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Extraction, Phytochemical Profiling, and Antibacterial Activity of n-Hexane Crude Extracts of Macroalgae *Padina australis*

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ABSTRACT
Padina australis, brown macroalgae, has potential bioactive compounds that possess remarkable bioactivities including antimicrobial. The present study extracts macroalgae *P. australis* using Ultrasound assisted extraction (UAE) with n-hexane solvent, followed by screening phytochemical compounds. Moreover, the antimicrobial activity and MIC value of the n-hexane crude extracts of *Padina australis* was tested against *Shigella sonnei, Escherichia coli, Salmonella typhi, and Bacillus cereus* by using the disc diffusion method. This result demonstrates the optimum time of UAE process is at 10 minutes that yields 0.117 %. The phytochemical screening of n-hexane crude extracts of *Padina australis* macroalgae revealed the presence of secondary metabolites such as alkaloids, flavonoids, saponins, tannins, steroids and phenols. Antibacterial activity of this extract against *Shigella sonnei, Escherichia coli, Salmonella typhi, and Bacillus cereus* at 100 ppm concentration with inhibitory diameter range of 10.97 to 13.86 mm. Then, the n-hexane crude extracts of *P. australis* is potent against *Bacillus cereus* with MIC value of 6.25 ppm.

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1. INTRODUCTION

Macroalgae known as seaweed belong to thallophytes group (plant-like organisms) which generally grow in coastal areas. Based on their pigment content, macroalgae are classified as brown, red, and green macroalgae. Agar, carrageenan, and alginate are common products from macroalgae where they can be used as raw materials of various industries, such as medicine, food, and cosmetics etc (Salosso et al., 2020; Kemer et al., 2015; Puasa et al., 2018).

Padina australis is a macroalgae of the Phaeophyceae class (brown algae) that grows widely and is found in Indonesian waters, including Tidung Island, Seribu Islands District which contains carbohydrate (8.78%), protein (1.05 %), fat (0.58 %) (Maharany et al., 2017). *P. australis* is commonly used in the food industry as a gelling agent, thickening, emulsifier, and stabilizer, as well as in the pharmaceutical business for anticancer and anti-obesity properties due to its hydrocolloid content, including fucoxanthine and alginate (Alhafizoh et al., 2024).

To reduce the danger of infectious diseases caused by bacteria, fungus, viruses, and parasites, extensive research has been conducted to identify compounds with strong antimicrobial activity. Medicinal properties of plants especially from marine have also been preferred globally, due to their potent pharmacological effects, low toxicity, and economic viability, compared to synthetic drugs (Atef et al., 2019). Medicinal plants contain numerous bioactive secondary metabolites such as tannins, terpenoids, alkaloids, saponins, flavonoids, and phenolic compounds that play an important role in determining of biological activities such as antimicrobial (Atef et al., 2019; Shakya A.K., 2016; Sholikhah, 2016).

There is a growing environmental interest in reducing the use of organic solvents using green technology. They enable for the development of efficient systems while minimizing negative environmental impacts.

Ultrasound assisted extraction (UAE), one of modern extractions, is a promising and widely accepted technology. This technology increases the yield and decrease the time of extraction, better preserving high extract quality, but it can decrease the stability of the extracted components (Palma et al., 2021; Alcántara et al., 2020). In this regard, this work assessed the effect of time at UAE process on the yield's percentage of *P. australis* using n-hexane solvent.

The objectives of this present study are the determination of optimum UAE time, screening the phytochemical compounds, and antibacterial assays from n-hexane crude extracts of *Padina australis* which collected from Tidung Island, Seribu Island District, North Jakarta Province, Indonesia.

2. RESEARCH METHOD

Materials

The macroalgae used in this research is *Padina australis*, collected from Tidung Island, Seribu Island District, North Jakarta Province, Indonesia (Figure 1) in December, 2024. Four test bacterial species used were *Shigella sonnei, Escherichia coli, Salmonella typhi, and Bacillus cereus* which obtained from collection of Pharmacy Faculty, Universitas Pancasila, Jakarta. The solvent used is n-hexane. Nutrient agar (NA) and Nutrient broth (NB) were used as the media growth of bacteria tested. Tetracycline antibiotic was used as positive control and the negative control is dimethyl sulfoxide (DMSO).

Procedure

Sampling and Identification of Macroalgae

Sampling of macroalgae was carried out in 14th Desember, 2023 from Tidung Island, Seribu Island District, North Jakarta Province, Indonesia (Figure 1). Padina sp. were collected by handpicking method. The sample is then separated and cleaned under running water to remove dirt, sand, dust and salt that is still stuck, and then dried to be further mashed into fine simplisia using a blender. The simplisia powder is weighed and kept at refrigerator (4^0C) for further analysis (Husni et al., 2014). Further, the macroalgae sample was identified by National Research and Innovation Agency, Indonesia to determine which species it belongs to.

Figure 1. Map of Tidung Island

Preparation of the crude extracts

The n-hexane crude extracts of *Padina australis* were prepared by *Ultrasound Assisted Extraction* (UAE) method of the dried macroalgae materials in organic solvent (n-hexane). 30 grams of *Padina australis* sample were extracted using 300 mL of n-hexane as solvent in an ultrasonic water bath apparatus (Branson 2510, USA) at 42 kHz and 50% amplitude. There were four different times (5, 10, 15 and 20 minutes) used in this study. After ultrasonic extraction process, the extracts were collected and evaporated by rotary evaporator at 40 ^{0}C to remove the solvent. The obtained extract was weighed and then stored in the refrigerator for further analysis. This UAE process was in triplicate and calculated its yield percentage obtained (Panjaitan & Farida, 2023).

Detection of Secondary Metabolites

Alkaloids Test

1 mL of n-hexane crude extracts of *Padina australis* was pippeted and placed into a test tube. Then 1 mL of Dragendorff's reagent (potassium bismuth iodide solution) was added and shaken. An orange red precipitate formed indicates the presence of alkaloids (Abubakar & Haque, 2020).

Flavonoids Test

1 mL of n-hexane crude extracts of *Padina australis* was pippeted and placed into a test tube. Moreover, few drops of sodium hydroxide solution were added and shaken. The appearance of intense yellow color that turns to colorless after adding dilute acid implies the existence of flavonoids (Abubakar & Haque, 2020).

Phenols Test

1 mL solution of n-hexane crude extracts of *Padina australis* was pippeted and placed into a test tube. Then 1% gelatin solution containing sodium chloride was added and shaken. The presence of phenols were indicated with the formation of bluish-black color (Abubakar & Haque, 2020).

Tannins Test

3 mL of n-hexane crude extracts of *Padina australis* was pippeted and placed into a test tube. Then, 3 drops of 1% $FeCl_3$ were added. The formation of blue or greenish-black color demonstrates the presence of tannins compounds (Ikalinus et al, 2015).

Saponins Test

3 mL of n-hexane crude extracts of *Padina australis* was pippeted and placed into a test tube, added 3 mL distilled water and mixed them vigorously. Then, the test tube was kept aside for 3-5 minutes. The formation of foam indicates the presence of saponins (Al-Bahrawee et al., 2023).

Steroids Test

2 mL of chloroform was added to 3 mL of n-hexane crude extracts of *Padina australis* was pippeted and placed into a test tube and mixed them. Then, 3 mL concentrated H_2SO_4 was added into test tubes and then a thin film on the surface was formed which divided two layers. A red orange ring appeared, indicating the presence of terpenoids. While, steroids can be found by the change in color of the upper layer, from yellow to blue or green (Mboneye et al, 2023).

Antimicrobial activity screening of n-hexane crude extracts from *Padina australis*

The antibacterial activity of n-hexane crude extracts from *Padina australis* were evaluated by disc diffusion method using four different bacteria tests namely *Shigella sonnei, Escherichia coli, Salmonella typhi, and Bacillus cereus*. The apparatus and bacterial growth media (NA and NB) to be used are first sterilized by autoclaving at 121 ^oC for 15 minutes at 1 atm.

Preparation of the bacteria culture

All the test bacteria were inoculated on nutrient broth (NB). The bacterial strains were incubated at 37 ^0C for 72 hours for *Shigella sonnei* (Aini, 2018); for 16-18 hours for *E. coli* and *Bacillus cereus* (Rusli et al., 2018 & Datta et al., 2019); for 12-36 hours for *Salmonella typhi* (Normaidah, 2020). After the incubation, the bacterial cultures were stored in the refrigerator at 4 ^0C till used.

Antibacterial activity assay of the n-hexane crude extracts of Padina australis by disc-diffusion method

The paper disc diffusion method was used to determine the antimicrobial activity of the n-hexane crude extracts, and performed by using nutrient agar (NA) according to the National Committee for Clinical Laboratory Standards. 180 µL of the test bacterial suspension were swabbed on the surface of petri dish containing nutrient agar (NA) and the inoculums were allowed to dry for 5-10 minutes. Six-millimetre diameter sterilized paper discs impregnated with 10 µL of 100 ppm of n-hexane crude extracts from *Padina australis* solution were placed on the surface of the inoculated plate. 30 µg of tetracycline antibiotic and DMSO solution were used as positive control and negative control, respectively. Then, the petri dishes were incubated based on incubation time of each test bacteria. The diameters (mm) of the clear zones (referred to inhibition zones) were measured using callipers with three replicates for each test bacteria (Nava-Solis et al., 2022).

Determination of the minimum inhibitory concentration (MIC)

The Minimum inhibitory concentration (MIC) assay was carried out by disc-diffusion method. The principle of MIC testing is the lowest concentration of the drug or extract which will inhibit growth of test microorganisms. The n-hexane crude extract of *Padina australis* were serial diluted at 50 ppm; 25 ppm; 12.5 ppm; 6.25 ppm and 3.125 ppm. The four bacterial tested were growing in broth overnight to contain 10^8 CFU/mL. The petri dish containing agars which inoculated with test bacterial (*Shigella sonnei, Escherichia coli, Salmonella typhi, and Bacillus cereus*) were prepared in the series of decreasing concentrations of the plant extract (from 50 ppm – 3.125 ppm). Moreover, all the petri dishes were incubated based on incubation time of each test bacteria. The procedure of inhibition zone followed the antibacterial activity assay above. The MIC test were examined visually in the NA-petri dish as the lowest concentrations of the extracts in which no bacterial growth was visible.

3. RESULT AND DISCUSSION

Padina australis

The macroalgae was identified as belonging to the class Phaeophyceae, order Dictyotales, family Dictyotaceae, genus Padina, and species *Padina australis* Hauck, 1887. On visual observation, the macroalga *Padina australis* taken from the waters of Tidung Island, Seribu Islands, has a brownish-green tallus with a length of around 5-7 cm, fan-shaped, and lobbed. This macroalgae is in agreement with the description by Bhuyar et al. (2020) that reported *Padina* sp. had "ear-like" blades and thalli which were irregularly cleft into narrow lobes as seen in Figure 1. *Padina australis* typically lives on dead coral substrates (Kemenangan et al., 2017). The

macroalgae can grow in deeper sublittoral region (5- 10 m deep) (Kadi et al., 2015). This sample was identified by National Research and Innovation Agency with genus: Padina and species: *Padina australis* Hauck, 1887.

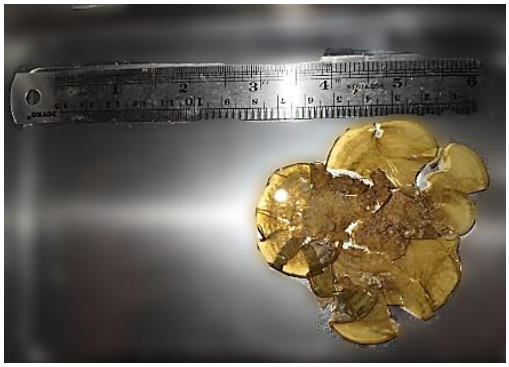


Figure 2. *Padina australis*

The yield percentages of the n-hexane crude extracts of *Padina australis*

Data in Table 1 demonstrate the yield percentages of the n-hexane crude extracts of *Padina australis* using UAE method. The n-hexane crude extracts of *Padina australis* is described in the form of moderate thick extract, a specific smell, and a blackish brown (Fig. 2). UAE process involves application of sound energy at a very high frequency greater than 20 KHz to disrupt macrolagae cell all and increase the drug surface area for solvent penetration. As a result, secondary metabolites will be released. The advantages of ultrasound-assisted extraction (UAE) is applicable to small sample; it minimizes the time of extraction and amount of solvent consumption, maximizes the yield (Abubakar & Haque, 2020) and have positive implication on environmental impact (Alcántara, 2020). The effect of UAE in liquid media is mainly attributed to cavitation phenomena, thus promoting stirring, creating high local turbulence, and also to temperature increase associated to gas bubble implosion (Alcántara, 2020).

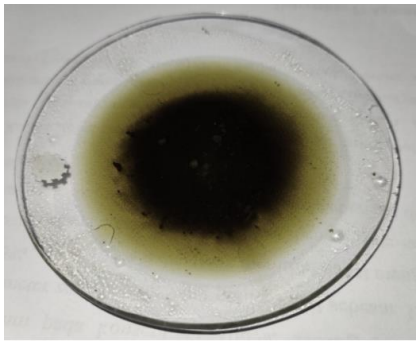


Figure 2. n-hexane crude extracts of *Padina australis*

According to Febrina (2015), the factors that influence the extraction yield are time, temperature, stirring, and solvent, and sample size. The smaller the sample's surface area, the stronger the contact and interaction with the solvent (Sineke et al., 2016). From Table 1, the optimum time of UAE is at 10 minutes that yields 0.117 %. After 10 minutes of extraction, the yield percentage decrease gradually.

Table 1. The result obtained between increasing extraction time and percentage yield.

Extraction Time (minutes)	% Yield
5	0.100
10	0.117
15	0.084
20	0.059
30	0.049

Time was one of the variables that positively influenced the increase of percentage yield, which can be explained as mass transfer is a time dependent process (Palma et al., 2021). Regarding temperature, Gullón et al. (2017) have reported that high temperatures favor the extraction efficiency of bioactive compounds by increasing acoustic cavitation, surface contact area, and decreasing solvent viscosity and density.

Preliminary Phytochemical Compounds Screening of n-hexane crude extracts of *Padina australis*

Medicinal plants and herbs having active phytochemicals can have therapeutic benefits. Different phytochemicals have been discovered to have a wide spectrum of effects, potentially aiding in disease prevention and therapy. Phytochemical screening is aimed to investigate the compounds of the plant extracts, and their predomination, along with the search for bioactive compounds that may be useful in the production of therapeutic drugs (Adil et al., 2024). The secondary metabolite groups that have been detected from n-hexane crude extracts of *Padina australis* using UAE extraction method can be seen in Table 2. This result is in agreement with other reports by Hidayah et al. (2024) where alkaloids, flavonoids, saponins, tannins, steroids and phenols were detected.

Compared to other studies by Maharany et al. (2017) and Nuzul et al. (2017) using maceration method, the phytochemical compounds obtained showed the same results with UAE method. The data outlined in Table 2 indicated that there are no different phytochemical compounds phytochemical compounds resulted between UAE and maceration method. Moreover, *Padina* sp. has potential as a natural antioxidant and contains active compounds in it such as flavonoids, alkaloids, tannins, triterpenoids, saponins, phenolics and pigments such as chlorophyll a, chlorophyll c, carotenoids, fucoxanthin, fucoxantol and β-carotene and fatty acids (Sari, et al., 2016; Hassan et al., 2021). Furthermore, Al-Zahrani et al., (2014), Salosso & Jasmanindra (2018) and Salosso et al., (2021) reported that *P. australis* contains alkaloids, saponins, steroids, terpenoids, tannins and flavonoids. This result is different with study from Gazali & Safutra (2016) that reported n-hexane crude extracts of *Padina australis*, collected from Labuhan Haji, South Aceh, Indonesia, did not contain flavonoids, tannins and saponins.

Table 2. Phytochemical Compounds of n-hexane crude extracts of *Padina australis*

Phytochemical Compounds	**n-hexane crude extracts of *Padina australis***	**References**		
		Extracted by Maceration Method		**Extracted by UAE Method**
		Maharany et al., 2017	**Nuzul et al., 2017**	**Hidayah et al., 2024**
Alkaloids	+	+	+	+
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+
Steroids	+	+	+	+
Phenols	+	+	+	+

Notes:
(+) = presence
(-) = absence

Terpenoids demonstrates multiple bioactivities i.e., working as active agents against inflammation, cancer, viruses, and bacteria along with hindering cholesterol synthesis. While, flavonoids have antioxidant effects, inhibiting the initiation, promotion, and progression of tumors. Tannins showes antiviral, antibacterial, and antitumor activity. Alkaloids was from to beta-carboline group have strong antimicrobial, anti-HIV, and antiparasitic activities (Adil et al., 2024). Tannins are a group of polyphenols which commonly occur in nature and easily extracted from plants (Kaczmarek, 2020).

Antibacterial activity of the n-hexane crude extracts of *Padina australis*

In our study, the n-hexane crude extract of *Padina australis* was tested its antibacterial activity against four different tested bacteria using disc-diffusion method (Kirby Bauer method) where tetracycline (30 µg) and DMSO were used as positive and negative control, respectively. The n-hexane crude extracts of *Padina australis* using UAE extraction method showed varying degrees of antibacterial activity against the tested bacteria (*Shigella sonnei, Escherichia coli, Salmonella typhi, and Bacillus cereus*) (Table 3). The results were expressed in terms of the diameter of the growth-inhibition zone (clear zones).

It can be seen in Table 3, n-hexane crude extracts of *Padina australis* showed high activity against *Shigella sonnei* (13.86 mm) and lowest activity against *Salmonella typhi* (10.97 mm). Compare to other studies, the 96% ethanol extract of *Padina australis* has been tested and proven to be able to inhibit the growth of *E. coli* (Haryani et.al., 2014), *Salmonella typhi*, and *Vibrio cholera* bacteria (Haryani et al., 2015).

The potential antibacterial activity of *P.australis* is related to the content of phenolic compounds and their derivatives (flavonoids). This compound can inhibit bacterial growth by disrupting the function of the cytoplasmic membrane. The presence of phenolic compounds causes damage to the cytoplasmic membrane, resulting in leaks

in the membrane (Kumar et al., 2013 & Salosso et al., 2011). Further, tannin is an active secondary metabolite compound that is useful as an astringent, antidiarrheal, antibacterial, and also antioxidant (Maharany et al., 2017). Tetracycline is an antibiotic and also as positive control that can inhibit cell wall synthesis (Wardani, 2024).

DMSO is used as a test extract solvent because it dissolves both polar and nonpolar compounds. The negative control, DMSO, was utilized to demonstrate that DMSO, which is used to dissolve the test extract, has no antibacterial action (Husni et al., 2020).

Table 3. Antimicrobial activity of n-hexane crude extracts of *Padina australis*

Bacterial Tested	**Inhibition diameter (mm)**		
	Extract (100 ppm)	[a]Positive Control (+)	[b]**Negative Control (-)**
Bacillus cereus	13.28	7.1	**0.00**
Shigella sonnei	13.86	4.45	**0.00**
Salmonella typhi	10.97	5.58	**0.00**
Escherichia coli	12	6.6	**0.00**

Note:
a = tetracycline
b = DMSO

Based on Davis and Stout's classification, the strength of antibacterial activity, that has inhibition zone diameter of 5 mm or less, as weak; 5–10 mm as moderate; 10–20 mm as strong, and 20 mm or more as very strong (Ariyani et al. 2018). From Table 3, we can conclude that n-hexane crude extract of *Padina australis* has strong antibacterial activity (10.97 – 13.28 mm) against all bacteria tested *Shigella sonnei, Escherichia coli, Salmonella typhi, and Bacillus cereus.* Interestingly, the antibacterial activity of this extract is stronger than positive control (tetracycline (30 µg)).

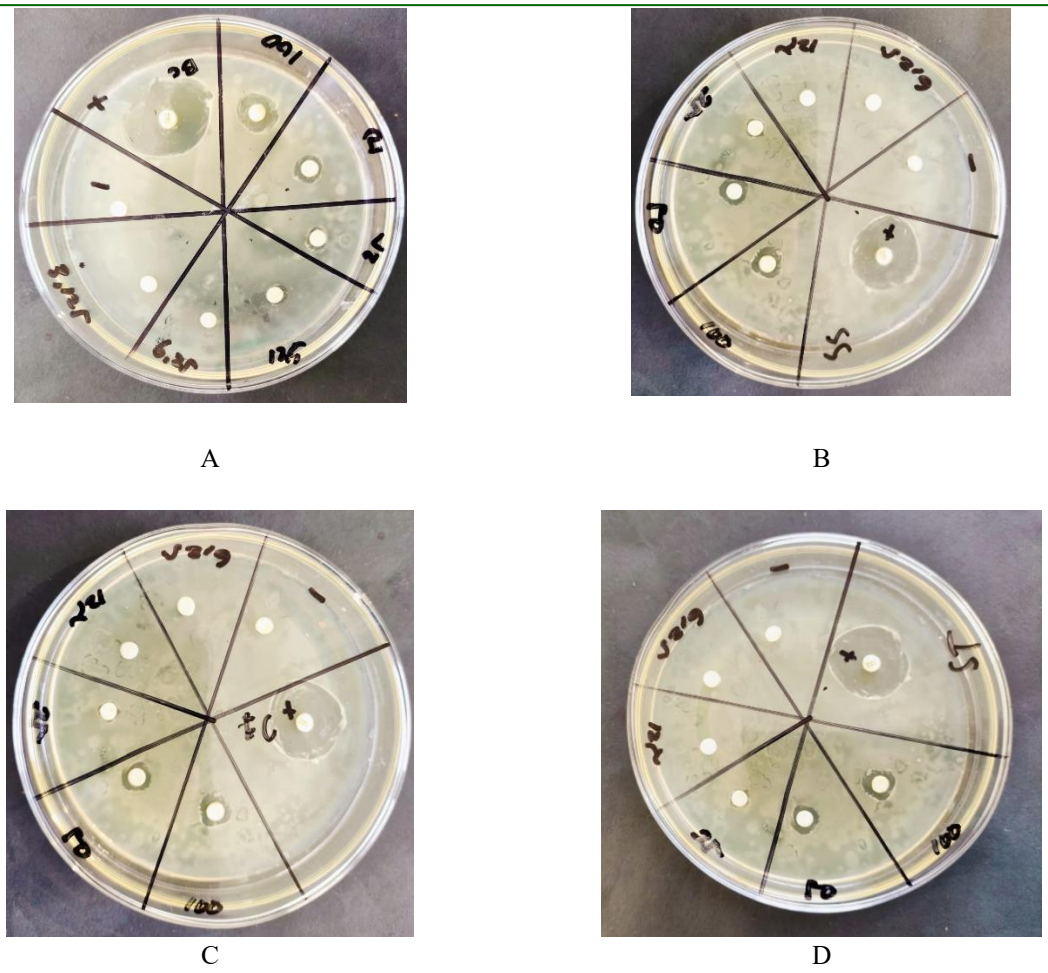
Minimum inhibitory concentration (MIC) of n-hexane crude extracts of *Padina australis*

Minimum inhibitory concentration (MIC) is the lowest concentration of an extract that visually inhibit the growth of the test organism after incubation (Ibrahim et al., 2017). The results of MIC presented in Table 4 showed that all microorganisms were very susceptible to the minimum inhibitory concentration of n-hexane crude extracts of *Padina australis* (12.5 ppm) except for *E. coli* and *Bacillus cereus* the MIC value were 25 ppm and 6.25 ppm. Important to note, n-hexane crude extracts of *Padina australis* strongly inhibit the growth of *Bacillus cereus* with the MIC value was 6.25 ppm. However, this extract has the lowest antibacterial activity against *E. coli* (MIC value = 25 ppm).

Table 4. MIC of n-hexane crude extracts of *Padina australis* against Four Different Bacterial Tested

Bacterial Tested	**Concentrations of crude extracts (ppm)**				
	50	**25**	**12.5**	**6.25**	**3.125**
Bacillus cereus	6.05	4.15	3.28	2.62	**0**
Shigella sonnei	3.78	3.16	2.4	0	**0**
Salmonella typhi	4.7	2.86	2.6	0	**0**
Escherichia coli	4.3	3.1	0	0	**0**

The concentration of the active compounds in the *P. australis* extract, the sensitivity of the bacteria tested to the extract, and the speed of the active compounds' diffusion into the agar medium all influence the diameter of the inhibition zone that forms (Gazali & Safutra, 2016). Many antibacterial medicines work by inhibiting pathways necessary for the synthesis of peptidoglycan, a key component of the bacterial cell wall (Biswas et al., 2014).



A B C D

Figure 3. MIC of n-hexane crude extracts of *Padina australis* against *Bacillus cereus* (A), *Shigella sonnei* (B), *Escherichia coli* (C), and *Salmonella typhi* (D)

4. CONCLUSION

In conclusion, n-hexane crude extracts of *Padina australis* from Tidung Island, Seribu Island District, North Jakarta Province, Indonesia contains alkaloids, flavonoids, saponins, tannins, steroids and phenols. Moreover, n-hexane crude extracts of *Padina australis* have antibacterial activity against four different bacterial tested (*Shigella sonnei, Escherichia coli, Salmonella typhi, and Bacillus cereus).* From this study, it can be concluded that n-hexane crude extracts of *Padina australis* is potent against *Bacillus cereus* with MIC value was 6.25 ppm.

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