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Potential of Antioxidant Activity *Caulerpa racemosa* Extract Using DES Solvent and Different Sonication Times as An Antibacterial Against Pathogenic Bacteria

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Abstract

Caulerpa racemosa, a type of green algae, has been studied for its potential health benefits, including antioxidant and antibacterial properties. The extraction of bioactive compounds from marine algae using green solvents like Deep Eutectic Solvents (DES) has gained interest due to their efficiency and environmental friendliness. This research aimed to determine the potential antioxidant activity of *C. racemosa* extract using DES solvents and different sonication times as an antibacterial against pathogenic bacteria. The research employed a Factorial Completely Randomized Design (CRD) with two variables: sonication time (10, 20, and 30 minutes) and different DES solvents (ethanol and ethylene glycol (1:20) with 90% aquades, and methanol and glycerol (1:10) with 90% aquades. The highest antioxidant activity was found in DES Methanol-Glycerol (36.39 mg TE/g dw), followed by DES Ethanol-Ethylene glycol (28.30 mg TE/g dw), both significantly different ($p<0.05$). The *C. racemosa* extract inhibited the growth of *V. parahaemolyticus* with an inhibition zone of 8.08 mm at 50 ppm and 13.13 mm at 1,000 ppm. For *V. harveyi*, inhibition started at 6.37 mm at 50 ppm, reaching 11.70 mm at 1,000 ppm. DES Methanol-Glycerol with a 10-minute sonication provided the highest antioxidant activity and effectively inhibited *V. parahaemolyticus* and *V. harveyi*. Extraction of *C. racemosa* with DES-MG solvent and varying sonication times influences its antioxidant activity and antibacterial properties.

INTRODUCTION

Many fish cultivation failures are caused by disease outbreaks. Control of diseases caused by bacterial infections is generally done by antibiotics. However, the use of antibiotics is starting to be reduced because it harms the environment and the emergence of bacterial resistance (Kumar *et al*., 2019). Considering growing concerns over antibiotic resistance, there has been increasing interest in exploring alternative sources for antimicrobial compounds, leading researchers to investigate the potential of seaweed commodities for their unique bioactive properties (Lomartire and Gonçalves, 2023).

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Seaweed commodities are often used by the community for Pharmaceutical and Food Industries (Syam *et al*., 2018). *Caulerpa* has high mineral, protein, carbohydrate, crude fiber content, and low fat content (Abbott *et al*., 2003). The bioactive components contained in *C. racemosa* have the potential to act as antioxidants and antibacterials. Bioactive compounds include tannins, terpenoids, sulfated polysaccharides, phenolic compounds, saponins, glycosides, steroids, and flavonoids (Kandhasamy dan Arunachalam, 2008). *C. racemosa* also contains the alkaloids, caulerpin, caulerpicin, and caulerpenin (Cameron and Wang, 2006). In the context of antioxidant activity, some of the compounds mentioned, such as phenols, tannins, steroids, and flavonoids contained in *C. racemosa*, are reported to have antioxidant activity. These compounds can fight the damaging effects of free radicals (Marraskuranto *et al.*, 2021). *C. racemosa* extract can be further explored for its activity to determine the type of antibacterial compound according to the solvent that attracts the active compound.

Extraction of *C. racemosa* requires a solvent separation process which involves moving the solute into the solvent. The sonication method using ultrasonic wave energy can be used for extraction (Mokoginta *et al*., 2021). One of the benefits of the extraction method is using wave ultrasonics to speed up the extraction process (Kuldiloke, 2002). Secondary metabolite compounds contained in seaweed have different levels of polarity. This extraction process requires the use of graded solvents from low polarity to high polarity. One of the solvents is Deep Eutectic Solvent (DES), a liquid in a condition close to the eutectic composition of a mixture. DES is a type of solvent related to ionic liquids consisting of natural and renewable ingredients. This mixture contains hydrogen bonds as the main driving force of eutectic phenomena (Kurniaji *et al*., 2020). DES is a potential substitute because it has characteristics such as ionic liquids in the form of low vapor pressure, cheap production costs and raw materials, good biodegradability, and no toxic properties (Hao *et al*., 2019). The application of *C. racemosa* extraction using DES is still relatively new. The raw materials for HBA from ethanol and HBD from ethylene glycol can use a ratio of 1:2 (Benítez-Correa *et al*., 2023)

Vibrio harveyi and V. parahaemolyticus are opportunistic pathogens known to frequently infect shrimp. Extracts from *Caulerpa racemosa* have shown the potential to inhibit the growth of these pathogens, as evidenced by various studies utilizing different solvents for compound identification (Palaniyappan *et al*., 2023). References for these studies are needed for further validation. A study conducted by Hao *et al*. (2019) showed that *C. racemosa* contains a relatively high content of tannins, phenol, sterols, and triterpenoids. A study states that the ethanol extract of the microalga *Tetraselmis chuii* shows that there are phytochemical compounds such as alkaloids, flavonoids, and glycosides. These compounds are also contained in *C. racemosa* extract which is a source of strong antibacterial agents (Yap *et al*., 2019). Various mechanisms have been reported for the antibacterial effect of flavonoids and phenol, these mechanisms include inducing damage to the cell membrane, inhibiting enzymes involved in nucleic acid synthesis, disrupting the bacterial respiratory chain, and interfering with the synthesis of the cell envelope (Yuan *et al*., 2021).

The antibacterial mechanism of phenolic compounds can disrupt the peptidoglycan components in the cell wall of the Gram-positive bacterium *S. aureus* by preventing the incorporation of N-acetylmuramic acid residues into the muropeptide structure, which typically confers rigidity to the cell wall. Consequently, bacterial cell wall synthesis is disrupted and not formed properly, leading to the loss of rigid cell walls and leaving the cell membrane vulnerable to damage and leakage (Hidayah *et al*., 2017). DES as an alternative solvent using the ultrasonic extraction method is relatively new. There is a need for scientific studies for research regarding the exploration of antioxidant compounds in *C. racemosa* as a potential to inhibit pathogens. It is hoped that the difference in DES solvent concentration can be

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used as the latest reference to determine optimal *C. racemosa* extract results.

METHODOLOGY

Ethical Approval

This research did not use animal tests therefore ethical clearance was not necessary.

Place and Time

The research was carried out in June-November 2023. The experiment was conducted at the Laboratory of Microbiology and Laboratory of Chemistry, Faculty of Fisheries and Marine, Airlangga University, located in the city of Surabaya, Indonesia.

Research Materials

Research equipment includes grinder, Erlenmeyer, Beaker glass, sonicator, refrigerator centrifuge, rotary evaporator (Hahnvapor HS-2005S-N), oven, cotton swab, paper disc, yellow tip, laminar airflow, incubator, and UV-Vis (Human Corporation® X-ma 1200).

Research materials including *C. racemosa* obtained from the Center for Brackish Water Aquaculture Fisheries (BBPBAP) Jepara - Central Java, ethanol, ethylene glycol, distilled water, methanol, glycerol, *V. parahaemolyticus*, *V. harveyi,* TSA (Tryptic Soy Agar), and Tryptic Soy Broth (TSB) media.

Research Design

The research design utilizes a Factorial Completely Randomized Design with 2 variables: sonification time (10, 20, and 30 minutes) and different solvents DES 1 (ethanol and ethylene glycol (1:20) with the addition of 90% Aquades), and DES 2 (methanol and glycerol (1:10) with the addition of 90% Aquades).

Work Procedure

Procedure for Making Simplicia

C. racemosa was obtained from the Center for Brackish Water Aquaculture Fisheries Jepara, Central Java. *C. racemosa* samples were cleaned from waste and sea water using flowing tap water 5 times. Next, the samples were air-dried for 7 days or until *C. racemosa* reached a dry weight indicated by a water content of less than 10%. Then cut into small pieces and mash with a grinder. Once smooth, then weigh 10 grams for processing extraction.

Procedure for Making DES Solvent

Several solvents are used in the *C. racemosa* extraction process type of solvent for DES 1 using a mixture of Ethanol (HBA) and Ethylene glycol (HBD) with a molar ratio of 1:2 with Ethanol calculations measured as 59 ml for 1 mole while ethylene glycol is 112 ml for 2 mole, then the DES concentration is adjusted by adding distilled water until it reaches a concentration of 100%. While DES 2 using Methanol (HBA) and glycerol (HBD) in a ratio of 1:2 Methanol calculations were measured as 40 ml for 1 mole while glycerol was 146 ml for 2 mol, then adjust the DES concentration by adding distilled water to reach a concentration of 100%.

Extraction Procedure using The Sonication

The *C. racemosa* extraction method uses the sonication or Ultrasonic assisted method extraction (UAE) where this method uses ultrasonic waves with frequency more than 20 kHz. *C. racemosa* that has been refined 2 grams were weighed and put into a 100 ml Erlenmeyer flask then added from each DES solvent, namely ethanol and ethylene glycol (powder comparison *C. racemosa*: DES solvents ethanol and ethylene glycol namely 1: 20) as well as DES solvents Methanol and glycerol (ratio of *C. racemosa* powder: DES Methanol and glycerol solvent, namely 1:10). *C. racemosa* powder was placed in a 200 ml beaker containing DES solvent and put into a sonicator to carry out the extraction process for a respective length of time 10 minutes, 20 minutes, and 30 minutes. The ultrasonic results are then placed in a refrigerator centrifuge at a speed of 3500 rpm with a temperature of 4°C for 15 minutes. Results from the centrifuge are obtained from the supernatant. The *C.*

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racemosa extract was then evaporated using a Rotary evaporator at a temperature of 35 °C until the DES solvent evaporated completely and became a paste by drying in the oven at 35°C for 6-7 hours.

Antibacterial Test

The bacterial inhibition test was carried out using the disc diffusion method, where in this study the disk test was used to find the best fraction of extract *C. racemosa* to inhibit the *V. parahaemolyticus* and *V. harveyi* bacteria. How the diffusion method works using disc paper 6 mm in diameter to determine antibacterial sensitivity which can be determined by measuring the inhibition zone or clear zone that forms around the paper disc that has been containing fractions. Test of the inhibitory power of *C. racemosa* extract fractions against *V. parahaemolyticus* and *V. harveyi* starting with bacterial culture using TSA or TSB media (Huyyirnah and Fitriyani, 2020).

The next step is carried out by inoculating the bacteria on solid media by spreading it evenly using a sterile cotton swab aseptically. After that, paper discs were dripped with 50 µl of each fraction and left for 20 minutes so that the remaining fluent evaporated, then placed gently on the solid media (Rosmania and Yuniar, 2021). Then incubated at 35ºC for 24 hours. Next, observed clear zone forms around the disc were measured. Then observations were carried out again 24 hours later. If visible bacterial growth around the disc means that the extract fraction used has properties bacteriostatic or inhibits the growth of bacteria. The clear zone formed from disc diffusion assay will later be classified based on (Wilapangga and Syaputra, 2018). Calcification of inhibitory responses consists of categories of very strong resistance of ≥ 20 mm, strong of 10 – 20 mm, medium of 5 – 10 mm, and weak ≤ 5 mm.

DPPH Method

The DPPH (1,1-diphenyl-2-picrylhydrazyl) method is a technique used in studying antioxidant activity. DPPH is a stable free radical compound with a dark purple color and absorbs at a wavelength of 517 nm (Sunarni *et al.*, 2007). The basic principle of this method is that antioxidant compounds will react with DPPH, reducing it, and thus changing the color of the solution from purple to yellow. When antioxidant compounds react with DPPH, the unpaired electron on the DPPH radical will pair with hydrogen from the antioxidant compound, forming a colorless reduced compound (Li *et al.*, 2011). This color change is observed as an indication of antioxidant activity. The parameters used to interpret the results of the DPPH method are IC50 (Inhibitor Concentration), Gallic acid Equivalent (GAE), and Trolox Equivalent (TE) (Panda, 2012).

Data Analysis

The data analysis used is experimental research which is included in inductive statistics by drawing conclusions and making decisions based on facts *C. racemosa* using a Factorial Completely Randomized Design with two variables namely sonification and different solvents variables.

RESULTS AND DISCUSSIONS

C. racemosa is a type of green seaweed from the Chlorophyceae group that is widely distributed in Indonesian waters (Jayusri *et al.*, 2023). *C. racemosa* seaweed is often called sea grapes because its shape is round like grapes and can be consumed directly without going through cooking and other stages (Damayanti *et al*., 2023). *Caulerpa* sp. contains protein, carbohydrates, crude fiber, fat, and minerals (Tapotubun *et al.*, 2021). The bioactive components contained in *C. racemosa* have the potential to act as antioxidants and antibacterials (Kandhasamy and Arunachalam, 2008).

According to Singkoh (2011), *C. racemosa* extract contains antibacterial compounds that can inhibit the growth of *Bacillus subtilis*. Apart from being an antibacterial, *C. racemosa* can be used as a source of antioxidants (Belkacemi *et al*., 2020). Antioxidants are chemical compounds that can ward off free radicals.

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DES is a solvent that is in a state close to eutectic in a mixture. Eutectic composition is a condition where the molar ratio of the components provides the lowest melting point (Niawanti, 2017). DES is a type of solvent related to ionic solutions consisting of natural and renewable ingredients. This mixture contains hydrogen bonds as the main driving force of eutectic phenomena (Abbott *et al*., 2003). DES is a potential alternative solvent replacement because it has characteristics such as ionic liquids in the form of low vapor pressure, cheap production costs, and raw materials, good biodegradability, and no toxic properties (Sander *et al*., 2016). The extraction application using DES solvent is still relatively new.

Sonication is a process of breaking down the cell walls of plants or animals to help the process of extracting chemical compounds run efficiently. The advantages of the sonication method are that it speeds up extraction time, is more efficient in using solvents, and is safe to use because the process does not result in significant changes to the chemical structure of the particles and the compounds used (Cameron and Wang, 2006).

The application of *C. racemosa* extraction using DES is still relatively new. The raw materials for HBA from ethanol and HBD from ethylene glycol can be used in a ratio of 1:2. Ethylene glycol was chosen because this organic compound is colorless, odorless, and has low viscosity. Ethylene glycol can lower the freezing point of the solvent by inhibiting the process of forming ice crystals (Latifah, 2015). In addition, DES solvents can use methanol as HBA and glycerol as HBD. Glycerol (1,2,3 propanetriol) is a colorless, odorless, and viscous liquid that has a sweet taste (Pagliaro and Rossi, 2008).

The results of the ANOVA test show that the duration of sonication and the use of different DES solutions have significantly different effects ($p<0.05$) and the results of the Duncan test give significantly different results between treatments ($p<0.05$) (Table 1).

Table 1. Average antioxidant activity (mgTE/g dw) of *C. racemosa* extract with different periods and DES solvents.

DES Solvents	Duration		
	10 (S1)	20 (S2)	30 (S3)
EE (A1)	25.5603 ± 4.3690^{b}	28.2970 ± 1.5008^{b}	17.1900 ± 0.9270^{a}
MG (A2)	36.3993 ± 0.6385^{c}	26.5230 ± 0.5260^{b}	23.8570 ± 1.0140^{b}

Note: Different superscript notations show significantly different results ($p<0.05$).

The highest antioxidant activity was obtained in the sonication treatment of 10 minutes with the DES solvent Methanol-Glycerol (A2S1) followed by the sonication duration of 20 minutes with the DES solvent Ethanol-Ethylene glycol (A1S2)) amounting to 36.3933 mg TE/g dw and 28.29700 mg TE/g dw and significantly different ($p<0.05$). However, the A1S2 treatment was not significantly different from the A1S1, A2S2 and A2S3 treatments.

Next, in the second stage, testing was carried out on the ability of antioxidant activity against the bacteria *V. harveyi* and *V. parahaemolyticus*.

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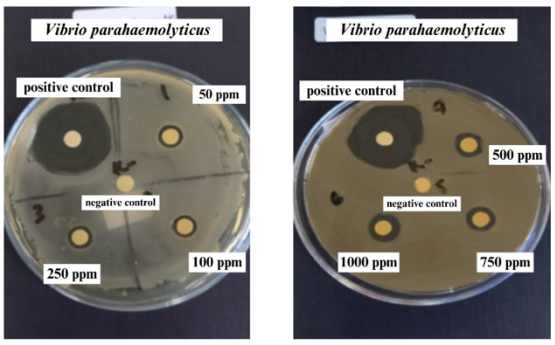

Figure 1. Testing of antibacterial extracts against *V. parahaemolyticus*.

Table 2. Inhibitory Activity of *C. racemosa* extracts against *V. parahaemolyticus* (mm).

Treatments	Repetition 1	Repetition 2	Repetition 3	Average
K+	19.45	19.90	20.60	19.98
K-	0.00	0.00	0.00	0.00
50 ppm	8.00	9.40	6.85	8.08
100 ppm	8.75	9.70	7.85	8.77
250 ppm	9.40	10.25	9.45	9.70
500 ppm	10.65	11.85	10.35	10.95
750 ppm	11.55	12.55	12.65	12.25
1000 ppm	12.30	12.65	14.45	13.13

Note: K+ uses oxytetracycline (6,25 ppm) as the treatment, while K- uses Dimethyl Sulfoxide (DMSO) (5%).

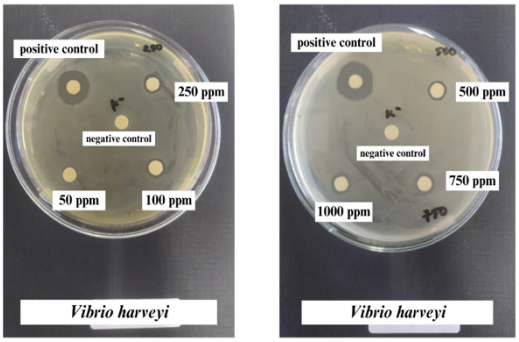

Figure 2. Antibacterial test of extracts against *V. harveyi*.

Table 3. Inhibitory Activity of *C. racemosa* extracts against *V. harveyi* (mm).

Treatment	Repetition 1	Repetition 2	Repetition 3	Average
K+	17.30	17.05	16.10	16.82
K-	0.00	0.00	0.00	0.00
50 ppm	6.90	7.15	5.05	6.37
100 ppm	7.85	6.60	6.85	7.10
250 ppm	8.70	9.45	8.65	8.93
500 ppm	9.10	9.65	9.45	9.40
750 ppm	10.70	11.65	10.45	10.93
1000 ppm	11.50	11.85	11.75	11.70

Note: K+ uses oxytetracycline (6,25 ppm) as the treatment, while K- uses Dimethyl Sulfoxide (DMSO) (5%).

The ability of *C. racemosa* extract to inhibit the growth of *V. parahaemolyticus* bacteria began to appear at a dose of *C. racemosa* extract of 50 ppm, namely 8.08 mm and the highest at a dose of 1000 ppm at 13.13 ppm. Extra inhibition of *C.*

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racemosa on *V. harveyi* showed inhibition starting at a dose of 50 ppm of 6.37 and the highest at a dose of 1000 ppm of 11.70 mm.

C. racemosa is a seaweed from the green algae group. *C. racemosa* seaweed is used by coastal communities as a vegetable or as a complement to staple foods. *C. racemosa* is a type of seaweed from the phylum Chlorophyta that is widely consumed by humans. Based on dry weight, *C. racemosa* has a chemical composition, namely a protein content of 21.730%; fat content of 8.681%; ash content of 20.91%; carbohydrate content of 48.679%; and crude fiber of 8.429%. Meanwhile, based on wet weight, *C. racemosa* has a water content of 92.375% (Ma'aruf *et al*., 2013). Apart from that, *C. racemosa* produces antioxidant active ingredients consisting of polyphenol, alkaloid, terpenoid, and alkaloid compounds.

Withdrawing a compound from a substance or material, whether derived from plants or animals, can be done by extraction. Factors that influence the extraction results include extraction time, temperature, and solvent. *C. racemosa* extracted using Ethanol solvent using the maceration method has antioxidant activity at a concentration of 0.7 mg/L after being tested using the DPPH method (Mokoginta *et al*., 2021). Solvents are substances that can dissolve gases, liquids, or solids without forming chemical bonds (Noack *et al*., 2010). Several conventional solvents that are often used to extract active antioxidant compounds include ethanol, methanol, and n-hexane. However, most of the solvents used have high toxicity and are not environmentally friendly (Ahmad *et al*., 2020).

Deep Eutectic Solvents (DES) are a new generation of solvents formed from a mixture of quaternary ammonium salts or hydrogen bond acceptor (HBA) with hydrogen bond donors (HBD) (Mannu *et al*., 2021). Ethylene Glycol and Glycerol act as HBD for the inductive effect on Ethanol and Methanol as HBA in this research. The results showed that the antioxidant activity from using DES-MG (Methanol Glycerol) solvent and sonication time gave the highest results compared to other treatments, namely 36.39933 mg TE/g dw. This is because the DES solvent contains methanol which is capable of dissolving polar and nonpolar analytes. Methanol can attract analytes in the form of alkaloids, steroids, saponins, and flavonoids originating from plants. Methanol has a wide polarity range so it can extract larger amounts of active ingredients, ranging from non-polar, semi-polar to polar compounds (Marraskuranto *et al*., 2021). In addition, the presence of glycerol in DES helps speed up the dissolution reaction, because glycerol has a high boiling point, thus allowing the reaction to be accelerated or allowing reactions to occur that do not proceed in solvents with low points (Gu and Jérome, 2010).

The sonication treatment in this study was to break down the cell walls of *C. racemosa* seaweed so that the solvent could enter the cells more quickly. In this study, it was shown that a sonication time of 10 minutes gave better results than a sonication time of 20 or 30 minutes. This is because the longer the sonication time lasts, the higher the temperature, which results in the destruction of antioxidant compounds and a decrease in the value of antioxidant activity. Treatment with DES - Ethanol Ethylene glycol solvent during sonication with a duration of 30 minutes also showed lower antioxidant activity compared to sonication durations of 10 and 20 minutes (Table 3).

The antibacterial activity of *C. racemosa* extract against the bacteria *V. harveyi* and *V. parahaemolyticus* was shown by the formation of a clear zone around the paper disc. The results of the study showed that the inhibitory power value was greater with the greater dose of *C. racemosa* extract used. This is due to the active ingredients contained. The higher the dose of extract given, the greater the active ingredients contained in the extract (Kurniaji *et al*., 2020). When compared to research conducted by Hidayati (2020) which used hot water extract of *C. racemosa*, it produced an inhibitory power of 6.68 mm at a dose of 100 ppm, 6.94 mm at 250 ppm, 7.77 mm at 500 ppm and 8.65 ppm at 1000 ppm. Meanwhile, *C. racemosa* extract at a dose of 100 ppm to

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1000 ppm produced an inhibition zone of 8.08 -13.13 against *V. parahaemolyticus* and 7.10 - 11.70 mm against *V. harveyi*.

The formation of the inhibition zone is due to the cyclohexane extract of *C. racemosa* which can play an active role as an antibacterial due to the chemical components contained in the extract. *C. racemosa* extract with DES MG solvent contains secondary metabolite compounds such as phenol, alkaloids, steroids, and flavonoids which are thought to have antibacterial potential. This statement is under Hao *et al*. (2019) which states that chemical compounds that have the potential to act as antibacterials are alkaloids, steroids, and saponins.

Alkaloid compounds have several pharmacological effects such as antibacterial, anticancer, and antiasthmatic. Alkaloids can inhibit the growth of bacteria, namely by damaging the peptidoglycan component in the bacterial cell wall, which will result in the cell wall layer not forming completely and causing damage to the cell and undergoing lysis (Darsana *et al.*, 2012). Damage to the cell membrane allows nucleotides and amino acids to exit the cell. Apart from that, this damage can prevent the entry of important materials into the cell, because the cell membrane also controls active transport into the cell. This causes bacterial cell death or inhibits bacterial growth. *C. racemosa* extract with a concentration of 100 ppm to 5000 ppm is classified as resistance criteria, 750 ppm is classified as intermediate, while a concentration of 1000 ppm, and the positive control is classified as sensitive.

From the results of this study, it can be concluded that extraction of *C. racemosa* using DES solvent and long sonication time influences antioxidant activity. DES – MG solvent with a sonication time of 10 minutes provided the highest antioxidant activity of 36.39933 mg TE/g dw and could inhibit the growth of *V. parahaemolyticus* and *V. harveyi* bacteria.

CONCLUSION

DES – MG solvent with a sonication time of 10 minutes provided the highest antioxidant activity of 36.39933 mg TE/g dw and can inhibit the growth of *V. parahaemolyticus* bacteria and *V. harveyi* extraction of *C. racemosa* using DES solvent and long sonication time affect antioxidant activity.

CONFLICT OF INTEREST

The author declares there is no conflict of interest.

AUTHOR CONTRIBUTION

Woro Hastuti Satyantini: author correspondence, principal researcher, analysis, and writing of the manuscript, Saiful Bakhri: Observation and Writing analysis data, Akhmad Taufik Mukti: Collecting data and analysis.

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