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ORIGINAL RESEARCH PAPER

FATTY ACIDS CONTENT AND ANTIBACTERIAL ACTIVITY OF *Sargassum duplicatum* LIPID

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Abstract: *Propionibacterium acne, Bacillus cereus and Escherichia coli* are bacteria that cause infections of the skin and digestive tract in humans. *Sargassum duplicatum,* one of brown macroalgae species, is known to have the antibacterial activities. This study aims to prove antibacterial activities of *S. duplicatum* lipid against three different bacteria. The method used is the Folch method (using soxhletation, with a ratio of chloroform: methanol 2:1, v/v) to extract lipid. Furthermore, fatty acids content was identified by gas chromatography - mass spectrophotometry (GC-MS). Then, the disc diffusion method was used to measure the inhibition zone for antibacterial activity by using tetracycline as positive control. The lipid percentage of *S. duplicatum* was obtained about 2.502 % (v/w) where there were two lipid layers namely chloroform layer (0.388 % (v/w)) and methanol layer (2.114 % (v/w)). *S. duplicatum* lipid contained palmitic acid (in both layers) and oleic acid (only methanol layer). Moreover, methanol and chloroform lipid layer (1000 $mg \cdot mL^{-1}$) of *S. duplicatum* showed antibacterial activity against *B. cereus*, *E. coli* and *P. acne*

Keywords: *disc diffusion, extraction, Folch method, oleic acid, palmitic acid*

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INTRODUCTION

Nowadays, the rising rates of antimicrobial resistance have become a serious problem in health and medicine field. Centers for Disease Control and Prevention (CDC) published the estimation that two million people develop infections with antibiotic-resistant pathogens each year and nearly 23,000 people die each year due to the infections [1]. There are two main reasons of this problem namely misuse and overuse of antibiotics and the lack of development of new antibiotic agents by the pharmaceutical companies. Therefore, research on the antibacterial properties of macroalgae compounds has become a solution to tackle this challenge. For instance, phlorotannin demonstrated its antibacterial activity can inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) [2].

Some researchers have proved the antibacterial activity of Sargassum sp. For instance, four different Sargassum sp. (*S. polycystum, S. oligocystum, S. crassifolium* and *S. cristaefolium)* were extracted in five different solvents (ethanolic, n-hexane, dichloromethane, ethyl acetate, water) showed their inhibitory effect on the growth of eight different pathogen bacteria namely *Aeromonas hydrophila, Escherichia coli, Staphylococcus aureus, Pseudomonas aeroginosa, Micrococcus luteus, Bacillus subtilis, Streptococcus mutans, Pseudomonas flourescens* [3]. The results of Al Khazan et al. [4] have reported that the maximum inhibitory effect of *S. marginatum* was observed in petroleum ether extract against MRSA (27 mm), *E. coli* (23 mm), *B. Subtilis* (22 mm), *P. aeruginosa* (19 mm), *S. aureus* and *K. Pneumoniae* (18 mm). In addition, the petroleum ether of *U. lactuca* showed inhibition zone against MRSA (18 mm), *E. coli* (14 mm), *B. Subtilis* (17 mm), *P. aeruginosa* (15 mm), *S. aureus* and *K. Pneumoniae* (13 mm) [4]. Then, other researches showed *Sargassum polycystum* has antibacterial activities against marine fouling bacteria [5]. Moreover, crude lipid extracts of *S. polycystum* showed antibacterial activity against gram-positive and gram-negative bacteria such as *B. cereus*, Methicillin-resistant *S. aureus* (MRSA) and *Shigella dysenteriae* [6].

Foodborne diseases (also known as foodborne illness or food poisoning) are a widespread and growing public health problem worldwide which caused by consuming contaminated food, contaminated with pathogenic bacteria, viruses or parasites. *B. cereus* and *E. coli* are the general bacteria which cause diarrhea [7]. Diarrheal disease is the second leading cause of death in children under five years old and is responsible for killing around 525,000 children every year [8].

Acne vulgaris is a common skin disease which affects almost all teenagers and many adults. It is estimated as the eighth most prevalent global disease, with 650 million people reported to have had acne in 2010 [9]. One of the most prevalent bacteria found on human skin which causes acne is *Propionibacterium acnes*. *P. acnes* is involved in the development of inflammatory acne by activating complements and metabolizing sebaceous triglycerides into fatty acids that irritate the follicular wall and surrounding dermis [10]. To date, the investigation of antibacterial agent from seaweed (macroalga) was expanded to primary metabolite product such as lipid. The aim of this study is to test the antibacterial activities of *S. duplicatum* lipid against *B. cereus*, *E. coli* and *P. acne*.

MATERIALS AND METHODS

Materials

S. duplicatum was collected from Sayang Heulang Beach, Pameungpeuk Village, West Java Province, Indonesia. This macroalga taken was fresh sample from all parts of the plant, regardless of its age. Moreover, the bacteria test (*B. cereus, E. coli* and *P. acne*) which used in this research were obtained from Microbiology Laboratory of Universitas Indonesia.
Methanol (p.a) *(Merck, Germany)* and chloroform (p.a) *(Merck, Germany)* were as solvents in lipid extraction. Tetracycline *(Merck, Germany)* was used as positive control.

Methods

Lipid Extraction

Lipid extraction of *S. duplicatum* was carried out based on Folch method [11]. Lipid of macroalga was extracted by soxhletation process using combination of two organic solvents namely chloroform : methanol 2 : 1 (v/v). This process has taken about 16 - 18 hours. Changing color (from brown to colorless) is an indicator of the end of lipid extraction process. Furthermore, the extract obtained in two layers (chloroform and methanol layer) were separated using a separating funnel and concentrated using a rotary evaporator (RE-1000 HN (horizontal) model, China) to obtain crude lipid extract. Finally, crude lipid extracts were stored in refrigerator (exactly in 4 °C) for further experiment.

Identification of Lipid Content by Gas Chromatography-Mass Spectrometry

The extracted lipid was esterified to determine the fatty acid composition before GC-MS analyzing. Firstly, 20 mg lipid in each layers was dissolved with 4 mL of mixture solvent of KOH - chloroform separately. Secondly, they are put into the test tubes before being heated at 60 °C for 5 minutes. Thirdly, the test tube was closed with a smaller tube containing water to prevent loss of steam during heating. Afterwards, 5 mL of BF3-MeOH was added and heated for 30 minutes at 60 °C which would form fatty acid methyl esters (FAMEs).
Furthermore, fatty acid methyl esters (FAMEs) were analyzed by Gas Chromatography-Mass Spectrometry in Indonesian Customs and Excise Laboratory, Jakarta, Indonesia (Hewlett Packard, 6890 series GC system that coupled with a MS HP 6890 series). It was equipped with silica capillary column HP-5ms (30 m×0.25 mm×0.25 μm). The detector and injector temperature were set around 280 °C and 250 °C, respectively. The carrier gas was helium with flow rate 1 $mL \cdot min^{-1}$.

Antibacterial Activities Test

Testing of antibacterial activity was carried out by disk-diffusion method [5]. Three pathogenic bacteria were used in this study namely *B. cereus, E. coli* and *P. acne*. Then, these bacteria were cultured in Luria Bertani (for *B. cereus* and *E. coli*) and Blood Agar (for *P. acne*) media. There were five different concentrations used (100, 80, 60, 40 and 20 %) in this research. Lipid was dropped on paper disk in agar media which contained bacterial test. Then, all of them were incubated at 37 °C, according to their incubation

time. In addition, tetracycline is as positive control while negative control are solvents namely methanol and chloroform. Moreover, the antibacterial activity was evaluated by observing and measuring the inhibition zone around the paper disk. All of the tests were conducted in triplicate.

RESULTS AND DISCUSSION

Lipid of *S. duplicatum*

S. duplicatum sample has long brown thallus (about 28 cm), cylindrical thallus, an oval-shaped leaves and bladders (Figure 1) which is similar to [12] that *S. duplicatum* has bladders, brown-colored thallus, grown in sharp coral-sea and oval-shape.

Figure 1. *S. duplicatum*

The extraction process resulted two lipid layers namely chloroform (bottom) and methanol (top) layer (Figure 2) due to the difference in solvents density where the density of chloroform solvent is higher (1.49 $g \cdot cm^{-3}$) than methanol solvent (0.787 $g \cdot cm^{-3}$). Moreover, each phase was evaporated using a rotary evaporator to obtain pure lipids without solvent.

(a) *(b)*

Figure 2*. S. duplicatum lipid in methanol (a) and chloroform (b) layer*

Table 1. *Total Lipid of S. duplicatum*

Type of Layers	Total Lipid (% v/w)
Chloroform (bottom)	0.388
Methanol (top)	2.114

The amount of *S. duplicatum* lipid in methanol layer is higher than the chloroform one. Generally, the amount of lipid in macroalgae is very low namely about 1 - 5 % of dry weight [13, 14]. Following study found lipid of macroalgae was about 7 % of dry mass [15]. Then, *S. ilicifolium* from Central West Coast, India contained 5.7 % of lipid [16]. The percentage of macroalgae lipid can differ depending on two main factors namely environment factors (before extraction process such as temperature, *p*H, salinity, harvesting season, collection site) and extraction process (like temperature and solvent type) [17].
The differences between methanol and chloroform lipid layer of *S. duplicatum* can be seen in table below.

Table 2. *Difference Between Methanol and Chloroform Lipid Layer of S. duplicatum*

Parameter	Methanol Lipid Layer	Chloroforme Lipid Layer
Odor	Fishy	Fishy
Color	Dark Brown	Yellowish Brown
Taste	Bitter	Bitter
State	Liquid	Liquid

Fatty Acids Composition

According to result of Gas Chromatography-Mass Spectrometry (GC-MS) analysis, two fatty acid types were obtained in methanol lipid layer of *S. duplicatum* namely palmitic fatty acid (saturated fatty acids (SFA)) and oleic fatty acid (monounsaturated fatty acids (MUFA)) (Figure 3a). By contrast, chloroform lipid layer contained only one fatty acid type namely palmitic fatty acid (saturated fatty acids (SFA)) (Figure 3b).

Table 3. *Fatty Acids Content of S. duplicatum Lipid*

No	Type of Lipid Layer	RT [Min]	Percentage composition [%]	Name of Fatty Acid	Quality
1	Methanol	19.825	50.65	Palmitic Acid (C16:0)	98
		21.657	36.40	Oleic Acid (C18:1 n-9)	99
2	Chloroform	19.831	40.68	Palmitic Acid (C16:0)	99

Table 3 shows palmitic acid (saturated fatty acid (SFA), (16:0) was more abundant than oleic acid (monounsaturated fatty acids (MUFA), (18:1 n-9)).

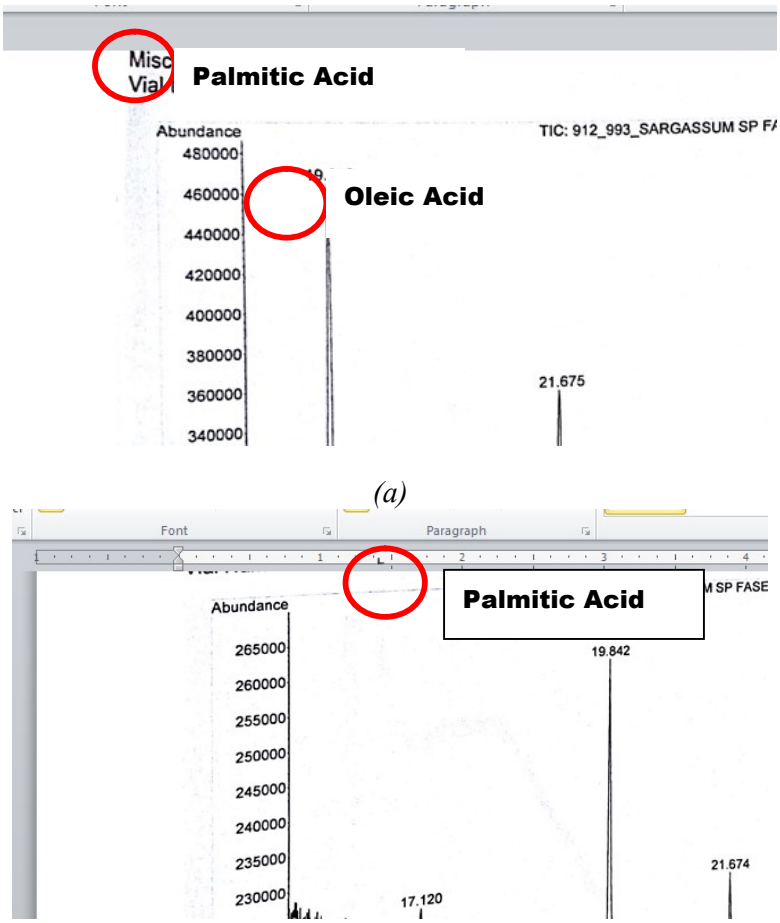

Figure 3. *GC-MS Analysis of S. duplicatum Lipid in methanol layer (a) and chloroform (b)*

The main fatty acid in macroalgae (including *S. polycystum)* or plant is palmitic acid (saturated fatty acid) [18, 19]. Moreover, Zailanie and Kartikaningsih [20] reported that lipid of brown macroalga (*S. duplicatum)* contained palmitic acid (saturated fatty acid) and oleic acid (monounsaturated fatty acid). Then, Thibane *et.al* [21] showed that lipids isolated from marine organisms are rich in polyunsaturated fatty acids. Further study showed that palmitic acid had the highest percentage from *Sargassum natans* [22]. In addition, palmitic acid (saturated fatty acids, (C16:0)) and oleic acid (unsaturated fatty acids, (C18:1 n-9)) were dominant fatty acids which found in four different species of *Sargassum* tested (*S. fusiforme*, *S. pallidum, S. horneri and S. thunbergii*) [23].

Antibacterial Activity

Overall, *S. duplicatum* lipid inhibited the growth of *B. cereus, E. coli* and *P. acne* in each lipid layer (Table 4). For *B. cereus* and *E. coli,* the antibacterial activity of chloroform lipid layer is larger than methanol lipid layer (Table 4). In contrast to *P. acne,* methanol lipid layer (15.32 mm) displays higher antibacterial activity than chloroform one (11.16 mm).

Moreover, there are five different concentrations of lipid used in this research (Table 4). Table 4 describes that the higher the lipid concentration used, the higher the antibacterial resulted. If, it is compared to tetracycline as positive control, the strength of antibacterial activity of *S. duplicatum* lipid was still weak because tetracyclines inhibited protein synthesis in bacteria by disrupting the function of the ribosome 30S subunit.

Table 4. *Antibacterial activity of S. duplicatum lipid*

Lipid concentration [mg·mL^{-1}]	The average diameter of inhibition zone (mm) in the lipid extract in layer					
	Chloroform	Methanol	Chloroform	Methanol	Chloroform	Methanol
	P. acne		*B. cereus*		*E. coli*	
1000	11.16	15.32	15.01	5.19	12.70	9.29
800	8.07	10.20	14.54	4.5	10.07	8.17
600	6.07	8.19	9.43	3.91	8.66	7.60
400	4.98	6.33	8.4	2.31	7.70	6
200	3.62	4.71	6.1	0	7.20	5.78
Control (-)	0.35	0.72	0	0	0	0
Control (+)	26.27		25.1		20.70	

In this study, *S. duplicatum* lipid which contained palmitic acid and oleic acid showed antibacterial activity (Figure 3). This result is similar to [24-26] which demonstrated that palmitic acid and oleic acid had antibacterial and antifungal activities. The saturated and unsaturated fatty acids which have more than ten carbon atoms can lyse bacterial protoplasts by changing the permeability of cytoplasmic membrane. Thus, it can release food material from bacterial cell membranes which inhibited the growth of bacteria tested [27].

Furthermore, similar finding was reported by Bazes *et al*. [28] saturated and unsaturated fatty acids with a predominance of myristic, palmitic, oleic and eicosapentaenoic acids were responsible for the antibacterial activities of different brown algal extracts. It is known that fatty acids can act as anionic surfactants and have antibacterial and antifungal properties at low *p*H [29]. Desbois and Smith [30] explained that the antibacterial activity of each fatty acid is influenced by its structure and shape. They also stated that mechanism of antibacterial activity as a function of the length of carbon chain and the presence, number, position and orientation of double bonds.

CONCLUSION

In this study we obtained the lipid percentage of *S. duplicatum* was 2.502 %. Besides, *S. duplicatum* lipid has two layers namely methanol lipid layer (2.114 %) and chloroform lipid layer (0.388 %). Furthermore, GC-MS analyzed result of methanol lipid layer demonstrated two different fatty acids namely palmitic acid (50.65 %) and oleic acid (36.40 %). On the other hand, chloroform lipid layer has only one fatty acid namely palmitic acid (40.68 %). The antibacterial activities test of *S.*

duplicatum lipid against *B. cereus, E. coli,* and *P. acne* showed that the methanol lipid layer has greater antibacterial activities than the chloroform lipid layer.

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