FOOD
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Date : 10th June 2020

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Please return by : 10th July 2020

Title of Manuscript : *In vitro* antibacterial activity and potential applications in food of *Sea Urchin (Diadema setosum)* from Cape of Palette, South Sulawesi

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1.	**Title** *It should reflect the article* OK	
2.	**Abstract** *Background, Aim, Methodology and Conclusion* All comments were given via comment boxes in the main text	We agree with the reviewer's assesment about redundancy and rephrasing sentence in abstract. We have made corrections related to it.
3.	**Keywords** *Min. 3 and Max. 6* OK	
4.	**Introduction** *Concise with sufficient background* OK	
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		results analysis).
6.	**Data Analysis** *Results well presented and discussed* All comments were given via comment boxes in the main text	We agree with the reviewer's assesment about volume or concentration. We use volume units in 20 µL. Questions: How many replicates? This would be best to be presented as an average -+ SD. Answer : two replicates being tested in agar plate and we have made corrections about it. Question: How do you make sure that there aren't any remnants of solvents in your working extract prior to bioassay etc? Prove it. Answer: We agree with the reviewer's assesment about remnants. The content of the impurities is likely to exist because we do not perform the isolation and purification stage of the extract. Isolation method to remove impurities can be done by thin layer chromatography method. Sample testing for applications on foodstuffs still in the form of crude extract. Question : Unless if you can prove that there isn't any solvent remnants in you extract. Otherwise this will be the reason the meat was badly damaged. Answer: The remaining solvent is lost at the evaporation stage and it can be seen from the results chromatogram from Gas Chromatography Mass Spectrometry. When there are compounds of methanol and ethyl acetate, chromatogram will show it both the amount and type of its compounds, and it is seen in the first peak of the chromatogrham.
7.	**Conclusion** *A clear summary of the study* OK	
8.	**References**	

	References should follow the journal's format OK	
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In vitro antibacterial activity and potential applications in food of _Sea Urchin (Diadema setosum)_ from Cape of Palette, South Sulawesi

Abstract

Marine invertebrates in support of his life's defence of sea predatory use an innate immune mechanism, namely the cellular component hemocytes by secreting the dissolved antimicrobial and cytotoxic substances. It shows that marine invertebrates are a potential sources and promising antimicrobial compounds. This research aims at determining the antibacterial activity of sea urchin _(Diadema setosum)_ extract against some bacterial isolates and its application to foodstuffs. The gonad and shell of sea urchin extracted by methanol and ethyl acetate, and then separated by ultrasound-assisted extraction. Screening of antibacterial compound use Gas Chromatography-Mass Spectrometry (GC-MS), and disc diffusion method was followed to determine the antimicrobial activity against _Salmonella, Escherichia coli, and Staphylococcus aureus_. The results of this study showed antibacterial activity against one or more strains. The gonad of sea urchin from methanol extract exhibited significant inhibitory effect and effective against _Salmonella, Escherichia coli, and Staphylococcus aureus_. Majority of gonad and shell of sea urchin extract showed antibacterial activity against the tested strains. However, gonad of methanol extract was found to be inhibiting microorganisms gram-negative _(Escherichia coli)_ and gram-positive _(Salmonella and Staphylococcus aureus)_. Gonad extract can be a good source of antibacterial agents.

Commented [SZ1]: Doesn't make any sense. Are you using the physical extraction method or using the microbes lysis to get the extract? Rephrasing - Those microbes are for antibac and the para was confusing

Keywords: antibacterial activity, diadema setosum, disc diffusion method, sea urchin

1. Introduction

Sea Urchin (Phylum Echinodermata, Class Echinoid) is a commodity traded in many countries, has a hard shell and the inside is five symmetrical. Certain types of Sea urchin have shells coated with black pigments. In addition to having a hard shell, 95% of the body parts of the pig are dominated by very fragile and toxic thorns. These thorns are used to move, protect themselves, and stimulate food, and for certain types contain toxins. The toxins produced by sea urchin can be utilized in the field of treatment that is potentially used as antibiotics new types to be developed in the pharmaceutical field because it contains bioactive compounds (L. Abubakar _et al_., 2012). The sea urchin has an innate immune system that is potentially an effective new antimicrobial. The need to find new antimicrobial material is increasing, because the growth and development of bacteria are currently able to be resistant to antibiotics, as well as the growing conventional antibiotics (Chun Li _et al._, 2008). The increasing number of studies showing that bacteria can be resistant to conventional antibiotics has encouraged researchers to find new antimicrobial agents derived from natural substances (vegetable and animal). As invertebrates, sea urchin does not have an adaptive immune system as it belongs to the type of vertebrates (Rast _et al_., 2006; Smith _et al._, 2006). The ability to defend itself in the form of resistance to infection attacks from attacking microorganisms, phagocytosis ability (the role of white blood cells in the immune system) and encapsulation (Coffaro and Hinegardner, 1977; Hildemann and Dix, 1972; Ito _et al_., 1992; Silva, 2000). In recent decades, there has been an increase in marine field research (crustaceans, molluscs and echinoderms) with an object of secondary metabolite content and type of antimicrobial (Casas _et al._, 2011; Haug _et al_., 2002).

Research from Bryan _et al._ (1997); Haug _et al._ (2002); Chun Li _et al._ (2008); Rinehart _et al._ (1981) exposing antimicrobial activity in several species of echinosderms acquired from the Caribbean,

Mexico, the Gulf of California, and the Norwegian coast. Studied Prokof'eva *et al*. (2003) obtained antimicrobial compounds such as steroidal glycosid, polyhydroxylated sterols (Zhou *et al.*, 2014), naphthoquinone pigments (Amarowicz *et al.*, 2012; Powell *et al.*, 2014; Yoon *et al*., 2019), lysozymes (Cong *et al*., 2009), and antimicrobial peptides (C. Li *et al*., 2010) are in isolation from animal echinoderms. This research suggests that marine animals are potentially developed as potential sources as antibiotics in the pharmaceutical field. Other studies reported by (Haug *et al.*, 2002), showed that antibacterial activity was found on different parts of green sea urchin using test bacteria *Vibrio anguillarum* serotype O2 (FT 1801), *Escherichia coli* (ATCC 15922) and *Staphylococcus aureus* (ATCC 9144) and *Corynebacterium glutamicum* (ATCC 13032). These results indicate that the phylum echinoderms one of them has potential as a natural antimicrobial (Uma and Parvathavarthini, 2010).

Foodborne microbial pathogens contaminating food have been human concern for a long time. Human attempts to control microbial food contamination have thus also been a long journey; from old-school methods such as heat-processing and cold-processing to chemically induced antimicrobial agents. Consumer awareness, concerns about the potential risk of synthetic food additives to human health, as well as urgent concerns about the freshness of food, have led to renewed interest in exploring more organic and natural methods of food preservation (Rahardiyan, 2019; Si *et al*., 2006). Research antimicrobial activity on sea urchin has been carried out, from all organs and tissues (gonads and shell). But testing antimicrobial activity by using extracts derived from ultrasonic-assisted extraction (UAE) has not been conducted by other researchers. Potential sea urchin as an antibacterial need to be developed because it can be utilized as a medicinal ingredient in the field of pharmacy. The study aims to obtain the swine fleece extract using the ultrasonic-assisted extraction methods, determining antibacterial activity using disc diffusion methods, and bioactive antibacterial components using Gas Chromatography-Mass Spectrometry (GC-MS). Research is now focused on screening and comparing antimicrobial and hemolytic activity in various organs/tissues of sea urchin *Diadema setosum (Echinoidea)* obtained from Cape of Palette, Bone Regency, South Sulawesi.

2. Materials and Methods

2.1. Materials

Sea urchin *(Diadema setosum)* collected from the Cape of Palette, Bone Regency, South Sulawesi, Indonesia. The gonads separated from the sea urchin shell (Figure 1), then washed to remove other components and taken to the laboratory by carrying in the coolbox and stored in the freezer (-10^{o}C) until the gonads and shell sea urchin processed in Food Science and Instrumental Analysis Laboratory, Chemical Engineering Department, Politeknik Negeri Ujung Pandang, Indonesia.

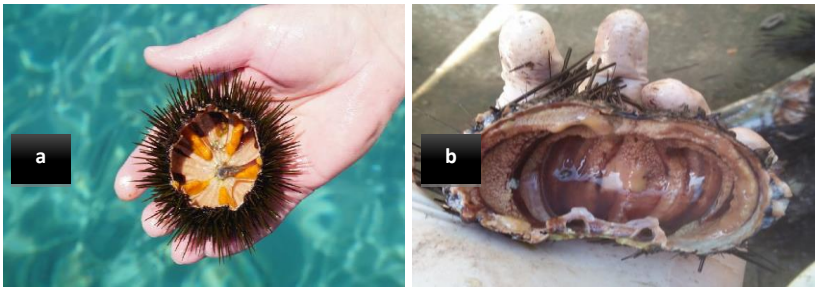

Figure 1. Sea urchin *Diadema setosum:* a) gonad and b) shell.

All chemicals were of analytical grade, ethyl acetate (CAS: 141-78-6), methanol (CAS: 67-56-1), aquadestilata (CAS: 7732-18-5), nutrient agar (CAS:105450), plate count agar (CAS: 105463), EMB agar (CAS: 101347), sodium chloride (CAS: 7647-14-5), pH paper, disc antibiotic blank (whatmann No.1 and No.5), dimethyl sulfoxide (CAS: 67-68-5) supplied by Merck Millipore (Burlington, Massachusetts, United States), Tetracycline hydrochloride (CAS: 64-75-5) supplied by Sigma Aldrich (St. Louis, Missouri, United States), beef and fish meat from carrefour, culture strains *Salmonella, Escherichia coli, and Staphylococcus aureus*. Tools used are water bath (Memmert WNB 7 Basic control) Hettich Zentrifugen EBA-20, rotary evaporator Buchi, Hitachi centrifuge brands, Ultrasonic Assisted Extraction instrument (Elmasonic P30), and Shimadzu GC-MS 2010 brand Gas Chromatography-Mass Spectrometry plus.

2.2. Preparation of extracts

Samples (200 g) gonads and shell of sea urchin were homogenized and extracted using Ultrasonic assisted extraction method with a ratio of Volume 1:1.5 (V/V) methanol or ethyl acetate for 30 and 60 min, then in the rotary evaporator at 39°C temperature, followed by a shaker at a temperature of 10°C for 24 hours. The Supernatant is produced for each sample, then in centrifugation for 10 min and stored at a temperature of 10°C, for use in testing the antibacterial disc diffusion method.

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2.3. Gas Chromatography-Mass Spectrometry(GC-MS)

Gonads and Shell extract of sea urchin were analyzed using GC-MS using capillary column DB-5 (30 µm, 0.25 mm, 0.25 µm film) and Flame Ionization Detector (FID) operated in EI mode at 70 eV. The injectors and detector temperature are set at 220 and 250°C. One sample was dissolved with 1 µl methanol, then injected and analyzed at 60°C for 2 min and then increased 3°C/min to 300°C, with Helium (He) is used as carrier gas (1 mL/min). The relative number of each component is expressed as a percentage peak area relative to the total peak area. Qualitative identification of different constituents is done by comparison of the relative retention time and the mass spectrum with authentic reference compounds, or by the retention index (RIS) and the mass spectrum.

2.4. Test microorganisms and culture media

Test microorganisms *Escherichia coli* (gram-negative), *Salmonella* (gram-positive), and *Staphylococcus aureus* (gram-positive), used in these studies were obtained from the Microbiology Laboratory, Department of Biology, State University of Makassar. The isolated bacteria will grow at a temperature of 32°C in nutrient broth (DIFCO Laboratories, Detroit, USA) in accordance with standard procedures (Keagle and Gersen, 2005).

2.5. Antimicrobial assay

Disc antibiotic blank (Whatman No. 1) cut to size and sterilized with other equipment using autoclaved at 121°C for 15 min. Growth media microorganisms are 5 g nutrient agar (NA), dissolved 250 ml of aquades in the Erlenmeyer 500 ml, then heated to homogeneous. The chloride sodium solvent is obtained by 0.9 g NaCl, then dissolved in a 100 ml volumetric flask, and inserted into the reaction tube 9 ml. Mc Farland solvent obtained, by mixing a solution of barium chloride ($BaCl_2$) 1.175% and a solution of sulphuric acid (H_2SO_4) 1%, so that the solvent obtained Mc Farland 0.5% to be used as standard turbidity. Media nutrient agar, chloride sodium solvent and Mc Farland solvent in sterilization using autoclaved at temperature 121°C for 15 min.

Bacteria *Salmonella sp, Escherichia coli, and Staphylococcus aureus*, as much as 20 ml of sterile nutrient agar was poured in Petri dishes, allowed to set at 37°C and then inoculate uniformly with 0.1 ml of a 24 hours broth culture of test bacteria (L. Abubakar *et al*., 2012; Syahirah and Rabeta, 2019). The gonad and shell extract sea urchin 0.25 g was dissolved in 1 ml aqueous dimethylsulfoxide (DMSO) with Tween 80 (0.5% v/v for easy diffusion) and sterilized by filtration through a 0.45 µm membrane filter. Under the aseptic condition, each sterile disc (Whatman no. 5, 6 mm dia) was then dipped in 20 µl of the gonad and shell of extracts sea urchin and carefully placed on the agar plate using flame sterilized forceps, ensuring the discs were at least 2 cm separate from one another. After 30 min, plates were inverted and incubated at 37°C for 24 hours, followed by measuring the inhibitory zone for each sample and the type of bacteria in mm. The experiment was carried out in duplicate and the averages diameter of zone of inhibition was recorded. Negative controls use a 10% DMSO solvent, and one paper disc is given a tetracycline of HCl as a positive control, antibacterial activity was classified as highly active (>10 mm), mild active (7-10 mm) and slightly active (6-7 mm) and less than 6 mm was taken as inactive (Chandra *et al*., 2011).

2.6. Application in beef and fish meat

Extracts that have the best inhibitory zone against bacteria *Escherichia coli, Salmonella sp and Staphylococcus aureus* are applied to beef and fish meat. 0.75 g of samples were dissolved in 3 ml aqueous dimethylsulfoxide (DMSO), beef and fish were cut in rectangular form 4 g, soaked into extract solution, meat was stored on 48 hours. The sample control is meat without the addition of extracts, as long as storage is observed physical parameters colour, aroma, texture and slime. After 48 hours of storage, 1 ml each of sterile plate count agar (PCA) and eosin methylene blue agar (EMBA) was poured in Petri dishes, meat dissolved in the NaCl and inoculate uniformly with 0.1 ml (dilution 10^{-1},10^{-2},10^{-3} and 10^{-4}) at 37°C for 24 hours of test bacteria.

3. Results and Discussion

3.1. GC-MS analysis

The presence of antibacterial components in the methanol and ethyl acetate extract of gonad and shell of sea urchin was characterized by GC-MS analysis (Table 1).

Table 1. Antimicrobial composition of gonad and shell extract of sea urchin.

Antibacterial Compound	Molecular formula	Methanol extract (% of Total)		Ethyl acetate extract (% of Total)	
		The gonad of sea urchin	The shell of sea urchin	The gonad of sea urchin	The shell of sea urchin
Cholest-5-EN-3-OL (3. Beta.)/Steroid (M. Abubakar & Majinda, 2016)	$C_{27}H_{46}O$	-	44.2	9.2	51.6
n-Hexadecanoic acid (Palmitic acid) (Rahman et al., 2014)	$C_{16}H_{32}O_2$	39.7	19.4	8.4	2.7
Ergosta-5,22-dien-3-ol, (3.beta.,22E) (Putra & Hadi, 2017)	$C_{28}H_{46}O$	-	6.3	-	10.7
Pentadecanoic Acid (Putra & Hadi, 2017)	$C_{15}H_{30}O_2$	1.7	1.1	0.3	-
1,2-Benzenedicarboxylic Acid	$C_{24}H_{38}O_4$	-	0.1	8.1	0.6

Commented [SZ3]: what is the concentration of each component analysed? This value will be used prior to bioassay

Commented [SZ4]: What total is this? w/w? with respect to the weight of supernatant? Be specific

(Phthalic Acid) (Hussain et al., 2011)					
9-Octadecenoic acid (Z) -, methyl ester (stearic acid methyl ester) (M. Abubakar & Majinda, 2016)	$C_{19}H_{36}O_2$	23.1	4.8	1.2	0.4
Arachidonic acid	$C_{20}H_{32}O_2$	-	3.9	-	-
9-Hexadecenoic acid, methyl ester, (Z)- (palmitoleic acid methyl ester)	$C_{17}H_{32}O_2$	3.7	2.4	1.1	0.6
Tetradecanoic acid (Myristic acid) (McNeil et al., 2010)	$C_{14}H_{28}O_2$	21.01	-	4.34	1.11
Oleic acid (M. Abubakar & Majinda, 2016)	$C_{18}H_{34}O_2$	0.97	-	-	-
Pentacosane (Brusotti et al., 2012)	$C_{25}H_{52}$	-	-	35.61	15.45
Tetratetracontane (M. Abubakar & Majinda, 2016)	$C4_4H_{90}$	-	-	6.88	6.53
Stigmast-5-EN-3-OL, (3.Beta.,24S)- (M. Abubakar & Majinda, 2016; Djouossi et al., 2015) / gamma.-Sitosterol	$C_{29}H_{50}O$	-	-	5.9	-

Thirteen constituents were predominantly found in the gonad and shell extract of sea urchin (Table 1). Six of these were from the methanol extract of gonad sea urchin. They include n-hexadecanoic acid (39.66%), Pentadecanoic Acid (1.73%), 9-Octadecenoic acid (Z) -, methyl ester (23.1%), 9-Hexadecenoic acid, methyl ester, (Z)- (3.74%), Tetradecanoic acid (21.01%), oleic acid (0.97%). While the remaining eight—i.e., Cholest-5-EN-3-OL (3. Beta.) (44.19%), n-Hexadecanoic acid (19.44%), Ergosta-5,22-dien-3-ol, (3.beta.,22E) (6.32%), Pentadecanoic Acid (1.06%), 1,2-Benzenedicarboxylic Acid (0.10%), 9-Octadecenoic acid (Z) -, methyl ester (4.84%), Arachidonic acid (3.85%) and 9-Hexadecenoic acid, methyl ester, (Z)- (2.9%) were from the methanol extract of shell sea urchin (Table 1). Most of these constituents have been found to show interesting biological activity against certain illnesses and/or pathogens. For instance, the antioxidant, anti-inflammatory, antibacterial, hypocholesterolemic activities reported for n-hexadecanoic acid, 9-Octadecenoic acid (Z) -, methyl ester and Tetradecanoic acid, with the dominant amounts of the three components.

There were ten antimicrobial compounds that were present in considerable amounts (80.93%) found in ethyl acetate extract of gonad sea urchin, and another nine antimicrobial compounds comprised 89.59% (Table 1). A component 9-Hexadecenoic acid, methyl ester, Pentacosane, -Octadecenoic acid (Z) -, methyl ester and Cholest-5-EN-3-OL (3. Beta.) have been reported to have antimicrobial activity. The presence of these compounds makes gonad and shell extract of sea urchin leaves a source of bioactive compound. Other minor compounds like n-Hexadecanoic acid have antibacterial properties, whereas Tetradecanoic acid, Tetratetracontane and Stigmast-5-EN-3-OL,(3.Beta.,24S) has antimicrobial activity.

3.2. Antibacterial Activity

Disc diffusion method was followed to determine the antimicrobial activity. Antimicrobial activities of gonad and shell of sea urchin *(Diadema setosum)* extracts were evaluated against three

bacterial (two gram-positive, one gram-negative) strains. The ethyl acetate solutions of the extracts were found to have potent antimicrobial activity against all the gram-positive and gram-negative bacteria tested by disc diffusion assay as shown in Table 2.

Table 2. Effect of gonad and shell extract of sea urchin against different pathogens.

Samples	Extraction time (min)	Concentration	Zone of inhibition (mm)		
			Escherichia coli	*Salmonella*	*Staphylococcus aureus*
Methanol extract of gonad sea urchin	30	20 µL	6.7	9.6	8.5
	60		6.9	10.5	11.2
Methanol extract of shell sea urchin	30	20 µL	6.5	8.8	7.6
	60		6.8	9.5	11
Ethyl acetate extract of gonad sea urchin	30	20 µL	7.4	12.6	11.8
	60		7.2	12.3	12.1
Ethyl acetate extract of shell sea urchin	30	20 µL	6.8	11.5	10.9
	60		6.9	10.3	10.6
Control positive	30	20 µL	17.3	35.7	32.5
Contol negative	30		0	0	0
Control positive	60	20 µL	17.2	33	33
Control negative	60		0	0	0

Commented [SZ5]: This is the volume used not concentration. What is the concentration used for each extract. Which component that give the effect on the bioassay attained? Be specific. Pick of any prominent component and work out with the concentration and volume to get the amount (mg) used for the bioassay.

3.2.1. Antibacterial activity against *Escherichia coli*

Methanol and ethyl acetate extract of sea urchin for extraction 30 and 60 min, exhibited a broad-spectrum antimicrobial activity with a minimum zone diameter of 6.5 mm for methanol extract of a shell with 30 min extraction and ethyl acetate extract of gonad with 30 min extraction a maximum zone diameter of 7.4 mm against on *Escherichia coli*. Ethyl acetate and methanol extract showed slightly active activity against *Escherichia coli* with a diameter of inhibitory zone 6 – 7 mm (Table 2 and Figure 2). The diameter of the inhibition zone (mm) was measured and the activity index was also calculated.

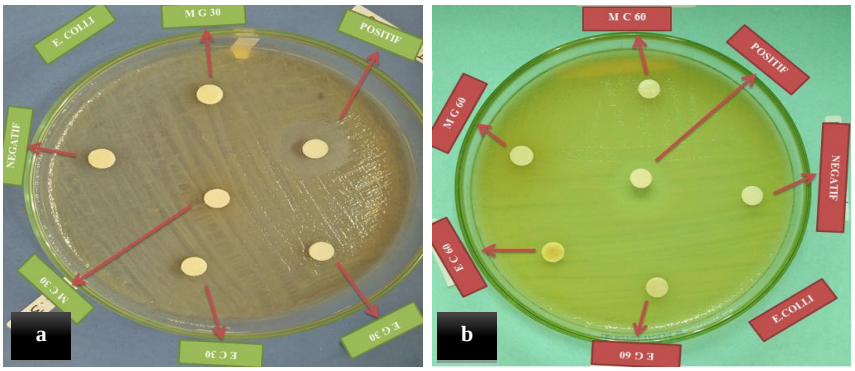

Figure 2. Antibacterial activity of methanol and ethyl acetate extract (gonad and shell) against *Escherichia coli*: a) 30 min extraction; b) 60 min extraction.

Notes: Sample EG30 (ethyl acetate extract of gonad, 30 min); EC30 (ethyl acetate extract of shell, 30 min); MG30 (methanol extract of gonad, 30 min); MC30 (methanol extract of shell, 30 min); Sample EG60 (ethyl acetate extract of gonad, 60 min); EC60 (ethyl acetate extract of shell, 60 min); MG60 (methanol extract of gonad, 60 min); MC60 (methanol extract of shell, 60 min).

3.2.2. Antibacterial activity against Salmonella

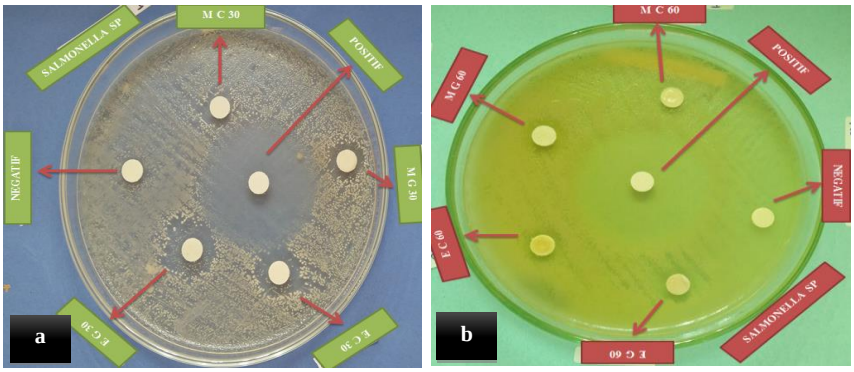

Figure 3. Antibacterial activity of methanol and ethyl acetate extract (gonad and shell) against *Salmonella*: a) 30 min extraction; and b) 60 min extraction.

Notes: Sample EG30 (ethyl acetate extract of gonad, 30 min); EC30 (ethyl acetate extract of shell, 30 min); MG30 (methanol extract of gonad, 30 min); MC30 (methanol extract of shell, 30 min); Sample EG60 (ethyl acetate extract of gonad, 60 min); EC60 (ethyl acetate extract of shell, 60 min); MG60 (methanol extract of gonad, 60 min); MC60 (methanol extract of shell, 60 min).

Methanol and ethyl acetate extract of sea urchin for extraction 30 and 60 min, exhibited a broad-spectrum antimicrobial activity with a minimum zone diameter of 8.8 mm for methanol extract of a shell with 30 min extraction and ethyl acetate extract of gonad with 30 min extraction a maximum zone diameter of 12.6 mm against on *Salmonella*. These result also showed ethyl acetate and methanol extracts as highly active activity against *Escherichia coli* with a diameter of inhibitory zone greater than 10 mm (Table 2 and Figure 3).

Commented [SZ6]: How many replicates? This would be best to be presented as an average -+ SD

3.2.3. Antibacterial activity against *Staphylococcus aureus*

Methanol and ethyl acetate extract of sea urchin for extraction 30 and 60 min, exhibited a broad-spectrum antimicrobial activity with a minimum zone diameter of 7,6 mm for methanol extract of a shell with 30 min extraction and ethyl acetate extract of gonad with 60 min extraction a maximum zone diameter of 12,1 mm against on *Staphylococcus aureus*. These result also showed ethyl acetate and methanol extracts as highly active activity against *Staphylococcus aureus* with a diameter of inhibitory zone greater than 10 mm (Table 2 and Figure 4).

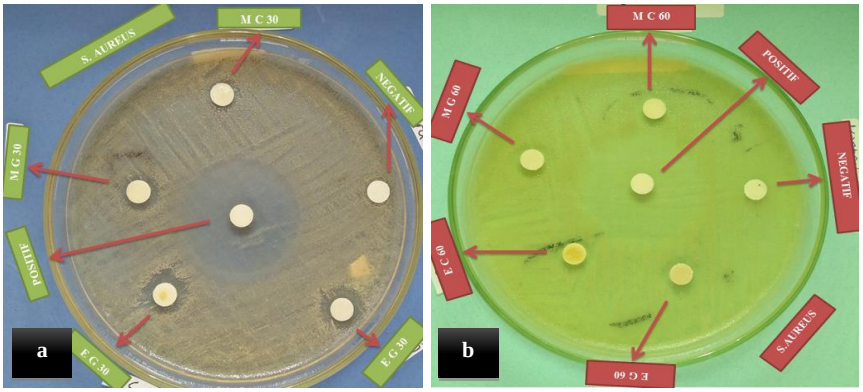

Figure 4. Antibacterial activity of methanol and ethyl acetate extract (gonad and shell) against *Staphylococcus aureus*: a) 30 min extraction; and b) 60 min extraction.

Notes: Sample EG30 (ethyl acetate extract of gonad, 30 min); EC30 (ethyl acetate extract of shell, 30 min); MG30 (methanol extract of gonad, 30 min); MC30 (methanol extract of shell, 30 min); Sample EG60 (ethyl acetate extract of gonad, 60 min); EC60 (ethyl acetate extract of shell, 60 min); MG60 (methanol extract of gonad, 60 min); MC60 (methanol extract of shell, 60 min).

The preliminary antimicrobial assay of the extracts showed different responses to the test organisms with the best activity observed for both methanol and ethyl acetate extracts of gonad and shell sea urchin against bacteria gram-positive (*Salmonell*a and *Staphylococcus aureus)*. The gonad and shell sea urchin extracts were observed to show poor activity against the test pathogens bacteria gram-negative (*Escherichia coli).*

3.3. Application in beef and fish meat

The results showed, that ethyl acetate extract of gonads with 30 min extraction is the best condition to produce antibacterial compounds found in the sea urchin because it can highly activity inhibitory zone against bacteria *Escherichia coli, Salmonella and Staphylococcus aureus.* The result of this extract will be applied to fresh food i.e. beef and fish meat (Figure 5 and 6).

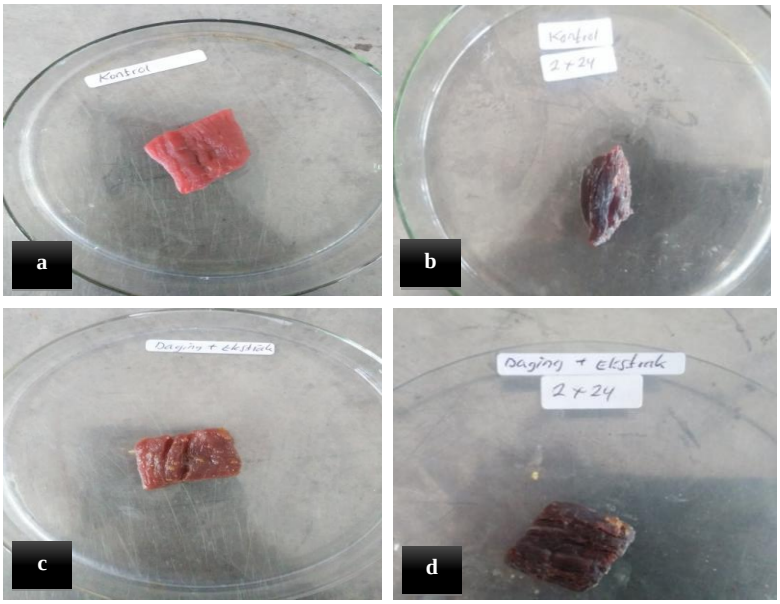

Figure 5. Application ethyl acetate extract of the gonad in beef meat: a) sample of beef control at 0 hours; b) sample of beef control after 48 hours; c) sample of beef with ethyl acetate extract of gonad at 0 hours; and d) sample of beef with ethyl acetate extract of gonad after 48 hours.

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Table 3. Amount of microbial after 48 hours of storage using Total Plate Count Test (TPC).

Sample	Dilution (colony/g)				TPC (colony/g)
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
Control beef meat	TNTC	TNTC	252	18	2.5×10^5
Sample ethyl acetate extract of gonad in beef meat	0	0	0	0	-
Control fish meat	TNTC	TNTC	145	28	1.4×10^5
Sample ethyl acetate extract of gonad in beef meat	8	-	-	-	8×10^1

Notes: TNTC = To Numerous To Count

The amount of bacteria that grows in beef meats that are not soaked in extracts (sample control) is 2.5×10^5 colony/g. Beef meat that is not added ethyl acetate extract of gonads sea urchin has suffered damage due to bacterial growth. In samples of beef meat given ethyl acetate extract of gonads, sea urchin is not damaged and overgrown by pathogenic bacteria, due to the presence of antibacterial compounds that inhibit the growth of bacteria during storage of 48 hours. The same thing on a sample of fish meat (sample control), overgrown by a microbial of 1.4×10^5 colony/g, while fish meat is given ethyl acetate extract of gonads sea urchin only overgrown microbes amounting to 8×10^1 colony/g during storage 48 hours. Fish meat is more rapidly damaged than beef because the protein contains very few tendons or binders so that easy to digest the body and easier to decompose (rot). High Protein (about 15 to 20% of other types of meat), as well as meat almost without tendons, makes fish meat a good medium for the growth of the microorganisms (Zhang *et al*., 2016). Damage by pathogenic microbes can be seen clearly when both samples in isolation on the media plate count agar and eosin methylene blue agar (Figure 7 and 8).

Commented [SZ8]: Unless if you can prove that there isn't any solvent remnants in you extract. Otherwise this will be the reason the meat was badly damaged

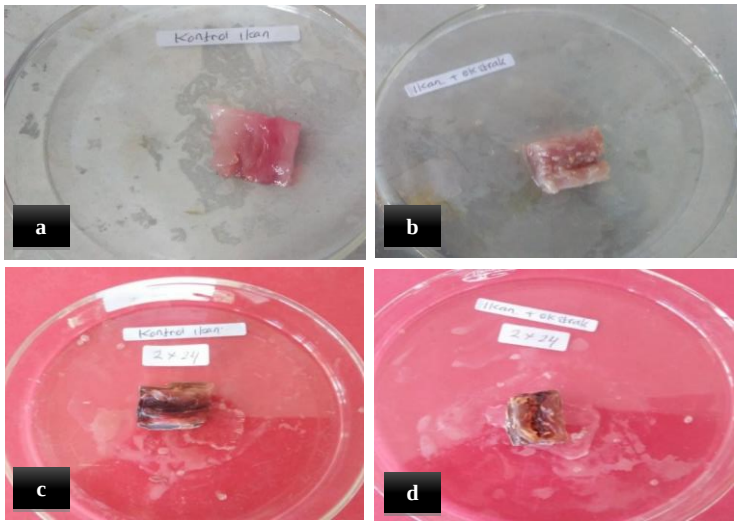

Figure 6. Application ethyl acetate extract of the gonad in fish meat: a) sample of fish control at 0 hours; b) sample of fish control after 48 hours; c) sample of fish with ethyl acetate extract of gonad at 0 hours; and d) sample of fish with ethyl acetate extract of gonad after 48 hours.

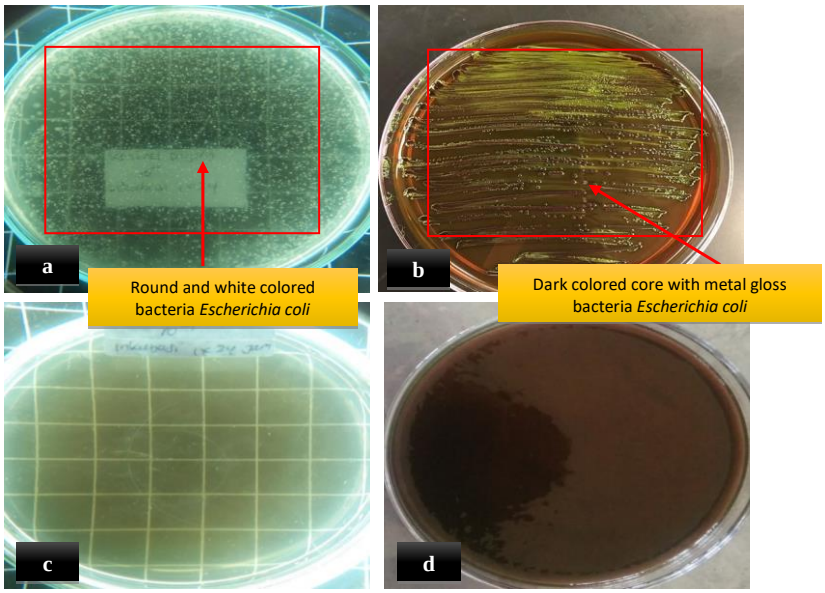

Figure 7. Observation of samples after 48 hours of storage: a) sample of beef control in the media plate count agar (dilution $^{10-1}$); b) sample of beef control in the media eosin methylene blue agar; c) sample of beef with ethyl acetate extract of the gonad in the media plate count agar (dilution $^{10-1}$); and d) sample of beef with ethyl acetate extract of the gonad in the media eosin methylene blue agar.

The results showed that Eosin Methylene Blue Agar (EMBA) is a medium used to determine the presence or absence of coliform bacteria in a sample. The medium of EMBA for this has the privilege of containing lactose and serves to distinguish microbes that fermenting lactose such as *Staphylococcus aureus, Pseudomonas aerugenosa,* and *Salmonella.* Microbes that ferment lactose produce colonies with dark nuclei with metal gloss. Other microbes that can grow their colonies are colourless. The functions of eosin and methylene blue help sharpen the colour difference. Nevertheless, if this medium is used in the early stages, other germs can also grow mainly *Pseudomonas aerugenosa,* and *Salmonella sp*, so this can lead to doubts. This media is very good to confirm that the contaminants are *Escherichia coli*. This solid shaped medium is useful for keeping cells from moving places so that it will easily be counted and separated by type when growing into a colony. Solid media also reveals the diffusion of bacterial metabolites to facilitate the testing of a metabolite result.

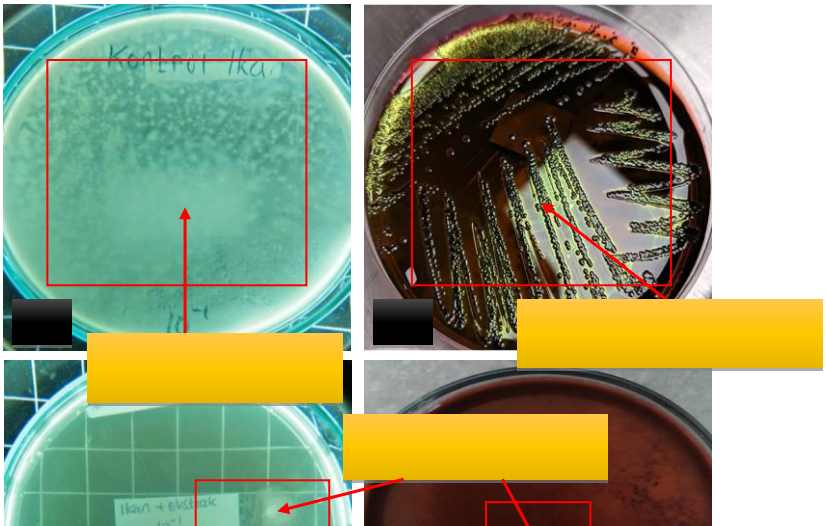

Figure 8. Observation of samples after 48 hours of storage: a) sample of fish control in the media plate count agar (dilution $^{10-1}$); b) sample of fish control in the media eosin methylene blue agar; c) sample of fish with ethyl acetate extract of the gonad in the media plate count agar (dilution $^{10-1}$); and d) sample of fish with ethyl acetate extract of the gonad in the media eosin methylene blue agar.

4. Conclusion

Gonad and shell sea urchin are the sources of secondary metabolites; n-hexadecanoic acid, Pentadecanoic acid, 9-Octadecenoic acid (Z) -, methyl ester, 9-Hexadecenoic acid, methyl ester, (Z)-, Tetradecanoic acid, oleic acid, Cholest-5-EN-3-OL (3. Beta.) (44.19%), Ergosta-5,22-dien-3-ol, (3.beta.,22E), 1,2-Benzenedicarboxylic Acid (0.10%), Pentacosane, Tetratetracontane, and Stigmast-5-EN-3-OL, (3.Beta.,24S)-/ gamma sitosterol, that contributes to the antioxidant and antibacterial properties. Ethyl acetate extracts give the highest number of those antibacterial components for both gonad and shell samples. *Staphylococcus aureus* and *Salmonella* (gram-positive bacteria) are more sensitive to gonad and shell sea urchin ethyl acetate extract because it has an inhibitory zone diameter greater than 10 mm, and not sensitive to *Escherichia coli* (gram-negative bacteria), with inhibitory zone diameter 6–7 mm. However, further studies on the specific antimicrobial compound activities need to be tested and, possibly, a double or triple combination of the gonad and shell extracts could enhance each of their potencies.

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