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ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *Caulerpa racemosa* EXTRACT WITH A COMBINATION OF SOLVENTS AND VARIATION OF MACERATION TIME

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Abstract

Seaweed from the Chlorophyta and Rhodophyta groups is widely used as food in Indonesia. *Caulerpa racemosa*, a type of Chlorophyta, contains bioactive antioxidant and antibacterial compounds, including terpenoids, polyphenols, alkaloids, and flavonoids. This study aimed to determine the optimal extraction conditions (concentration, solid:solvent ratio, and duration) of *C. racemosa* using Deep Eutectic Solvents (DES) based on antioxidant and antibacterial activity. This study used independent variables (5%, 10%, and 15%), solid:solvent ratio (1:10, 1:15, and 1:20); and maceration time (24, 48, and 72 h maceration times). The optimization process was carried out using the response surface methodology (RSM) with a Box–Behnken design using Design Expert software v13. The antioxidant activity of *C. racemosa* extract ranged from 28.46±0.067 to 50.50±0.067 mg TE/g dry weight (DW). The concentration of the DES solvent and maceration time had a significant effect (p<0.05), whereas the solid-to-solvent ratio did not show a significant effect (p>0.05). Antioxidant activity decreased at 15% DES owing to differences in solvent polarity and viscosity, whereas lower viscosity increased the extraction efficiency. Optimal conditions were obtained at 10% DES, a ratio of 1:20, and a maceration time of 72 h. Antibacterial activity against *Vibrio harveyi* (13 mm) and *Vibrio parahaemolyticus* (14.8 mm) was also highest under these conditions, possibly due to an increase in the bioactive compound extracts. These results indicate that the optimized extraction method is suitable for developing aquaculture and functional food products.

Keywords: bioactive compounds, DES, response surface methodology, *Vibrio harveyi*, *Vibrio parahaemolyticus*

Aktivitas Antioksidan dan Antibakteri Ekstrak *Caulerpa racemosa* dengan Kombinasi Pelarut dan Variasi Waktu Maserasi

Abstrak

Rumput laut dari kelompok Chlorophyta dan Rhodophyta banyak dimanfaatkan di Indonesia sebagai bahan pangan. *Caulerpa racemosa*, salah satu jenis Chlorophyta, mengandung senyawa bioaktif

antioksidan dan antibakteri di antaranya terpenoid, polifenol, alkaloid, dan flavonoid. Tujuan penelitian untuk menentukan kondisi ekstraksi optimal (konsentrasi, rasio padatan:pelarut, dan lama waktu) *C. racemosa* menggunakan Deep Eutectic Solvents (DES) berdasarkan aktivitas antioksidan dan antibakteri. Penelitian ini menggunakan variabel bebas (konsentrasi DES 5, 10, 15%), padatan : pelarut (1:10, 1:15, 1:20) dan lama waktu maserasi (24, 48, 72 jam). Proses optimasi dilakukan menggunakan metode response surface methodology (RSM) dengan rancangan Box–Behnken melalui perangkat lunak Design Expert versi 13. Aktivitas antioksidan ekstrak *C. racemosa* berkisar antara 28,46±0,067 hingga 50,50±0,067 mg TE/g DW. Konsentrasi pelarut DES dan waktu maserasi berpengaruh signifikan (p<0,05), sedangkan rasio padatan terhadap pelarut tidak menunjukkan pengaruh signifikan (p>0,05). Aktivitas antioksidan menurun pada 15% DES akibat perbedaan polaritas dan viskositas pelarut, sementara viskositas yang lebih rendah meningkatkan efisiensi ekstraksi. Kondisi optimum diperoleh pada 10% DES, rasio 1:20, dan waktu maserasi selama 72 jam. Aktivitas antibakteri terhadap *Vibrio harveyi* (13 mm) dan *Vibrio parahaemolyticus* (14,8 mm) juga tertinggi pada kondisi tersebut, kemungkinan karena peningkatan ekstrak senyawa bioaktif. Hasil ini menunjukkan bahwa metode ekstraksi teroptimasi ini dapat direkomendasikan untuk pengembangan produk akuakultur dan pangan fungsional.

Kata kunci: DES, response surface methodology, senyawa bioaktif, *Vibrio harveyi*, *Vibrio parahaemolyticus*

INTRODUCTION

In Indonesia, water covers approximately 70% of the archipelago, presenting significant potential for seaweed cultivation (Priono 2016). Zhang *et al.* (2022) reported that total seaweed production (aquaculture and wild catch) increased nearly threefold, from 118,000 tons to 358,200 tons, during the period 2000–2019. Seaweed is widely used by coastal communities in Indonesia as a vegetable or to complement staple foods. These people commonly consume seaweed from the Chlorophyta and Rhodophyta, with *Caulerpa racemosa* being a widely consumed seaweed from the Chlorophyta phylum (Ma'aruf *et al*., 2013).

C. racemosa is known for its ability to produce antioxidant compounds, such as terpenoids, polyphenols, alkaloids, and flavonoids (Nufus *et al*., 2017; Erfani, 2019; Hidayat *et al*., 2020). Previous studies have indicated that the ethanol extract of *C. racemosa* prepared by maceration has the highest antioxidant activity at a concentration of 0.7 mg/L, as estimated by the DPPH method, with an inhibition rate of 62.63% (Mokoginta *et al*., 2021). Antioxidants in seaweeds can combat free radicals in the body, which are molecules with one or more unpaired electrons in their outer orbit, rendering them highly labile and reactive (Sari *et al*., 2018). The instability and reactivity of free radicals can damage proteins, DNA, nucleic acids, and cell membranes in humans and other animals (Erfani, 2019). Diseases caused by exposure to free radicals include heart attacks, cancer, cataracts, and decreased kidney function (Fakhriah *et al*., 2019). In addition to its antioxidant properties, *C. racemosa* contains other bioactive components that can act as antibacterial agents (Tapotubun *et al*., 2016). These bioactive components include tannins, terpenoids, sulfated polysaccharides, phenolic compounds, saponins, glycosides, steroids, and flavonoids, among others. *C. racemosa* extracted using ethanol by the maceration method exhibited the greatest antibacterial activity at a concentration of 0.05 mg/mL (Ragunath & Ramasubramanian, 2024).

Maceration is a widely applied extraction method for seaweeds because it is simple, inexpensive, and avoids the degradation of heat-sensitive bioactive compounds (Martins *et al.,* 2023). However, the choice of solvent significantly influences the extraction efficiency. Conventional organic solvents such as methanol, ethyl acetate, and n-hexane are often used; however, their volatility, toxicity, and unsuitability for food applications limit their use (Erfani, 2019). Therefore, alternative solvents that are safer and more environmentally friendly are needed. In recent years, Deep Eutectic Solvents (DES) have emerged as promising green solvents for the extraction of bioactive compounds from natural resources, including antioxidants (Ivanović *et al.,* 2020). Despite their potential advantages, the application of DES in the extraction of *C. racemosa* has not yet been reported, creating an opportunity for further investigation (Prayogo *et al.,* 2024).

DES are generally prepared by mixing a hydrogen bond donor (HBD), such as an alcohol or carboxylic acid, with a hydrogen bond acceptor (HBA), such as a quaternary ammonium salt (Wojciechowski *et al.*,2020). Although various combinations can be formulated, trial and error remains the most common approach for identifying suitable solvents, especially for natural product extraction. In this study, a DES combination of ethanol (as HBA) and ethylene glycol (as HBD) was used for the maceration of *C. racemosa* (Alshammari, 2022). Ethanol was chosen because it is effective in preserving and extracting bioactive compounds, particularly those with antioxidant activity, whereas ethylene glycol provides a low-freezing-point DES with relatively low viscosity (Widarta & wiadnyani, 2019). This study aimed to determine the optimal extraction conditions (concentration, solid:solvent ratio, and duration) of *C. racemosa* using Deep Eutectic Solvents (DES) based on antioxidant and antibacterial activity.

MATERIAL AND METHODS

Study Area

C. racemosa samples were collected from the Jepara Brackish Water Aquaculture Center (BBPBAP), Jepara, Central Java, Indonesia (Figure 1). The species was identified at the Faculty of Fisheries and Marine Sciences of the Airlangga University. Preparation of DES (ethanol:ethylene glycol), extraction, and antioxidant activity assays (2,2-diphenyl-1-picrylhydrazyl [DPPH] method) were conducted at the Food and Analytical Chemistry Laboratories, Faculty of Fisheries and Marine Sciences, Airlangga University, while viscosity testing of DES was performed at the Basic Physics Laboratory, Faculty of Science and Technology, Airlangga University.

Experimental Design

The research was designed using the Design Expert v13 program with the Response Surface Methodology Box-Behnken design and five central points. This study used the independent variables of DES concentration (X1), solid:liquid ratio (X2), and extraction time (X3). The independent variables were determined based on previous research by Putraisya *et al.* (2021), who used DES for maceration extraction of antioxidants and antibacterials from *Paracaudina australis* at DES concentrations of 5, 10, and 15% and maceration times of 24, 48, and 72 h. The additional independent variable of the solid-to-liquid ratio was included following Putraisya *et al.* (2021), suggesting further studies to improve the antioxidant and antibacterial activities. The main raw material used was *C. racemosa,* obtained from the Centre for Brackish Water Fisheries (BBPAP), Jepara. Equipment included a Fomac® FCT Z200 grinder for grinding the seaweed into fine particles to maximize surface area during extraction, a 2,100 g analytical balance (Ohaus® PA2102 Pioneer) for weighing, 50–250 mL measuring cups, a 250 mL volumetric flask, a 10 mL pipette, and a 100–1,000 µL micropipette (Dragon Lab Scientific® MicroPette) for precise solvent measurement.

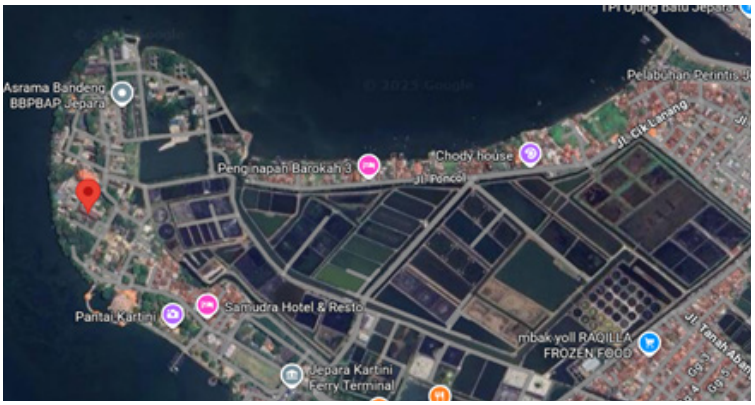

Figure 1 The Jepara Brackish Water Aquaculture Center (BBPBAP) is located at Cik Lanang St, Rw. IV, Bulu, Kec. Jepara, Jepara Regency, Central Java 59418, Indonesia

Extraction Process

Deep eutectic solvents (DES) were prepared by mixing ethanol as a hydrogen bond acceptor (HBA) and ethylene glycol as a hydrogen bond donor (HBD) in a molar ratio of 1:2. A total of 10 mL of ethanol and 19.15 mL of ethylene glycol were mixed in an Erlenmeyer flask and heated at 60°C for ±5 min until a clear and homogeneous solution was obtained. The resulting pure DES solution was diluted with distilled water to obtain concentrations of 5, 10, and 15%, which were used in the extraction stage.

The dried *C. racemosa* powder was mixed with the DES solution using solid-to-solvent ratios of 1:10, 1:20, and 1:30 according to the Box–Behnken design. The maceration process was carried out at room temperature (26–29°C) to prevent the degradation of heat-sensitive bioactive compounds. Homogenization was performed using a Dragon Lab® MX-S vortex, and the separation between the extract and residue was performed using a Hettich® EBA 20 centrifuge.

Antioxidant Activity Assay

Baliyan *et al.* (2022) described that the antioxidant activity of *C. racemosa* extracts was evaluated using the DPPH radical scavenging assay. The DPPH stock solution was prepared in methanol and adjusted to an absorbance of 517 nm using a UV-Vis spectrophotometer (Human Corporation® X-ma 1200). The extract samples were diluted to the appropriate concentrations, mixed with the DPPH solution, and incubated in the dark at room temperature for 30 min. The decrease in absorbance at 517 nm was measured using a methanol blank. Antioxidant activity was expressed as Trolox Equivalent (TE) per gram of dry weight (mg TE/g dw), based on a calibration curve constructed using Trolox as the standard. All measurements were performed in triplicate, and the results are expressed as the mean ± standard deviation (SD). The levels of the independent variables are listed in Table 1, and the combinations of treatments are listed in Table 2.

The values of the independent variables, namely DES concentration (5, 10, and 15%), solid: liquid ratio (1:10, 1:15, 1:20), and extraction duration (24, 48, and 72 h), were input into the Design Expert V13 program with the Response Surface Methodology Box-Behnken Design method to randomize and obtain a combination of 17 treatments (Table 2).

Antibacterial Assay

Antibacterial tests were performed using the disk diffusion method to determine the best *C. racemosa* extract fraction that inhibited *V. parahaemolyticus* and *V. harveyi*. The diffusion process used a paper disc with a diameter of 6 mm to evaluate the antibacterial sensitivity by observing the inhibition zone around the disc containing the extract. Observations began with bacterial cultures using Tryptic Soy Agar (TSA) or tryptic Soy Broth (TSB) media (Huyyirnah & Fitriyani, 2020). The bacteria were evenly plated on solid media using sterile cotton. The paper discs were wetted with each fraction and placed on top of the solid medium after the liquid evaporated. Next, the medium was incubated at 35 °C for 24 h, and the zone of inhibition was measured. Bacterial growth around the disk indicated that the extract fraction inhibited the bacterial growth. The results of the disk diffusion test were classified based on the size of the inhibition zones.

Table 1 Levels and independent variables of *C. racemosa* extraction experiment with DES

Level	Independent variables		
	DES concentration (%)	Solid : Liquids (g : mL)	Duration time (h)
1	5	1:10	24
2	10	1:15	48
3	15	1:20	72

Table 2 Box-Behnken Design of the research

No	DES concentration (%)	Solid : Liquids	Duration time (hour)
1	5	1:20	48
2	10	1:15	48
3	10	1:20	72
4	10	1:15	48
5	15	1:10	48
6	5	1:15	24
7	10	1:10	24
8	5	1:10	48
9	10	1:10	72
10	5	1:15	72
11	10	1:15	48
12	15	1:15	24
13	15	1:20	48
14	10	1:15	48
15	15	1:15	72
16	10	1:15	48
17	10	1:20	24

Data Analysis

The antioxidant activity data from the experiment were analyzed using Response Surface Methodology (RSM) with Box–Behnken Design (BBD) in Design Expert v13 software to evaluate the effect of DES concentration, solid:liquid ratio, and extraction time on antioxidant activity. Additionally, the average antioxidant activity values of each treatment were compared using analysis of variance (ANOVA) at a 95% confidence level to determine the effect of treatment. If the ANOVA results showed a significant difference ($p < 0.05$), a post-hoc Duncan's Multiple Range Test (DMRT) was performed to examine the differences between treatments. The data from the antioxidant activity measurements were analyzed using analysis of variance (ANOVA) at a 95% confidence level to determine whether the treatment had a significant effect. Significant differences between treatments are indicated by different superscript letters in the results table. Mean values with the same superscript letters indicate no significant difference, while values with different superscript letters indicate significant differences between the treatments.

RESULTS AND DISCUSSION

C. racemosa Characteristics

This study used *C. racemosa* seaweed obtained from the Centre for Brackish Water Fisheries (BBPAP) Jepara. *C. racemosa* used in this study has a dark green colour both before and after drying with wind away from sunlight (Figure 2). The characteristics of *C. racemosa* analyzed show that the moisture content is very high in fresh conditions, namely 91.4%, while after drying, it decreases to 14.48%. The extraction process yielded 1.24%, indicating that only a small portion of the dry biomass was obtained as an extract.

Ma'aruf *et al.* (2013) reported that fresh (wet) samples of *C. racemosa* contained 92.38% moisture, whereas dried samples had a moisture content of 13.39%. This indicates that the analytical results obtained in this study are not significantly different from those of previous studies. The difference in the

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Figure 2 Fresh *C. racemosa* from BBPAP Jepara

moisture content can occur due to differences in the place of origin during growth and development. The proximate value of one species can differ from place to place (Wisnuaji, 2021). The final weight produced from 4 kg of fresh *C. racemosa* was a powder of 48 g and from 7.45 kg of fresh *C. racemosa* was a powder of 94.77 g, so the percentage yield obtained was 1.24%. Research conducted by Wisnuaji (2021) showed that *C. racemosa* washed 12 times with water and dried in the sun with 85% paranet had a yield of 1.75%. The wet weight of *C. racemosa* is dominated by water, some of which is lost during washing and drying. Water loss in food ingredients causes a decrease in the water content and yield value (Jumsurizal *et al*., 2021).

Viscosity Test of DES

In this study, DES made from ethanol and ethylene glycol with a molar ratio of 1:2 were prepared at 60 °C until a homogeneous liquid was obtained and diluted to concentrations of 5%, 10%, and 15% using distilled water. The viscosity of 100% DES was higher than that of 15%, 10%, and 5% DESs. This demonstrates that the addition of distilled water to DES can increase the extraction ability by decreasing the viscosity and increasing the polarity of the solvent (Ozturk *et al*., 2018). The viscosity test results are presented in Table 3.

The characterization of DES aims to determine the general properties of DES from ethanol and ethylene glycol raw materials and to determine the differences in the physical properties of each DESs at different concentrations. The solvent viscosity is also affected by temperature, solution concentration, solute molecular weight, and pressure. Viscosity is directly proportional to the solution concentration; however, other factors, such as temperature and pressure, greatly affect the final value of viscosity. The viscosity decreased with increasing temperature. The tendency of a liquid to evaporate is greater, or the vapor pressure is greater, with increasing temperature. This makes the distance between liquid molecules more tenuous, and the viscosity decreases (Lumbantoruan & Erislah, 2016). Ethanol, a

Table 3 Viscosity of DESs

DES concentration (ethanol : ethylene glycol)	Viscosity (mPa.s)
5%	0.1
10%	0.77
15%	0.86
100%	4.56
Ethanol	1.04*
Aquades	0.282**

*Smith *et al*. (2014), **Pátek *et al*. (2009)

component of DES, has a viscosity of 1.04 cP (Smith *et al*., 2014). Water has a viscosity of 0.282 cP (Pátek *et al.,* 2009). One cP is one-hundredth of a poise, equal to one millipascal-second (mPa.s). This shows that 10% DESs has the lowest viscosity (0.77 mPa.s) compared to other DES concentrations as well as ethanol (1.04 cP) and water (0.282 cP), thus facilitating mass transfer from sample to solvent.

Antioxidant Activity

The antioxidant activity of the *C. racemosa* DES ethanol–ethylene glycol extracts was expressed in mg Trolox equivalents (TE)/g DW. TE can measure the antioxidant activity of crude extracts, as it is difficult to measure the individual antioxidant components of complex mixtures (Wojciechowski *et al*., 2020). The antioxidant activity of *C. racemosa* is presented in Table 4. were plotted using Design Expert v13 software to study the effects of the parameters and their interactions, resulting in the response surface plot shown in Figure 3.

Data were analyzed using the Box–Behnken design in Design Expert v13 to evaluate the effects of DESs concentration, solid:liquid ratio, and extraction time on antioxidant activity. The suggested model was quadratic and capable of analyzing both individual and interactive effects, supported by a high R^2 value. ANOVA indicated that DES concentration and extraction time had significant effects ($p<0.05$), whereas the solid:liquid ratio did not ($p>0.05$). No significant interaction effects were observed among the independent variables. The quadratic term for the DES concentration

Table 4 Antioxidant activity of *C. racemosa* extract

Treatments	Concentration DES (%)	Solid:Liquid	Time (h)	Antioxidant activity (mg TE/g DW)
Control with distilled water	0	1:20	72	28.6±0.067[a]
Control with ethanol*	0	1:2	24	32.16±0.000[b]
1	5	1:20	48	47.62±0.067[h]
2	10	1:15	48	50.07±0.134[k]
3	10	1:20	72	50.50±0.067[l]
4	10	1:15	48	50.12±0.067[k]
5	15	1:10	48	39.30±0.263[c]
6	5	1:15	24	40.41 0.133[d]
7	10	1:10	24	49.36±0.201[i]
8	5	1:10	48	43.99±0.133[e]
9	10	1:10	72	49.98±0.134[k]
10	5	1:15	72	49.69±0.200[j]
11	10	1:15	48	50.12±0.201[k]
12	15	1:15	24	32.19±0.066[b]
13	15	1:20	48	44.60±0.263[f]
14	10	1:15	48	50.02±0.067[k]
15	15	1:15	72	45.20±0.066[g]
16	10	1:15	48	50.02±0.067[k]
17	10	1:20	24	49.41±0.134[i]

*Mokoginta *et al.* (2021). The results of antioxidant activity as affected by DESs concentration, solid: liquids, and time.

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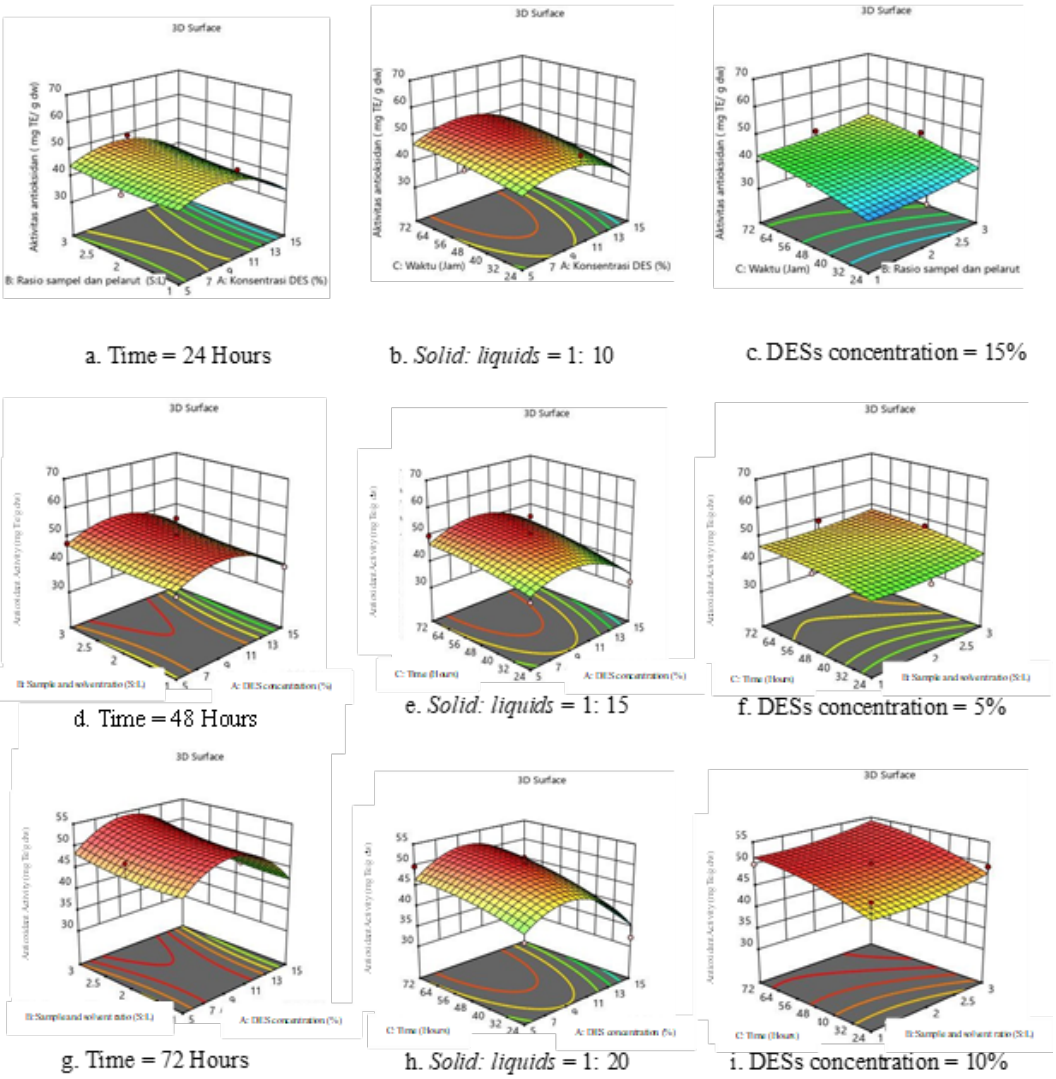

Figure 3 Response Surface (3D) showing the effect of (a) (d) (g) time (b) (e) (h) solid:liquids and (c) (f) (i) DESs concentration

was highly significant. The coefficient of variation (CV%) was <10, indicating model reproducibility. The R^2 value of 0.8475 suggested that 84% of the variation could be explained by the model, while the adjusted R^2 of 0.6514 indicated that 65% of the antioxidant response was influenced by the studied factors, with the remainder being affected by other variables. The Adeq Precision of 7.997 confirmed that the model had an adequate signal. Figures 3 (a), (d), and (g) show significant differences in the time effects, whereas Figures 3(b), (e), and (h) indicate no significant differences for the solid:liquid ratio. Figures 3(c), (f), and (j) reveal significant differences in the effects of DES concentration. The highest antioxidant activity was achieved at 10% DES, a solid:liquid ratio of 1:20, and 72 h. The 10% DES had the lowest viscosity (0.77 mPa·s), which facilitated mass transfer and compound dissolution. Comparisons with control solvents showed that distilled water and ethanol extracts had lower antioxidant activities (28.46 ± 0.067 and 32.16 ± 0.020 mg TE/g DW, respectively) than DESs extracts. The higher polarity range and lower viscosity of the DES (0.81, 0.77, and 0.86 mPa·s for 5%, 10%, and 15%, respectively) improved the extraction efficiency.

Antibacterial Activity

The antibacterial activity of *C. racemosa* extracts evaluated using ethanol–ethylene 10% DESs 1:20 for 72 h against *V. harveyi* and *V. parahaemolyticus*. The antibacterial activity of *C. racemosa* extracts was measured as the zone of inhibition (mm). The zone of inhibition was used to measure the antibacterial potential of the *C. racemosa* extracts. The antibacterial activity of *C. racemosa* is presented in Table 5.

Figure 4 shows that the extract of *C. racemosa* exhibited clear inhibition zones against *V. harveyi* after 72 h of incubation. The increasing diameter of the inhibition zones at higher extract concentrations indicates enhanced antibacterial efficacy, likely associated with the greater availability of the bioactive compounds in the extract. These findings confirm that *V. harveyi* is sensitive to the active constituents of *C. racemosa* and suggest the potential application of this extract for bacterial disease control in aquaculture.

Figure 5 shows that *C. racemosa* extract also produced distinct inhibition zones against *V. parahaemolyticus*, demonstrating its effective antibacterial activity. The gradual increase in the diameter of the inhibition zone with increasing extract concentration indicates that the antibacterial effect is dose-dependent. This result confirms the susceptibility of *V. parahaemolyticus* to the extract and supports the potential use of *C. racemosa* as a natural antibacterial agent for the management of pathogenic bacteria in aquaculture.

The antibacterial activity of *C. racemosa* extract against *V. parahaemolyticus* and *V. harveyi* was evident from the formation of inhibition zones, with inhibition increasing with increasing extract concentration, likely due to higher active compound levels at higher doses (Kurniaji *et al.*, 2019). A previous study by Hidayati (2020) demonstrated that *C. racemosa* extract produced inhibition zones of 6.68 mm at 100 ppm, 6.94 mm at 250 ppm, 7.77 mm at 500 ppm, and 8.65 mm at 1,000 ppm. In the present study, *C. racemosa* extract at doses ranging from 100–1,000 ppm generated larger inhibition zones of 8.08–

Table 5 Antibacterial activity of *C. racemosa* extract against *V. harveyi* and *V. parahaemolyticus*

Types of bacteria	*C. racemosa* extract (ppm)	Inhibition zone diameter (mm)						Inhibition zone			Average (mm)
		R1		R2		R3		R1	R2	R3	
		H	V	H	V	H	V				
V. harveyi	K-	0	0	0	0	0	0	0	0	0	0
	K+	20	19.2	19	18.7	20.3	19.5	20	18.9	19.9	19.6
	1,000	12.7	12.4	12.1	12.5	13	12.6	12.6	12.3	12.8	12.6
	750	11.5	12	11.2	11.4	11.4	11.9	11.8	11.3	11.8	11.6
	500	10.4	11.4	10.2	10.5	10.3	10.6	10.9	10.4	10.5	10.6
	250	11.2	9.5	10	10.4	9.8	10.8	10.4	10.2	10.3	10.3
	100	10.1	9.6	9.8	10	9.4	10.4	9.9	9.9	9.9	9.9
	50	9.8	9.6	9.4	9.1	9.5	9.0	9.7	9.3	9.3	9.4
V. parahaemolyticus	K-	0	0	0	0	0	0	0	0	0	0
	K+	20.6	19.4	19.8	19.1	20.1	19.7	20	19.5	19.9	19.8
	1,000	14.8	13.2	13.9	14	14.3	13.7	14	13.9	14	14
	750	13	12.7	12.5	12.3	13.1	12.6	12.9	12.4	12.9	12.7
	500	12.0	12.4	12	11.9	12.1	11.7	12.2	11.9	11.9	12.7
	250	11.8	11.0	11.5	11.4	11.1	11.6	11.4	11.5	11.4	11.4
	100	10.2	10.0	10.3	10.1	10.4	9.8	10.1	10.2	10.1	10.1
	50	9.6	9.2	9.4	9.2	9.3	9.7	9.4	9.3	9.5	9.4

H (Diameter horizontal); V (Diameter vertical); R1–R3 (measurement replicates).

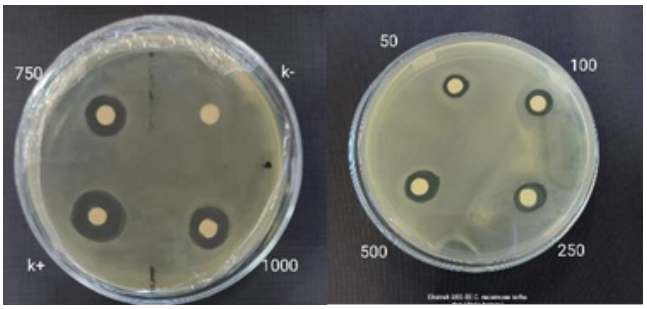

Figure 4 Antibacterial activity at 72 h of 10% DES ethanol–ethylene glycol extract (1:20) of *C. racemosa* against *V. harveyi*

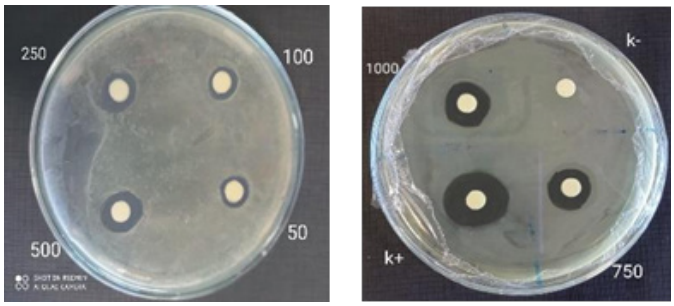

Figure 5 Antibacterial activity at 72 h of 10% DES ethanol–ethylene glycol extract (1:20) of *C. racemosa* against *V. parahaemolyticus*

13.13 mm against *V. parahaemolyticus* and 7.10–11.70 mm against *V. harveyi,* indicating higher antibacterial activity than previously reported, possibly due to differences in the extraction solvents. For *V. parahaemolyticus*, the inhibition began at 50 ppm (9.2 mm) and peaked at 1,000 ppm (14.8 mm), whereas for *V. harveyi*, the inhibition began at 50 ppm (9.0 mm) and peaked at 1,000 ppm (12.8 mm). The formation of these inhibition zones was attributed to the cyclohexane extract of *C. racemosa,* which contains secondary metabolites such as phenols, alkaloids, steroids, and flavonoids, all of which are known antibacterial agents (Hao *et al.,* 2019). Alkaloids, for example, can disrupt bacterial cell walls by damaging peptidoglycan, leading to impaired wall formation, leakage of nucleotides and amino acids, and cell death. Damage to the cell membrane also hinders nutrient uptake, thereby inhibiting bacterial growth. In this context, the inhibition classifications indicated that concentrations of 100–500 ppm were resistant, 750 ppm were intermediate, and 1,000 ppm along with the positive control were sensitive.

CONCLUSIONS

The combination of the DES solution, solid:solvent ratio, and effective maceration time that yielded the highest antioxidant activity value was 10% DES, a ratio of 1:20, and 72 h of maceration time. This combination produced the strongest antibacterial effects against *V. harveyi* and *V. parahaemolyticus*.

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