvent (Shukla et al., 2019). The efficacy of UAE is ascribed to its capacity to improve mass transfer and augment the solubility of extracted compounds, which is advantageous for the extraction of important chemicals from seaweed (Shayen et al., 2023). The choice of solvent and extraction conditions greatly influence the yield of seaweed extracts, whereby the use of dried seaweed, compared to fresh varieties, can markedly improve extraction efficiency due to greater solvent accessibility to cellular structures (Gomes et al., 2024), the implementation of UAE facilitates the utilization of eco-friendly solvents, aligning with the tenets of green chemistry (Dulanlebit & Hernani, 2023; Muhammad et al., 2023)

Table 2: Zone of Inhibition of *C cylindracea* extract of *M. morganii* bacteria

Solvent Concentrations	Extraction Time															
	Stolon						Ramuli						Whole			
	25'				15'		25'		15'		35'		25'		15'	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
A	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6.44±0.75b	8.11±2.13d	6±0.00a	7.56±1.91c	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a
B	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	7.22±1.61c	6±0.00a	6±0.00a
C	6±0.00a	6±0.00a	6±0.00a	8.11±2.13d	8.46±2.27d	8.51±2.34d	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6.26±0.46b	6±0.00a	6±0.00a
D	6±0.00a	6±0.00a	6±0.00a	6±0.00a	7.21±1.51c	7.28±1.87c	6±0.00a	6±0.00a	6±0.00a	6.82±1.44b	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a
E	6±0.00a	6±0.00a	6±0.00a	7.27±1.81c	7.12±1.44c	7.23±1.67c	6±0.00a	8.91±2.52d	6±0.00a	6.71±1.22b	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a	6±0.00a

Ratio of solvent concentrations between methanol and acetone: A (5:5), B (7.5:2.5), C (2.5:7.5), D (pure methanol), E (pure acetone). Different superscript letters within the same column or row signify statistically significant differences ($P<0.05$) among the groups

Antibacterial Activity of *C. Cylindracea*

The inhibitory zone produced by the crude extract of *C. cylindracea* on the stolon, ramuli, and whole at concentrations of 500 and 1000mg/ml, respectively (Table 2). The results indicated that the stolon segment, subjected to an extraction duration of 15min and a solvent ratio of methanol to acetone at 2.5:7.5, exhibited optimal antibacterial efficacy, demonstrating moderate activity at 8.46 and 8.51mm, respectively.Other treatments had marginal antibacterial action at a concentration of 500mg/ml, whereas *C. cylindracea* crude extract at 1000mg/ml demonstrated predominantly marginal bacterial activity, with the remainder being mildly active.

The solvent concentration C (methanol: acetone 2.5:7.5) exhibited the most significant antibacterial activity against *M. morganii* at a concentration of *C. Cylindracea* crude extract 500 mg/ml, categorized as mildly active. At the same time, other treatments demonstrated mildly active antibacterial activity ranging from 6 to 7mm. This suggests that the semi-polar solvent (acetone) predominates. In contrast, the polar solvent (methanol) is secondary in achieving optimal antibacterial activity of the crude extract of *C. cylindracea* against *M. morganii*. The antibacterial efficacy of seaweed is affected by various factors, such as its habitat, growth stage, extraction technique, and solvent polarity (Godlewska et al. , 2016; Vimala & Poonghuzhali,

2017; Nofal et al., 2022), with methanol crude extract yielding the most significant gram-positive antibacterial activity, while acetone crude extract demonstrates gram-negative antibacterial activity (Perez et al., 2016; Moubayed et al., 2017).

Acetone is a more appropriate solvent for extracting antibacterial chemicals from green seaweeds that exhibit antibacterial activity against diverse gram-negative bacteria (Manivannan et al., 2011; Madkour et al., 2019). Acetone is recognized for its ability to extract a broader spectrum of polar and non-polar molecules, perhaps augmenting its antibacterial efficacy against Gram-negative bacteria, which are often more resistant due to their thicker cell walls (Perez et al., 2016; Saleh et al., 2019). The efficacy of solvents employed in extraction is contingent upon the species of seaweed and the specific bacteria targeted, suggesting that no singular solvent is ideal for the extraction procedure (Yap et al., 2019).

LCHRMS Analysis

LC-HRMS was performed to detect bioactive compounds in the crude extract of *C. cylindracea* from the stolon part, which was the most effective component against the antibacterial *M. morganii* (Table 3). The extract contains bioactive compounds, including betaine (Fig. 3), 5-Fluoro-2-hydroxybenzoic acid, and Isoamylamine (Fig. 4), which exhibit antibacterial properties through mechanisms that disrupt bacterial membranes and metabolism, leading to bacterial death, as shown by SEM results in Fig. 5. Betaine has antibacterial bioactivity against gram-positive and gram-negative microorganisms, such as *Escherichia coli*, *Salmonella typhimurium,* and *Staphylococcus aureus* have been reported (Lindstedt et al., 1990; Ahlström et al., 1999; Birnie et al., 2000; Liu et al., 2020), Betaine exerts its antimicrobial effect primarily by interacting with the negatively charged bacterial membrane surface, where its positively charged groups bind, leading to membrane destabilization and subsequent bacterial cell death (Derwin et al., 2022).

Betaine demonstrates antibacterial properties by disrupting bacterial membranes and metabolism (Massai et al., 2019; Derwin et al., 2022). Betaine engages with the anionic surface of the bacterial membrane. The positively charged groups in betaine can adhere to these surfaces, leading to membrane instability and bacterial cell death. High quantities of betaine can alter osmotic pressure, releasing cellular contents and subsequent cell lysis (Stadmiller et al., 2017).

SEM Analysis

The application of crude extract from *C. cylindracea* against *M. morganii* demonstrated that bacterial cells treated with 500mg/mL (Fig. 2) exhibited shrinkage. Normal cells measure 3.70µm in length and possess a diameter of 0.81µm, whereas cells exposed to crude extract of *C. cylindracea* measure 0.48µm in length and have a diameter of 0.12µm (Fig. 6). The antibacterial mechanism of seaweed crude extract can lead to the lysis of cell walls, releasing substantial fluid and causing cellular shrinkage and death (Saleh et al., 2019). Furthermore, the cell membrane cannot endure the cytoplasm's internal pressure, resulting in leakage. The discharge of cytoplasmic constituents, such as proteins and nucleic acids, intensifies the decline in cellular function, resulting in cell death (Sutharshan et al., 2021).

Conclusion

The crude extract of *C. cylindracea*, obtained via the *UAE* method, exhibits inhibitory activity against *M. morganii*. LC-HRMS analysis reveals the presence of three bioactive compounds with antibacterial properties: betaine, 5-Fluoro-2-hydroxybenzoic acid, and Isoamylamine, among the 24 bioactive compounds identified in the crude extract of *C. cylindracea*. The stolon extract of *C. cylindracea*, prepared with a methanol:acetone (2.5:7.5) solvent ratio and a 15min extraction time, demonstrated the highest antibacterial

Table 3: Bioactive compounds identified from *Caulerpa cylindracea* crude extract through LC-HRMS

No	Bioactive	Formula	Calc. MW	RT [min]	Area (Max.)
1	2-(1,3,5-Triazin-2-yl)-1,2-dihydro-1,2,3,4-tetrazine	C5 H5 N7	163.06017	1.542	7.46E+09
2	Nitromethanetriol	C H3 N O5	109.00139	1.539	1.48E+09
3	2-(3-Methyl-5-nitro-3H imidazol-4- ylamino)-ethanol	C6 H10 N4 O3	186.07611	1.526	1.03E+09
4	µ6004C2Z3	C7 H13 Cl O2	164.06105	1.542	9.40E+08
5	2-(1,3,5-Triazin-2-yl)-1,2-dihydro-1,2,3,4-tetrazine	C5 H5 N7	163.06017	1.239	7.62E+08
6	3-Oxa-2,4,6,8,9 pentaazabicyclo[3.3.1]nona- 1(9),5,7-trien-7-amine	C3 H4 N6 O	140.04445	1.542	6.64E+08
7	5-Fluoro-2-hydroxybenzoic acid	C7 H5 F O3	156.02196	1.638	5.99E+08
8	2-(1,3,5-Triazin-2-yl)-1,2-dihydro-1,2,3,4-tetrazine	C5 H5 N7	163.06017	1.703	3.29E+08
9	Betaine	C5 H11 N O2	117.07903	1.088	2.85E+08
10	1H-Imidazo[4,5 e][1,2,3,4]tetrazine	C3 H2 N6	122.03386	1.541	2.41E+08
11	2-Phenyl-2-[(1E)-1-propen-1-yl]- 1,3,5,2,4,6-trioxatrisilinane	C9 H14 O3 Si3	254.02399	1.514	2.30E+08
12	Nitromethanetriol	C H3 N O5	109.00142	1.217	1.66E+08
13	5 (Dimethylamino)tetrazolo[1,5- a][1,3,5]triazin-7(3H)-one	C5 H7 N7 O	181.07079	1.485	1.07E+08
14	2-Ethynyl-1-benzofuran	C10 H6 O	142.04201	1.542	9.84E+07
15	2-(3-Methyl-5-nitro-3H-imidazole-4- ylamino)-ethanol	C6 H10 N4 O3	186.07611	1.228	8.18E+07
16	N-Chloro-N-methyl-1-Butanesulfonamide	C5 H12 Cl N O2 S	185.02767	1.166	6.75E+07
17	BMEDA	C10 H24 N2 S2	236.13828	1.841	6.18E+07
18	Piracetam	C6 H10 N2 O2	142.07416	1.257	5.42E+07
19	Choline	C5 H13 N O	103.09988	1.353	3.93E+07
20	Piracetam	C6 H10 N2 O2	142.07416	1.093	3.27E+07
21	N,Ndiethylmethanesulfonamide	C5 H13 N O2 S	151.06659	1.03	2.37E+07
22	Tetraethynylethene	C10 H4	124.03138	1.544	2.37E+07
23	BMEDA	C10 H24 N2 S2	236.13828	1.713	2.04E+07
24	Isoamylamine	C5 H13 N	87.10511	1.388	5.95E+06

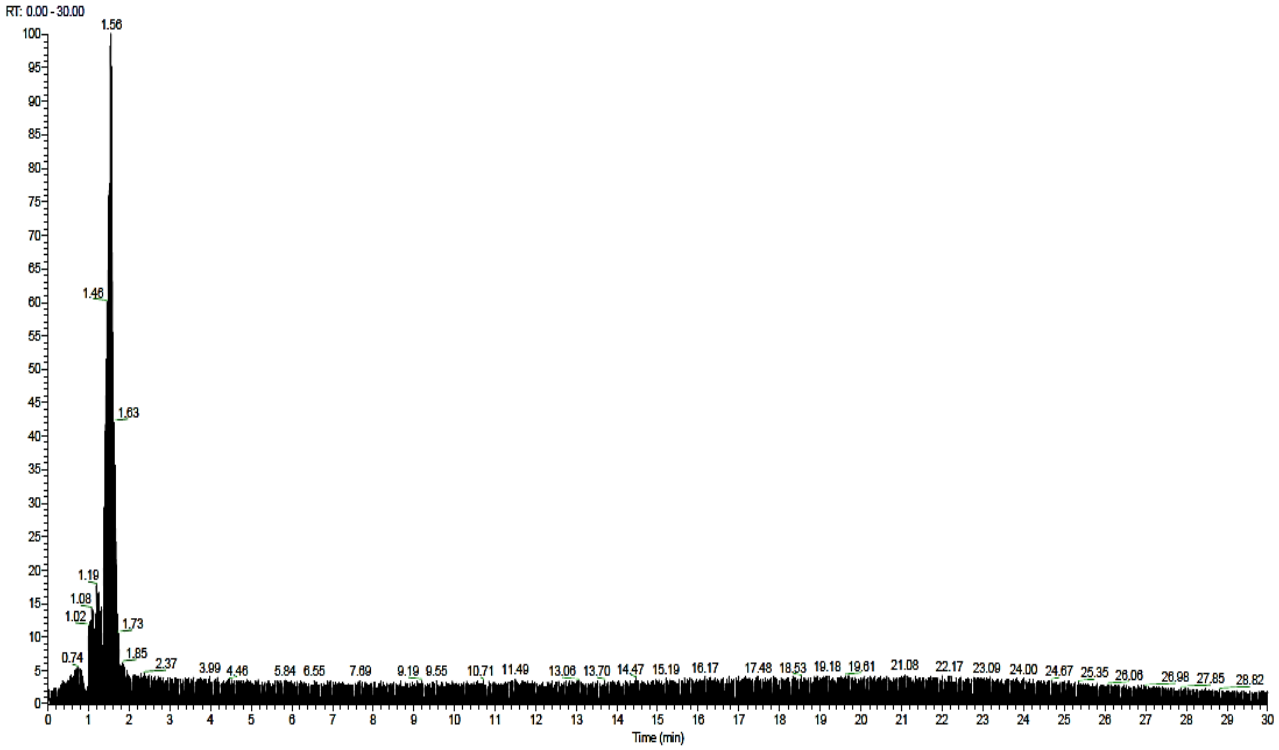

Fig. 2: Total ion chromatogram.

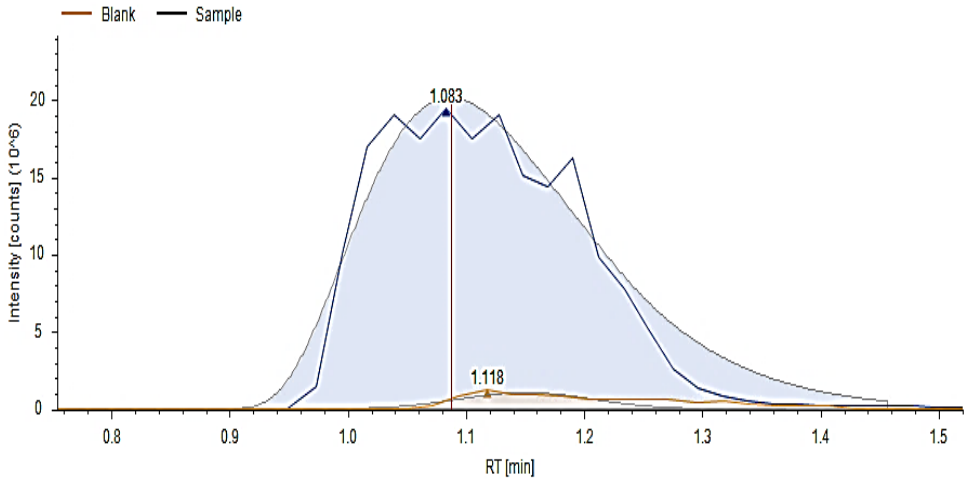

Fig. 3: Chromatogram of betaine.

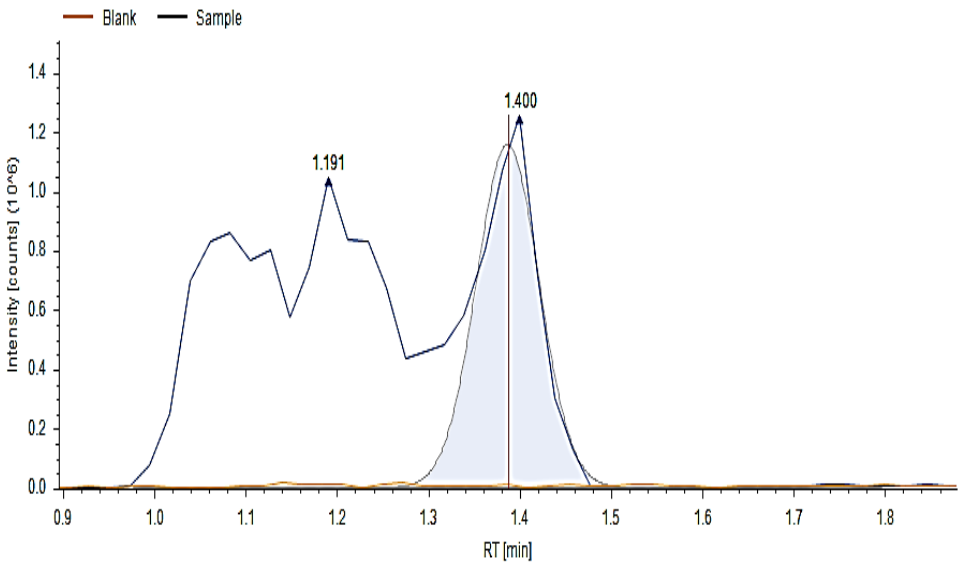

Fig. 4: Chromatogram of Isoamylamine.

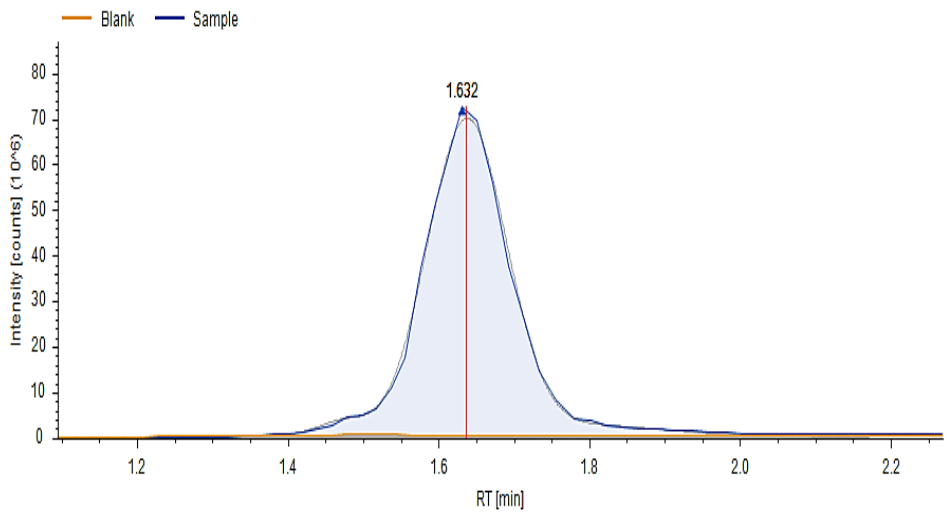

Fig. 5: Chromatogram of 5-Fluoro-2-hydroxybenzoic acid.

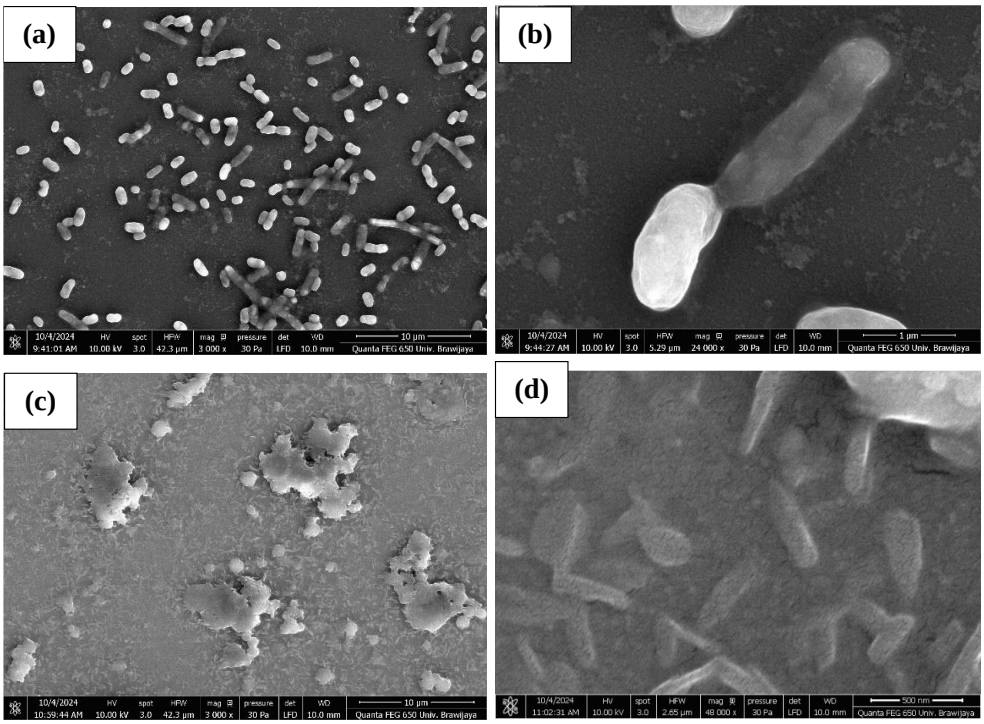

Fig. 6: SEM micrograph of *Morganella morganii* (a), (b) without treatment; (c), (d) with treatment.

efficacy compared to extracts from other plant parts and conditions, showcasing its potential as an effective bioactive agent. Alternative treatments exhibit marginal antibacterial action at 500mg/ml of crude extract *C. cylindracea*. In contrast, at 1000mg/mL, the bacterial activity is predominantly marginal, with the remainder being mildly active. Subsequently, to validate the antibacterial bioactivity of these three components, forthcoming research must undertake the fractionation or purification of the crude extract derived from *C. cylindracea*.

Author's Contribution: MM: Designed the research project, performed the experiments, and analyzed the data, writing-original draft preparation, and editing. AAP: Designed the research project, conceptualization, original draft preparation and editing, and supervision. MF: Designed the research project, wrote the original draft preparation, and validated it RN: Designed the research project, writing- original draft preparation, validation.

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