Bioedukasi
Jurnal Biologi dan Pembelajarannya
Vol. 22 No 3, October 2024, page 444-454
e-ISSN: 2580-0094; p-ISSN:1693-3931
DOI: 10.19184/bioedu.v22i3.50081
jurnal.unej.ac.id/index.php/BIOED

Ultrasound Assisted Extraction (UAE) of Macroalgae Padina australis From Tidung Island, Indonesia and Testing Its Antibacterial Activity

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Article Info
Article history:
Received July 12, 2024
Revised October 3, 2024
Accepted October 5, 2024

Keywords: (A-Z)
Antibacterial activity
Escherichia coli
Padina australis
Ultrasound Assisted Extraction (UAE) method

ABSTRACT
Padina australis is one of brown macroalgae that grows in Indonesian ocean. This study aimed to determine the optimum of time extraction of *Padina australis* ethanolic extract extracted by *Ultrasound Assisted Extraction* (UAE) method, its phytochemical compounds and the antibacterial activity of *Padina australis* ethanolic extract against on four different pathogenic bacteria (*Salmonella typhii, Shigella sonnei, Escherichia coli* dan *Bacillus cereus*). *Padina australis* were extracted by *Ultrasound Assisted Extraction* (UAE) method using ethanol solvent (70%) with three different times (10, 20 and 30 minutes). The ethanolic extract of *Padina australis* were evaluated its antibacterial activity and its minimal inhibitory concentration (MIC) by using disc diffusion method. The results showed that the optimum UAE time of *Padina australis* was 20 minutes which yielded 0.4%. Moreover, the ethanolic extract of *Padina australis* contained alkaloids, saponins, flavonoids, tannins, steroids, and terpenoids. Furthermore, this study indicates that *Padina australis* ethanolic extract demonstrated its antibacterial activity against all bacteria tested. MICs of *Padina australis* ethanolic extract determined for *S. typhi* and *B. cereus* were at 6.25 ppm and for *S. sonnei* was at 12,5 ppm. Whereas, this extract showed the MIC at 25 ppm on *E. coli*.

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1. INTRODUCTION

In recent decades, the number of antimicrobial-resistant diseases (bacteria and fungi) has increased. Because standard antibiotics and antifungals are no longer effective, this is now regarded as a public health problem (Roca et al., 2015; Silva et al., 2020). Besides, the widespread, inappropriate, irregular, and indiscriminate use of antibiotics has led in the establishment of antimicrobial resistance, rendering many currently available treatments ineffective. It is critical to explore new alternatives to solve this problem especially from marine macroalgae.

Medicinal plants, particularly marine ones, provide an almost unlimited source of bioactive chemicals, and their potential as antimicrobial agents has been investigated in a variety of methods. However, the chemicals have not yet been properly explored (Lampinen, 2021; Vaou, 2021). Indonesia is a mega biodiversity country including abundant macroalgae. Based on their pigment, macroalgae are classified as three types namely rhodophyta (red macroalgae), phaeophyta (brown macroalgae), and chlorophyta (green macroalgae) (Wiryato, 2015). There are many compounds derived from macroalgae with various biological activities, such as antibiotics, antivirals, antitumor, anti-inflammatory and neurotoxins (Dulger, 2014; Setha, 2013). *Padina australis* contains phytochemicals such as flavonoids, phenol hydroquinone, terpenoids, tannins, saponins, β-carotene, diadinoxanthin, diatoxanthin, fucoxanthin, chlorophyll a, and chlorophyll c (Hutapea et al., 2021; Maharany et al., 2017; Saptari, 2015; Hassan, 2021).

Padina sp. extract has previously been extracted using standard methods such as maceration (Harb & Chow, 2022; Maharany et al., 2017; Sari et al., 2016) and percolation (Al-Enazi et al., 2018). Traditional extraction methods were inefficient in terms of solvent and time used, leading to the development of more efficient procedures such as ultrasonic-assisted extraction (UAE). The UAE method is considered a cold extraction method, using acoustic waves in the kilohertz range (20 kHz to 100 kHz), as the temperature during the extraction process is relatively low and has no effect on the stability of extracted compounds (Silva, 2020). Futhermore, Ultrasonic

assisted extraction (UAE) involves the application of high-intensity, relatively low-cost, high-frequency sound waves and their interaction with materials (Tobgay, 2020).

Based on the explanation above, this research was designed to extract phytochemical content from 70% ethanol extract of Padina australis using the *Ultrasound-Assisted Extraction* (UAE) method, to determine the optimum extraction time, and to test its antibacterial activity against several pathogenic bacteria (*Salmonella typhii, Shigella sonnei, Escherichia coli* and *Bacillus cereus)*.

2. RESEARCH METHOD

Materials

All the chemicals and reagents were of analytical grade. The main material used was *Padina australis* macroalgae were collected from Tidung Island, Seribu Island District, Indonesia. The chemicals used were 70% ethanol (Merck, Germany), dimethyl sulfoxide (DMSO; Merck). Four test bacterial species obtained from collection of Pharmacy Faculty, Universitas Pancasila, Jakarta, Indonesia were *Salmonella typhii, Shigella sonnei, Escherichia coli* dan *Bacillus cereus.* The growth media for bacteria are nutrient broth (NB; Oxoid), nutrient agar (NA; Oxoid), and tryptone soya agar (TSA; Oxoid). 6 mm Diameter paper disk, Whatman No. 1 filter paper (Merck), and tetracycline antibiotic (30 µg/disc).

Collecting and Preparation of *Padina australis* Macroalgae

Padina australis macroalgae were collected in Desember, 2024 from Tidung Island, Seribu Island District, North Jakarta Province, Indonesia (Figure 1). The fresh macroalgae were stored in cool box during transportation time from Tidung Island to laboratory (Yunianto *et al.*, 2014). They were cleaned by tap water to remove sands, epiphytes, and other impurities and were identified by National Research and Innovation Agency for knowing their species. Moreover, the fresh and clean macroalgae were dried and blended. Then, the pulverized macroalgae was weighed and stored in refrigerator (4^0C) for further analysis (Husni *et al.*, 2014).

Ultrasound Assisted Extraction (UAE)

The extract was obtained by the *Ultrasound Assisted Extraction* (UAE) method with 70% ethanol as a solvent. UAE was carried out in an ultrasonic water bath apparatus (Branson 2510, USA). The pulverized macroalgae (30 gram) was extracted using 300 mL of 70% ethanol (ratio 1:10 (w/v)) in an ultrasonic water bath with three different times (10, 20, and 30 minutes) at 42 kHz and 50% amplitude. After ultrasonic extraction, the extracts were collected and evaporated by rotary evaporator at 40 ^{0}C to remove the solvent. The resulting extract was weighed and then stored in the refrigerator for further analysis. Then, the *Padina australis* ethanolic extract was extracted in triplicate and calculated its yield percentage (Panjaitan & Farida, 2023).

Qualitative Phytochemical Analysis

Tannins Testing

3 mL of *Padina australis* ethanolic extract was put into a test tube, then add 3 drops of 1% $FeCl_3$. The formation of blue or greenish-black color indicates the presence of tannins compounds (Ikalinus et al, 2015).

Alkaloids Testing

About 1 mL of Dragendorff's reagent was added to 3 mL of *Padina australis* ethanolic extract. The formation of orange-red precipitate proved the availability of alkaloids (Al-Bahrawee et al., 2023 & Adil et. al, 2024).

Saponins Testing

About 3 mL of extract obtained from the macroalgae sample was put into test tubes. Then, 3 mL distilled water was added into the test tube and mixed them vigorously. Then, the test tube was kept aside for 3-5 minutes. The presence of foam indicates the presence of saponins (Al-Bahrawee et al., 2023).

Flavonoids Testing

3 mL of *Padina australis* ethanolic extract was put into a test tube, were mixed by 0.1 gram of magnesium powder, 1 mL of concentrated HCl and 1 mL of amyl alcohol. The mixture was shaken vigorously and allowed the layers to separate. The formation of red in the amyl alcohol layer was interpreted as a sign of flavonoids's presence (Ikalinus et al, 2015).

Terpenoids and Steroids Testing

About 2 mL of chloroform was added to 3 mL of *Padina australis* ethanolic extract into test tubes 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).

Antibacterial Assay

Sterilization of tools and materials

The tools and materials were sterilized using an autoclave at 121 ^{o}C for 15 minutes. The tweezers and wire loops are burned directly over a fire. This seeks to eliminate germs found on instruments and materials that may interfere with the test.

Preparation of Media

For NA media, 23 gram nutrient agar (NA) were dissolved in 1 L aquadest. While, 13 g nutrient broth (NB) were dissolved in 1 L aquadest. Then, both media (NA and NB) had been sterilized in an autoclave at 121 ^{0}C for 15 minutes. The agar medium was cooled to about 50°C before being poured into the petri dish (Kumesan et al., 2017).

Serial of Concentration Test

Preparing a 100 ppm stock solution is carried out by dissolving 100 mg of the thick extract with DMSO (dimethyl sulfoxide), then diluting it until 100 mL using volumetric flask (100 mL). The ethanol extract solution of *Padina australis* was made with concentration series of 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm and 3.125 ppm using dimethyl sulfoxide (DMSO) solvent.

Preparing Inoculum Bacteria

Salmonella typhi

A single loop of *Salmonella typhi* was cultivated in nutrient broth (NB) and then incubated at 37°C for 12-36 hours (Normaidah, 2020).

Shigella sonnei

A single loop of *Shigella sonnei* was cultivated in nutrient broth (NB) and then incubated at 37°C for 72 hours (Aini, 2018).

Escherichia coli

A single loop of *Escherichia coli* was cultivated in nutrient broth (NB) and then incubated at 37°C for 16-18 hours (Rusli et al., 2018).

Bacillus cereus

A single loop of *Escherichia coli* was cultivated in nutrient broth (NB) and then incubated at 37°C for 16-18 hours (Datta et al., 2019).

Antibacterial activity of *Padina australis* ethanolic extract

The antibacterial activity of *Padina australis* ethanolic extract was assessed against pathogenic bacteria (*Salmonella typhii, Shigella sonnei, Escherichia coli* dan *Bacillus cereus)* by disc diffusion method. The positive control used was tetracycline antibiotic (30 µg/disc), whereas dimethylsulfoxide (DMSO) was the negative control. Petri dishes containing nutrient agar (NA) were inoculated with bacterial inoculums. The disc of Whatman paper of 6 mm in diameter was impregnated by *Padina australis* ethanolic extract (10 µL /disc). Tetracycline antibiotic disc, DMSO disc and *Padina australis* ethanolic extract disc were placed on the petri dishes containing nutrient agar (NA) surface were inoculated with bacterial inoculums and incubated at 37°C for 18-48 hours based on the incubation time of each bacteria tested. Antibacterial activity was examined by measuring the inhibition zone or clear zone diameter around the disc using digital vernier caliper. Each experiment was conducted in triplicate (Syafni *et al.*, 2012).

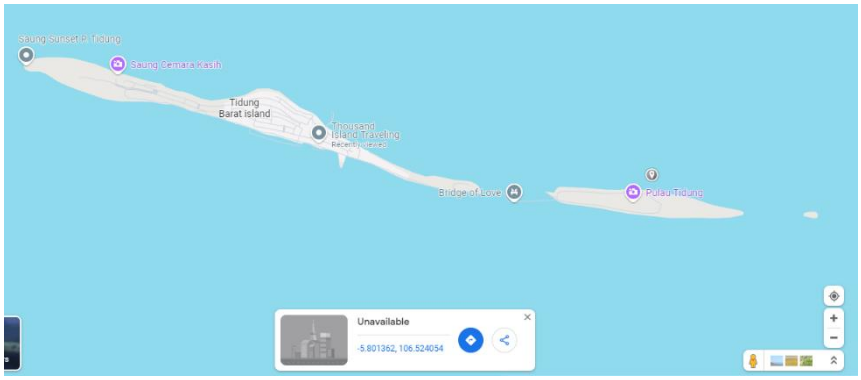
Determination of Minimum Inhibitory Concentration (MIC)

The MIC demonstrated the least concentration of extracts that totally inhibited bacterial growth. MIC assay was performed out by disc diffusion method. The ethanol extract of *Padina australis* were serial diluted at 100 ppm; 50 ppm; 25 ppm; 12.5 ppm; 6.25 ppm and 3.125 ppm. The discs (6 mm diameter) impregnated with 10 µL aliquots of serial diluted concentrations of extracts (100, 50, 25, 12.5, 6.25 and 3.125 ppm) were used, then placed on nutrient agar (NA) plates inoculated with test bacterial (*Salmonella typhii, Shigella sonnei, Escherichia coli* dan *Bacillus cereus)* and incubated for 18-48 hours at 37°C. Tetracycline antibiotic (30 µg/disc) was used as positive control and negative control was DMSO. After incubation, the inhibition zone was measured to determine the MIC value. The tests were carried out in triplicate (Syafni *et al.*, 2012).

3. RESULTS AND DISCUSSION

Sample of macroalgae *Padina australis* was collected from Tidung Island, South Seribu Island District, North Jakarta Province, Indonesia (106º19'30" - 106º44'50" East Longitude and 5º10'00" - 5º57'00" South Latitude) (Figure 1) on 14th December, 2023. The determination result showed that the sample is *Padina australis.*

(A)



(B)

Figure 1. Tidung Island (A) and Map of Tidung Island (B)

As visual observation, the thalli's colour of Padina australis which collected from Tidung Island are yellowish brown. It has a fan-shaped morphology in the form of thin, segmented sheets with lines that tend to be circular and light brown-green in color. The edges of the talus tend to curve inward, with an average diameter of 3–4 cm and a length of up to 7 cm. This visual observation is in agreement with Kepel et al., (2016) study.

Macroalgae were identified by National Research and Innovation Agency with genus: Padina and species: *Padina australis* Hauck, 1887.

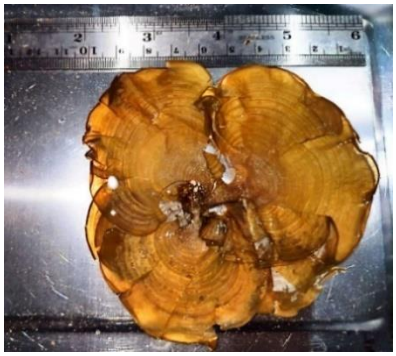
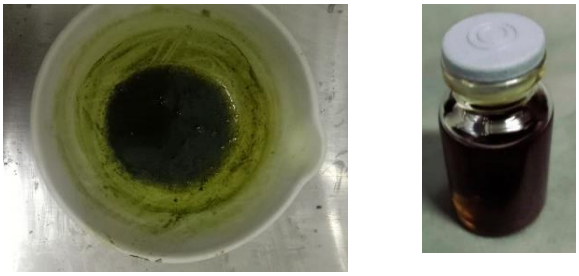


Figure 2. Padina australis from Tidung Island, Indonesia

Percentage Extraction Yield

Extraction of secondary metabolites from *Padina australis* was carried out by *Ultrasound Assisted Extraction* (UAE) method with 70% ethanol as a solvent with three different extraction times namely 10, 20 and 30 minutes. The result is presented in Table 1. Ethanol solvents have high polarity and are nontoxic. Besides, previous studies found that this solvent is the most effective for extracting brown seaweed (Hassan, 2021).



(A) (B)

Figure 3. Concentrated *Padina australis* ethanolic extract

According to Table 1, the optimum extraction time of *Padina australis* ethanolic extract is 20 minutes, which results in a yield of 0.4%. When the time was prolonged (to 30 minutes), the % yield declined (0.21%). Similar to Hassan et al. (2021) study, the yield of *Padina australis* ethanolic extract reached a maximum at 70 minutes (5.37 mg) and declined as the time increased (at 80 minutes) as 5.06 mg. Extended extraction time may cause oxidation, epimerization, and degradation of the metabolites of interest (Esparza, 2020; Fadzare, 2021).

Table 1. % Yield of *Padina australis* at Various Extraction Time

Time (minute)	% Yield
10	0.30
20	0.4
30	0.21

Ultrasound-assisted extraction (UAE) uses ultrasound power and solvents to extract target compounds from macroalgae extract. During the ultrasonic process, the cell wall is destroyed, while accelerating the release and diffusion of components within the cell (Chemat, 2017). Ultrasound-Assisted Extraction additionally employs a mechanical effect that enhances solvent penetration into the sample matrix, allowing for a faster diffusion rate when it passes the cell walls (Syahir et al., 2020). The working principle of ultrasound-assisted extraction involves high-frequency sound energy, specifically above 20 kHz, which can improve solvent penetration into the matrix due to cell wall breakage caused by acoustic cavitation (Zhang et al., 2018).

Phytochemical compounds of *Padina australis* ethanolic extract

Qualitative phytochemical screening is aimed to evaluate the bioactive compounds of the macroalgae extracts that may be useful in the manufacturing of medicinal or therapeutic drugs. In this study, the qualitative phytochemical analysis of *Padina australis* ethanolic extract is carried out as shown in Table 2. Alkaloids, flavonoids, saponins, tannins, steroids and terpenoids were detected in the ethanol extract of *Padina australis*. Compared to study which conducted by Hidayah et al (2024) that extracted *Padina* sp. using UAE method reported methanol extract of *Padina* sp. has alkaloids, saponins, steroids and phenols.

Maharani et al. (2018) reported three macerated extracts of *Padina australis* (methanol, ethyl acetate and n-hexane) contained alkaloids, flavonoids, saponins, tannins, steroids and terpenoids. Furthermore, the macerated 96% ethanol of *Padina australis* which collected from Bayah Beach, Banten, was confirmed the presence of alkaloids, flavonoids, saponins, tannins, steroids and terpenoids (Saptari et al, 2015). In addition, Hidayati et al (2022) study, flavonoid, steroid and tannins were also present in macerated methanol of *Padina* sp. which collected from Bintan Island, Indonesia. This finding is in agreement with the studies of Maharani et al. (2018), Saptari et al. (2015), and (Hassan et al., 2021) that are presented in Table 2. Furthermore, the phytochemicals constituents depend upon the types of extraction, seasons, habitat and species.

Table 2. Phytochemical Compounds of *Padina australis* ethanolic extract

Phytochemical Compounds	*Padina australis* ethanolic extract	References		
		Maharani et al., 2018	Saptari et al., 2015	(Hassan et al., 2021)
Alkaloids	+	+	+	+
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+
Steroids	+	+	+	+
Terpenoids	+	+	+	+

Notes:
(+) = detected
(-) = undetected

Flavonoids have antioxidant effects and inhibiting tumors while tannins exhibit antiviral, antibacterial, and anticancer activity (Bhattacharya, 2016). Flavonoids contained in plants have various benefits, including antioxidants, anti-inflammatory, antibacterial, and antifungal properties, with their respective mechanisms (Ningsih et al., 2023).

Antibacterial Activity of *Padina australis* ethanolic extract

In this study, the antibacterial activity of *Padina australis* ethanolic extract (100 ppm) was tested against four different pathogenic bacteria (*Salmonella typhii, Shigella sonnei, Escherichia coli* and *Bacillus cereus)* by disc diffusion method. Tetracycline antibiotic (30 µg) was as positive control, while the negative control used is DMSO. In this study, DMSO, as negative control, is a surfactant that can dissolve polar and nonpolar materials and also showed no inhibition activity in Table 3 and 4. The inhibition activity of *Padina australis* ethanolic extract (100 ppm) against four different bacteria is presented in Table 3. The 70% ethanol extracts of *Padina australis* are seen by the diameter of inhibition formed around the paper discs (Figure).

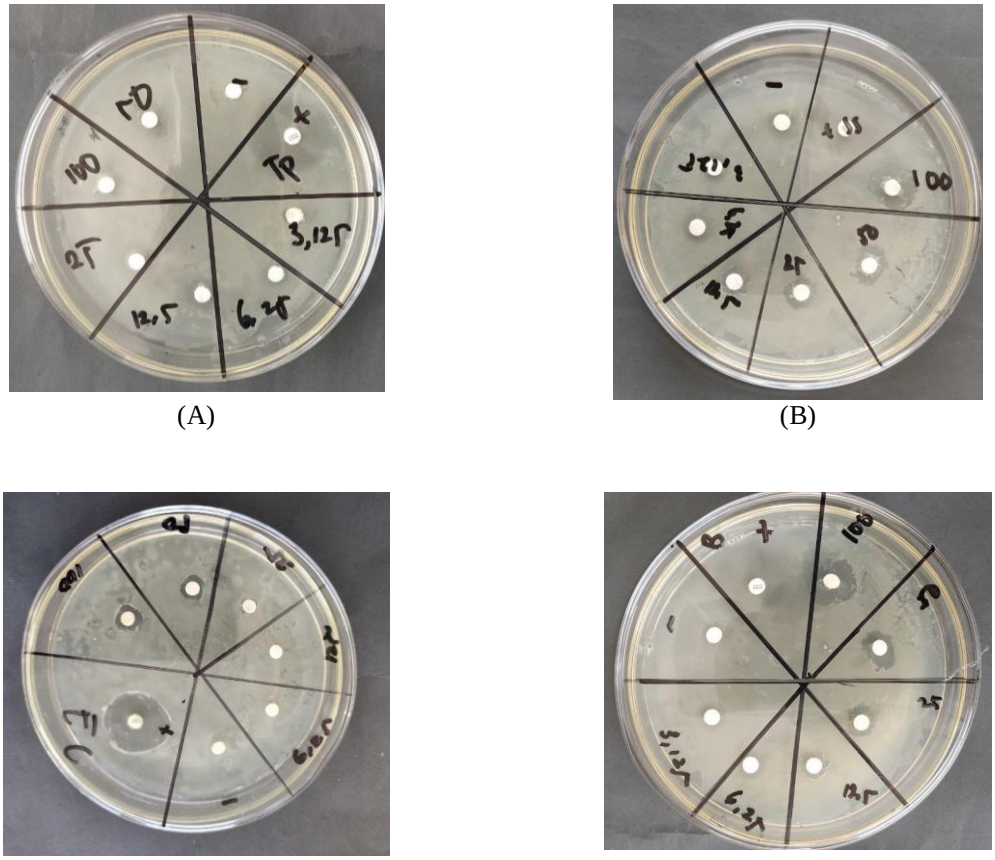
The important finding resulting from this current study is *Padina australis* ethanolic extract (100 ppm) showed the highest antibacterial activity against *Shigella sonnei* with the diameter of inhibition zone of 6 mm. Compared to positive control (tetracycline (30 µg)), the diameter of inhibition zone resulted was 11.67 mm which is still stronger than the inhibition activity of *Padina australis* ethanolic extract. On the other hand, *Padina australis* ethanolic extract (100 ppm) has the lowest antibacterial activity against *E. coli* (3.63 ppm) but tetracycline showed the highest antibacterial activity such as 12.93 mm. Compared to Warsidah et al. (2021) study, the 70 % ethanol extract of *Padina pavonica* demonstrated the antibacterial activity against *E. coli* (11.6 mm) at 20 ppm.

Davis and Stout's criteria classify the strength of antibacterial activity, namely an 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). Findings of the present study clearly demonstrate that the antibacterial activity of *Padina australis* ethanolic extract was weak against *Salmonella typhi, Escherichia coli, and Bacillus cereus* and moderate level against *Shigella sonnei* (Table 3).

Table 3. Antibacterial Activity of *Padina australis* ethanolic extract

Test Bacteria	Diameter of inhibition zone of *Padina australis* ethanolic extract (mm)	Category of Inhibition activity of *Padina australis* ethanolic extract (Ariyani et al. 2018)	Diameter of inhibition zone of Tetracycline (mm)	Diameter of inhibition zone of DMSO (mm)
Salmonella typhii	4.9	weak	11.8	0
Shigella sonnei	6	moderate	11.67	0
Escherichia coli	3.63	weak	12.93	0
Bacillus cereus	4.3	weak	8.75	0

The antibacterial activity or inhibition effects of *Padina australis* ethanolic extract against pathogenic bacterial (*Salmonella typhii, Shigella sonnei, Escherichia coli* and *Bacillus cereus)* can inform the new potential candidate of bioactive compounds for drug development especially for finding new antibiotic source candidate.



(A) (B)

(C) (D)

Figure 4. Antibacterial Activity of 70% ethanol extract of *Padina australis* against *Salmonella typhi* (A), *Shigella sonnei* (B); *E. coli* (C) and *B.cereus* (D)

Minimum Inhibitory Concentration

MIC was tested for the 70% ethanol extract of *Padina australis* against four different pathogenic bacterial (*Salmonella typhii, Shigella sonnei, Escherichia coli* and *Bacillus cereus)* and the results are presented in Table 4.

Table 4. Diameter of zone of inhibition (mm) against bacteria by *Padina australis* ethanolic extract

Test Bacteria	Concentration (ppm)	Mean zone of inhibition (mm)		
		Extract	Tetracycline	DMSO
Salmonella typhii	50	4.3	11.8	0
	25	3.4		
	12.5	2.73		
	6.25	1.6		
	3.125	0		
Shigella sonnei	50	4.53	11.67	0
	25	3.16		
	12.5	3.05		
	6.25	0		
	3.125	0		
Escherichia coli	50	2.76	12.93	0
	25	1.73		
	12.5	0		
	6.25	0		
	3.125	0		

Test Bacteria	Concentration (ppm)	Mean zone of inhibition (mm)		
		Extract	Tetracycline	DMSO
Bacillus cereus	50	4	8.75	0
	25	3.3		
	12.5	3.02		
	6.25	1.1		
	3.125	0		

According to the MIC values (Table 4), the 6.25 ppm crude ethanol extract of *Padina australis* suppressed the growth of *Salmonella typhi* and *Bacillus cereus*. Moreover, *Escherichia coli* has the lowest MIC value namely 25 ppm while *Shigella sonnei* had MIC value of 12.5 ppm.

The present study reports that the crude ethanol extract (70 %) of *Padina australis* has antibacterial activity against *Salmonella typhii, Shigella sonnei, Escherichia coli* and *Bacillus cereus*. This result is supported by the presence of alkaloids, flavonoids, saponins, tannins, steroids and terpenoids which might be responsible for the antibacterial activities (Hemant, 2013 and Krishnaiah, 2009). Specifically, tannins work with inactivating enzymes, microbial adhesins and cell envelope transport proteins. Moreover, flavonoids and alkaloids disrupt microbial membrane. Furthermore, saponin acts as a detergent and might increase the permeability of bacterial cell membranes (Khan et al., 2018).

Generally, the action mechanism of antibacterial activity disrupts cell membrane stability or permeability that causes cell lysis and inhibit enzymes and metabolic pathways (Silva et al., 2020). Antibiotic can inhibit or kill the pathogenic bacteria. Tetracyclines are broad-spectrum bacteriostatic drugs or antibiotic that can inhibit protein synthesis. They are effective against many gram-positive and gram-negative bacterials (Levine & O' Connor, 2012 and (Amalia, 2016).

4. CONCLUSION

From this study, it can be concluded that the optimum time of *Ultrasound Assisted Extraction* (UAE) that yield the highest yield percentage (0.4 %) is at 20 minutes. *Padina australis* ethanolic extract contained alkaloids, flavonoids, saponins, tannins, steroids and terpenoids. The ethanolic extract (70%) of *Padina australis* can inhibit the growth of four pathogenic bacteria *Salmonella typhii, Shigella sonnei, Escherichia coli* and *Bacillus cereus*, that the highest antibacterial activity is against on *Shigella sonnei* (6 mm). The minimum inhibitory concentration (MIC) value for each bacteria was found at a concentration of 25 ppm (*E. coli*), 12.5 ppm (*Shigella sonnei*), and 6.25 ppm (*Salmonella typhi* and *B. cereus*).

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