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The Potential of Sea Urchin (*Diadema* Setosum) Extracts as Antibacterial Against *Staphylococcus Aureus*

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Abstract

Secondary metabolites from sea urchin shells contain active substances with the potential of antibiotics. The purpose of this study was to investigate how sea urchin shell extract affected the development of the *Staphylococcus* bacterium. In this kind of research, the active chemical content of sea urchin shell extract was examined quantitatively, using scanning electron microscope (SEM) and Fourier Transform Infrared (FTIR), as well as the extract's effect on the development of *Staphylococcus aureus* bacteria. According to the study, the average level of total flavonoids was 1.29 %, total alkaloids were 0.12 %, and total tannins were 1.0 %. Sea urchin shell powder has an absorbance of 0.42 at a wavelength of 1,400.84 cm^{-1}. According to microbiological experiments, sea urchin shell ethyl acetate extract was able to stop *Staphylococcus aureus* growth in the category of the strong inhibition zone (inhibition zone diameter of 12 mm). Extract from sea urchin shells has antibacterial properties and may be used to make anti-diabetic ointments. This fabrication is a fantastic option for use in healthcare and medicine.

Keywords: Antibacterial, Fourier transform infrared, Sea urchin shell extract, Scanning electron microscope, *Staphylococcus aureus*

Introduction

Out of 198 countries, Indonesia is the 2nd-largest archipelagic country after Canada, with more than 18,000 islands and 81,000 km long [1]. In addition, Indonesia has a diverse population and a high percentage of its geographical area is covered by water. Because of how rich Indonesia's maritime biodiversity is, it is frequently referred to as the nation with the most biodiversity in the world, known as the "global highest biodiversity".

A promising and important location, in particular, is the sea area of Lombok, which is made up of bays, peninsulas and beaches over a length of 985 km and has a marine area that is 11 times greater than the mainland [2]. As soon as it is discovered that the sea may be a source of subsistence for people who are no longer dependent on the land, the richness of marine biodiversity needs to be harnessed. Therefore, addressing it necessitates a better comprehension of the genetic and biological variety of marine resources.

Sea urchin shells are one of the marine biotas that can be used as a source of bioactive substances with significant economic value [3]. Indicators of the distribution and quantity of marine plants in shallow seas include sea urchin shells. Sea urchin shells typically reside in groups or schools in coastal waters, depending on their habitat. There are many different species of sea urchin shells. Sea urchin shells are typically found in seagrass and coral reef habitats. Sea urchin shells exist as a rather hard substrate in this ecosystem, especially in seagrass ecosystems that are composed of sand and coral chips [4,5]. One of the various biotas found in Indonesian seas, including NTB, is sea urchin shells (*Diadema* stetosum) [6,7]. People have begun raising sea urchin shells for food and as a remedy for human ailments because of the quantity of them in Indonesian waters [8].

Highly resistant bacteria, fungi and viruses can become pathogens for these organisms and marine biota are susceptible to these microbes. As a result, these marine biotas produce a bioactive compound, a secondary metabolite, which is used to protect itself from environmental hazards and the animals in its environment [5,8]. The shells of sea urchin shells contain poisonous bioactive substances that may be used as antibiotics. The active chemicals found in the toxins produced by many species, such as sea urchin shells, can serve as antibiotics, making them useful as medicinal ingredients in the medical industry, particularly in the pharmaceutical industry [9]. According to multiple earlier research, sea urchin shells with ethanol extract included antibacterial and antioxidant components [10,11].

Diabetic foot ulcers are a consequence of diabetes mellitus brought on by neuropathy and Peripheral Arterial Disease (PAD). The risk of complications rises and wound healing is slowed down when diabetic foot ulcers are present [12]. Up to 25 % of people with diabetes mellitus run the risk of getting diabetic foot ulcers. Around 20 % of all diabetes mellitus therapies in the UK go toward treating this consequence of diabetic foot ulcers [13]. The prevalence of diabetic foot ulcers is 6.3 % worldwide, with America having the highest incidence (13 %), Asia having the 2nd-highest prevalence (5.5 %) and Oceania having the lowest prevalence (3 %) [14]. Up to 55.4 % of diabetic patients in eastern Indonesia have diabetic foot ulcers, and about 12.2 % are at risk of getting them [13,15].

According to Yusuf *et al.* [15], 12 % of Indonesian adults had diabetic foot ulcers. The prevalence of diabetic foot ulcers is rising, necessitating significant treatment approaches to wound care. Amputation risk is increased by either treating or not treating ulcerated Diabetic Foot Ulcer (DFU); this risk can be deadly. Foot Diabetic Wound is a skin-surface open wound that has the potential to get infected. Diabetes ulcer complications brought on by infection must be treated and managed according to industry standards. The development of antibiotic resistance mechanisms is a difficult issue in antibiotic therapy. To employ natural substances for antibiotic therapy, we must consider this. According to various research, *Staphylococcus aureus* is the predominant bacterium that is frequently discovered in diabetic foot ulcer cases [16,17].

By performing a proximate test with a UV-Vis Spectrophotometer instrument, a FTIR test, a SEM test to determine the particle size of the bioactive substances contained, and an inhibition test to determine the antibacterial activity of ethanol, methanol and ethyl acetate extra, this study seeks to identify the bioactive substances present in sea urchin shells and their concentration. Thus, our study wanted to elucidate the potential of sea urchin extract as an antimicrobial, especially those found in diabetic foot ulcer patients such as *Staphylococcus aureus.*

Materials and methods

Experimental design

The method used in this study, known as an experimental laboratory design, aims to identify the bioactive substances present in sea urchin shells and their concentrations selected and applied in this research that has the purpose to confirm the antibacterial activity of ethanol extract, methanol extract and ethyl acetate extract from sea urchin shells against *Staphylococcus aureus* through proximate analysis using a UV-Vis Spectrophotometer instrument and a FTIR test. SEM is also used to determine the particle size of the bioactive substances present and to examine their antimicrobial activity. The study design was shown in **Figure 1**.

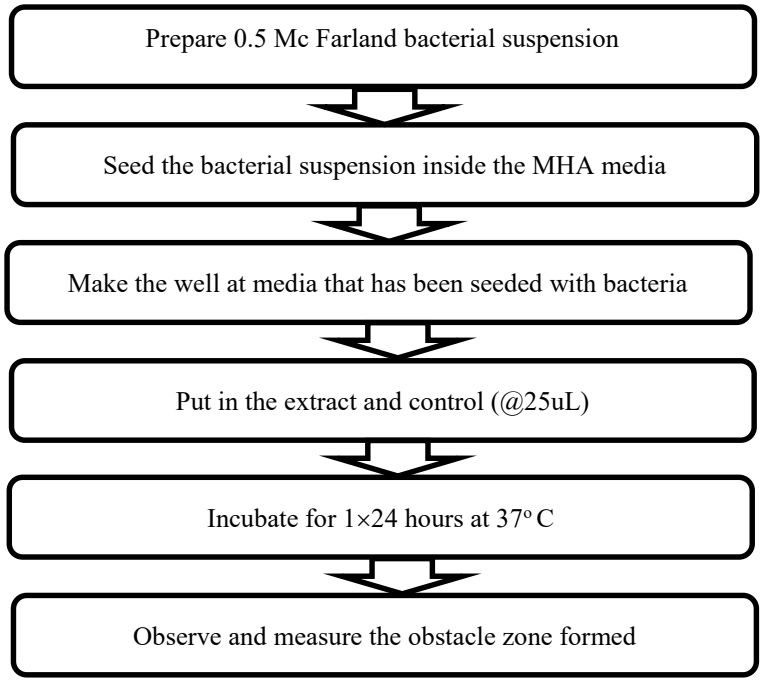

Figure 1 The diagram of the extract sensitivity test flow

The Ethical Clearance approval number for this study is 248/UNI18.F7/ETIK/2022. This study was conducted at the laboratories of the Medica Farma Husada Mataram Polytechnic, the Biomedical Research Unit (URB) of the NTB General Hospital, and the Integrated Research and Testing Laboratory of Gajah Mada University.

Preparation of powder and extract of sea urchin shells

Sea urchin shells from Ekas Beach in East Lombok were used as the research sample and were analyzed at the Integrated Research and Testing Laboratory at Gajah Mada University. The sea urchin shells were dried and powdered, then sieved to get a finer powder with 100 mesh. The majority of the obtained powders were then steeped for 7 days with daily stirring in a different solvent of ethanol, methanol and ethyl acetate, with a ratio of 1:3. The extract was filtered using Whatman filter paper once every 2 days until the 7th day when final filtering was done and the extract was then evaporated to create a pure viscous extract.

Determination of bioactive substance particle size by SEM

SEM is a type of electron microscope that uses an electron beam to describe the surface shape of the material being analyzed. The working principle of SEM is as follows, an electron gun produces an electron beam and is accelerated by the anode then a magnetic lens focuses the electrons towards the sample, and the focused electron beam scans (scans) the entire sample directed by the scanning coil. Then when the electrons hit the sample, the sample will emit new electrons which will be received by the detector and sent to the monitor Cathode Ray Tube (CRT) [18]. When observing the material, the location of the surface of the object being shot with the highest intensity electron beam is scanned over the entire surface of the observed material. Because of the wide area of observation, we can limit the location of our observations by zooming in or zooming out. By utilizing the reflected beam from the object, information can be found using an image processing program contained in the computer. SEM has a higher resolution than an optical microscope. This is caused by the de Broglie wavelength which has shorter electrons than optical waves. Because the smaller the wavelength used, the higher the resolution of the microscope [19].

FTIR test

FTIR spectroscopy is an excellent analytical technique in the process of identifying the molecular structure of a compound. The main component of FTIR spectroscopy is the Michelson interferometer which has the function of decomposing (dispersing) infrared radiation into frequency components. The use of the Michelson interferometer gives the FTIR method an advantage over conventional infrared spectroscopy methods and other spectroscopic methods. Among them is the molecular structure information that can be obtained precisely and accurately (has a high resolution). Another advantage of this method is that it can be used to identify samples in various phases (gas, solid or liquid). The difficulties found in identification with FTIR spectroscopy can be supported by data obtained using other spectroscopic methods [20].

Infrared spectroscopy is a method that observes the interaction of molecules with electromagnetic radiation in the wavelength region of 0.75 - 1,000 μm or at wave numbers of 13,000 - 10 cm^{-1}. The infrared spectroscopy method is a method that includes absorption techniques, emission techniques and fluorescence techniques. The electric field components that play a large role in spectroscopy are generally only electric field components such as in the phenomena of transmission, reflection, refraction and absorption. The absorption of electromagnetic waves can cause the excitation of energy levels in molecules. It can be in the form of electronic excitation, vibration, or rotation [21].

The working principle of an infrared spectrophotometer is photometry. Light from an infrared light source is a combination of different wavelengths. The light passing through the interferometer will be focused on the sample. The beam transmitted by the sample is focused on the detector. Changes in the intensity of the light produce an interference wave. This wave is converted into a signal by the detector, amplified by the amplifier, then converted into a digital signal. In the FTIR optical system, laser radiation has interfered with infrared radiation so that the infrared radiation signal is received by the detector intact and better. The operating technique of the FTIR is different from that of an infrared spectrophotometer. In FTIR, a Michelson interferometer is used as a substitute for the monochromator which is located in front of the monochromator. This interferometer will give a signal to the detector according to the intensity of the molecular vibration frequency in the form of an interferogram [22].

UV-Vis Spectrophotometer

Visible Spectrophotometry (UV-Vis) is the measurement of light energy by a chemical system at a certain wavelength [23]. Ultraviolet (UV) light has a wavelength between 200 - 400 nm, and visible light

has a wavelength of 400 - 750 nm. Spectrophotometric measurements use a spectrophotometer which involves a large amount of electronic energy in the molecule being analyzed so the UV-Vis spectrophotometer is more widely used for quantitative rather than qualitative analysis. The UV-Vis spectrum is very useful for quantitative measurements. The concentration of the analyte in the solution can be determined by measuring the absorbance at a certain wavelength using the Lambert-Beer law [24].

The working principle of the spectrophotometer is the absorption of light at a certain wavelength by the material being examined. Each substance has a unique absorbance at certain wavelengths. The wavelength with the highest absorbance is used to measure the level of the substance being examined. The amount of light absorbed by a substance is directly proportional to the level of the substance.

Antimicrobial activity test of sea urchin shell extract

The *Staphylococcus aureus* isolates utilized were purified from the NTB General Hospital from pure cultures of individuals with diabetic foot ulcers. Bacterial suspension of *Staphylococcus aureus* was made to reach a final bacterial concentration of 6×10^5 CFUs/mL of which 100 µL were pipetted onto the test surfaces. Spread the bacterial suspension onto the sterile cotton swab aseptically in the Muller Hinton Agar (MHA) medium (Merck, Germany). MHA media was prepared by weighing 38 g according to the composition on the package (2 g beef extract; 17.5 g casein hydrolysate; 1.5 g starch; 17 g agar) then dissolved in 1 L of distilled water, if necessary with heating assistance. Give the bacterial suspension 5 to 10 mins to properly diffuse in the media. Wells were made using a sterile cork borer (6 mm in diameter) into the agar plates containing the inoculum. add 50 µL of Sea Urchin Shell Extract was added to each of the 4 wells, and A commercial reference disc was used as a control (Ciprofloxacin 5 mg purchased from 500 mg). The media were incubated for 24 h at 36 ± 1 °C, under aerobic conditions. Measure the inhibition or clear zone that formed in mm. Tests were performed in duplicate.

Results and discussion

Sea urchin powder particle size by SEM and FTIR

In this study, proximate testing using the UV-Vis Spectrophotometer instrument, the FTIR test, and using a SEM were used to determine the antimicrobial activity of sea urchin shell ethanol extract, sea urchin shell methanol extract, and sea urchin shell ethyl acetate extract. SEM was carried out before the powder was made into an extract to see how homogeneous the powder particles were. The main objective of conducting SEM is to observe the surface morphological structure of the sample, in this case, sea urchin shell powder. The surface shape and shape homogeneity of the powder will greatly affect the extraction and withdrawal process of the targeted active compounds [25]. SEM is used to characterize alloy powders which aim to determine the morphology of the powder such as particle shape and size. Digital images from SEM characterization results were processed using ImageJ software to determine particle size and measurement results. SEM test results on samples of sea urchin shell powder (**Figure 3**) using magnifications of 100, 50, 10 and 5 µm, i.e. the average particle size is 100.6 nm. In general, the analysis of the particle size of sea urchin shell powder was carried out using the ImageJ application. Segmentation is based on edge detection, thresholding method, or region-based segmentation methods and the most frequently used technique is the watershed segmentation technique to determine particle sizes that are homogeneous and evenly distributed. As shown in **Figure 3**, ImageJ has been analyzed using 2 methods. In the watershed segmentation method, it can be seen that 1 particle can be divided into several smaller particles so that the known particle size is not suitable for particles that are not evenly distributed in SEM images. Whereas the segmentation based on the edge detection method allows the particles to unite with other particles as shown in **Figure 3**. The homogeneous particle size of the sea urchin shell powder sample is very decisive in the manufacture of ointment products. That is what makes the importance of sample testing using SEM.

While FTIR is done to see the absorbance ability of the extract later. This relates to how the preparation will be made, the administration of the preparation, and how many doses are given. FTIR can identify functional groups, and compounds and analyze mixtures present in samples without damaging the samples [26]. The infrared region of the electromagnetic spectrum starts from a wavelength of 14,000 cm^{-1} to 10 - 1. Based on these wavelengths the infrared region is divided into 3 regions, namely near IR (14,000 - 4,000 cm^{-1}) which is sensitive to overtone vibrations, medium IR (4,000 - 400 cm^{-1}) associated with the vibrational energy transitions of molecules which provide information about groups -functional groups in the molecule, and far IR (400 - 10 cm^{-1}) to analyze molecules containing heavy atoms such as inorganic compounds but requires special techniques [27,28]. Usually, the analysis of compounds is carried out in the moderate IR region [29]. In this case, FTIR can be used to analyze the presence of flavonoids, tannins

and alkaloids based on their absorbance spectra [30]. The FTIR results of sea urchin shell powder samples obtained several compounds, namely 0.12 % alkaloids, 1 % tannins and 1.29 % flavonoids. Based on the results of FTIR absorption spectroscopy, it can be seen that the FTIR spectra of sea urchin shell powder generally show significant differences at an absorption wavelength of 1,633.19 cm^{-1} with an amplitude of 0.287 and at an absorption wavelength of 1,400.84 cm^{-1} with an amplitude of 0.395 (**Figure 4**). FTIR is very important to do because samples from sea urchin shell powder will be made into ointment products.

The powdered shell of a sea urchin underwent an SEM test. With a magnification of up to 1,000,000 times, SEM is a tool for examining/viewing the surface morphology and cross-sectional structures of samples. The following is the SEM's operating principle: 1) An anode accelerates an electron beam that is produced by an electron cannon. 2) The electrons are directed toward the sample by the magnetic lens. 3) The scanning coil directs the concentrated electron beam to scan the entire sample. **Figure 3** displays the SEM test outcomes for sea urchin shell powder.

The absorbance of sea urchin shell powder is shown in **Figure 4**, with the maximum absorbance occurring at a wavelength of 1,400.84 cm^{-1} and a wave peak of 0.42. Sea urchin shell powder absorbs light at a wavelength between 600 and 3,700 cm^{-1}. The intensity of IR ray absorption is at this wavelength. The highest absorbance of sea urchin shell powder occurs at a wavelength of 1,400.84 cm^{-1}.

Etil asetete extract inhibits *Staphylococcus aureus* bacteria

Sea urchin shells inhibit the growth of *Staphylococcus aureus* bacteria in diabetic foot ulcer patients' pure cultures, using ciprofloxacin as a positive control. The results of the ethanol extract of sea urchin shells' antibacterial activity test revealed no antimicrobial activity (**Table 3**). **Table 1** displays the inhibition category based on the size of the inhibition zone that formed.

Alkaloids, tannins and flavonoids are present in the sea urchin shell extract, according to the results of the proximate test. Based on the results of the proximate test (**Table 2**) it is known that the total amount of tannin and flavonoid alkaloids is different in each solvent. This is due to the different nature or molarity of the solvent so the ability to attract active compounds is also different. Variations in the type of solvent are known to have a significant effect on antioxidant activity [31]. The difference in the ability to attract active substances in a natural substance of several solvents is determined by the dielectric constant which is expressed as the repulsive force between 2 electrically charged particles in a molecule. The higher the dielectric constant, the more polar the solvent. The dielectric constants for water, methanol, ethanol and ethyl acetate each have values of 80, 33, 24 and 6.0 [32].

Additionally, the extract's antibacterial activity against *Staphylococcus aureus* was tested on the media. **Figure 2** displays the outcomes of the sea urchin shell extract's antibacterial activity test. The potential of flavonoids and alkaloids as antibacterial agents against bacteria has also been proven in several studies, including celery extracts and randu honey which have been shown to inhibit *Staphylococcus aureus* and *Staphylococcus epidermidis* [33,34]. Research on the content of flavonoids in plant extracts of the Genus Artocarpus has also been shown to inhibit the growth of gram-negative bacteria [35].

Four different extract concentrations were used in this study: Ethanol extract, methanol extract and ethyl acetate extract from sea urchin shells at concentrations of 5, 10, 25 and 45 %. **Table 2** shows that neither the ethanol extract nor the methanol extract had an inhibitory zone or clear zone at any of the quantities utilized. Different results were shown in the study of [36] that the use of methanol as a solvent still shows the potential inhibiting bacterial growth. This is possible because the ethanol we use as a solvent is a polar protic solvent, while the bioactive compounds contained in sea urchin shells are dominant in both quality and quantity and are non-polar bioactive compounds.

Table 1 The inhibition category of Ciprofloxacin antibiotics based on the diameter of the inhibition zone formed.

Inhibition zone diameter	Inhibitory category
< 5 mm	Weak
6 - 10 mm	Intermediates
11 - 20 mm	Strong
> 21 mm	Very strong

Source: Fatimah, 2022.

Table 2 Proximate test results for sea urchin shell extract with uv-vis spectrophotometer.

Solvent	Test parameters	Results	Unit
Ethanol	Total flavonoids	1.29	% b/b
	Total alkaloids	0.15	% b/b
	Total tannins	1.24	% b/b
Methanol	Total flavonoids	0.91	% b/b
	Total alkaloids	0.13	% b/b
	Total tannins	1.41	% b/b
Ethyl Acetate	Total flavonoids	1.69	% b/b
	Total alkaloids	0.07	% b/b
	Total tannins	0.49	% b/b

Table 3 Results of sensitivity test of sea urchin shell extract on the growth of *Staphylococcus aureus*.

	Test material	Concentration 5 %			Concentration 10 %			Concentration 25 %			Concentration 45 %			Ciprofloxacin (+)			Aquadest (–)		
No		U1	U2	U3	U1	U2	U3	U1	U2	U3	U1	U2	U3	U1	U2	U3	U1	U2	U3
1	Ethanol	0	0	0	0	0	0	0	0	0	0	0	0	27	27	27	0	0	0
2	Methanol	0	0	0	0	0	0	0	0	0	0	0	0	30	30	35	0	0	0
3	Ethyl acetate	12	12	12	12	12	12	12	12	12	12	12	12	27	27	27	0	0	0

As a result, solvents such as ethanol and methanol cannot inhibit the growth of *Staphylococcus aureus* bacteria. Our result is following studies by Kresnamurti *et al.* [11], and Kresnamurti *et al.* [10]. Only at concentrations of 5, 10, 25 and 45 % from ethyl acetate did the inhibition zone form, and its average diameter was 12 mm (Strong), compared to 27 mm for the positive control (Very strong). According to El-Sayed [37], sea urchin crude extract demonstrated antibacterial efficacy against gram-positive bacteria but not against gram-negative ones. This results from variations in the gram-positive and gram-negative bacteria's cell membrane structures. On the media containing *Staphylococcus aureus* bacteria, an inhibition zone of 6.5 mm was created. The diameter created in our investigation is half of this diameter. The findings of this investigation diverge significantly from those of ours. This is likely because our extract was made from shells, whereas earlier research either employed crude extract or the whole sea urchin.

Ethyl acetate extract from sea urchin shells exhibits antibacterial activity as it is shown by several studies [37-40], antibacterial activity is made possible by flavonoid, alkaloid and tannin found in ethyl acetate extract. Based on the results by Purwitasari *et al.* [38], and Indriati *et al.*[33], the use of the ethyl acetate fraction as an extract solvent is proven to have a high content of flavonoids and terpenoid compounds which have the potential to inhibit virus growth and have a low toxicity effect.

The results of the study [37] are nearly identical to those of the study [39], where the ethyl acetate extract from sea urchin shells as a whole exhibits antibacterial activity against the growth of *Staphylococcus aureus* with an inhibition zone diameter of 7.67 mm, as opposed to the study Hardani *et al.* [40] , where the inhibition zone formed with sea by earlier research, *Staphylococcus aureus* growth is reported to be inhibited by ethyl acetate extract from sea urchin shells. This is a manifestation of the flavonoids, alkaloids and tannins found in sea urchin shells, which have antibacterial properties [41-43].

The mechanism by which flavonoids function as an antibacterial is by forming connections with extracellular proteins of bacteria, which eventually cause damage to the bacterial cell membrane [44]. Meanwhile, one of the factors that damage the bacterial metabolic system and impair the bacteria's ability to function is tannin toxicity [45]. Hyperglycemia is the primary symptom of the degenerative disease known as diabetes mellitus (DM) [46]. Having diabetic ulcers is one of the side effects of DM. When bacteria that are resistant to antibiotics infect diabetic ulcers, the lesions can enlarge and develop into chronic conditions. *Staphylococcus aureus* contributes to the growth of wounds and the development of ulcers [47,48].

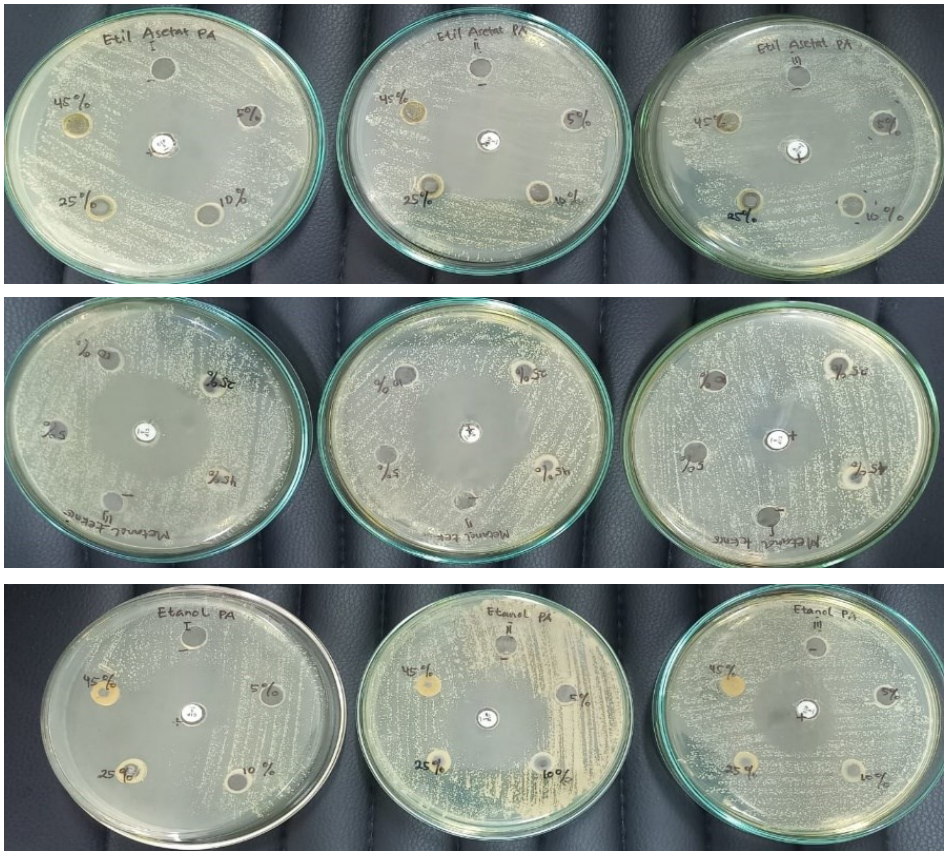

Figure 2 Test results for the antimicrobial activity of sea urchin shell extract against Staphylococcus aureus bacteria.

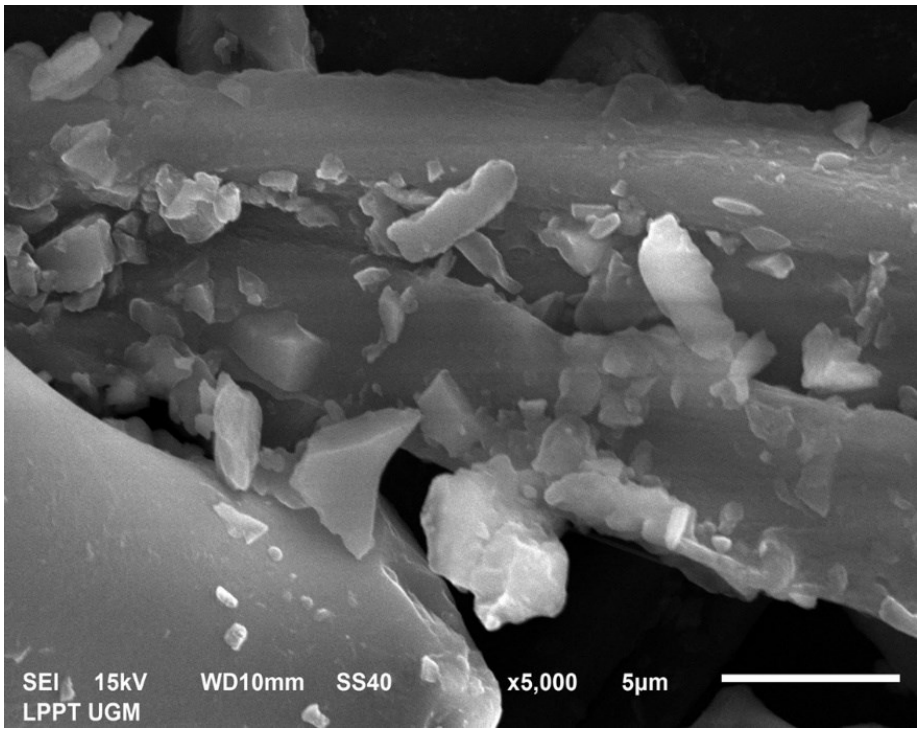

Figure 3 SEM test results for sea urchin shell powder.

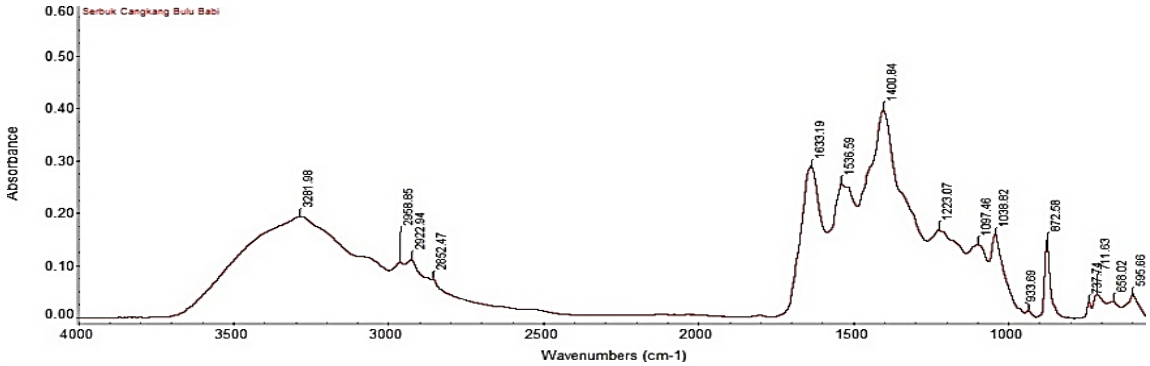

Figure 4 FTIR absorbance test results for sea urchin shell powder.

Conclusions

According to the study's findings, only the sea urchin shell extract with ethyl acetate solvent exhibited antimicrobial activity, as evidenced by the development of an inhibition zone around the sea urchin shell extract against *Staphylococcus aureus* bacteria. The extracts made using ethanol and methanol did not exhibit any antimicrobial activity.

Acknowledgments

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