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Submission date: 29-Dec-2020 09:20AM (UTC+0700)
Submission ID: 1481786038
File name: Cytotoxicity_and_Phytochemical_Profiling_of_Sargassum_Sp..pdf (315.21K)
Word count: 4065
Character count: 22007

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IOP Conf. Series: Earth and Environmental Science **116** (2018) 012024 doi:10.1088/1755-1315/116/1/012024

Cytotoxicity and Phytochemical Profiling of *Sargassum Sp.* Extract As Anti-Mdr Bacteria

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Abstract. *Sargassum* sp. contains bioactive compounds having the potential as an antibacterial agent. *Sargassum sp*. was collected from five different locations, i.e., Teluk Awur, Panjang Island, Bandengan, Ujung Piring, and Bondo. There were several different species of *Sargassum sp.* identified from each sampling locations. The collected seaweeds were washed, naturally dried, and ground to the powder-sized dry material. Dry seaweed was extracted gradually using n-hexane, ethyl acetate, and methanol (1:3 w/v). The antibacterial analysis was conducted based on Agar Diffusion method using Zobell as media. Resistance analysis was performed to evaluate the resistance of pathogen against commercial antibiotics, namely, chloramphenicol, ampicillin, erythromycin, amoxycillin, and tetracycline. Each *Sargassum sp.* extract was tested against three candidates of MDR bacteria, i.e., *Staphylococcus aureus*, *Escherichia coli,* and *S. epidermidis.* Results showed that *S. aureus* was resistant towards four out of five commercial antibiotics. *E. coli* and *S. epidermidis* were not susceptible to two and three out of five commercial antibiotics, respectively. N-hexane, ethyl acetate and methanol yielded 0.1-0.3%, 0.3-0.7 % and 0.8-4.7% of dry extract. Ethyl acetate extract of Sargassum from Teluk Awur performed the best antibacterial activity and contained an alkaloid, flavonoid, and phenolic compounds. Toxicity analysis showed that this ethyl acetate extract had LC_{50} at 463 ppm and categorized as chronic toxicity.

1. Introduction

Applications of drugs particularly synthetic antibiotics have been commonly used to control the disease. The excessive use of antibiotics becomes the single most important factor leading to a resistance increase of bacteria towards antibiotics. This is due to the adaptability of disease to the toxicity of antibiotic and bacterial resistance [1]. Bioactive compounds from marine resources offer a promising solution to overcome the resistance problem. Also, it also becomes an alternative replacement of synthetic drugs to treat infection disease. World Health Organization (WHO) has recommended the use of natural products in maintaining the well being of the community, in preventing and resolving chronic and degenerative disease [2]. *Sargassum* sp. is one of the marine resources that can be proposed as alternative natural products for disease control. *Sargassum sp*. has been reported to show interesting and promising pharmacological properties serving as anticancer, anti-inflammatory, antibacterial, and antivirus [3], antioxidant [4], antifouling [5]. And antifungal [6]. *S. fulvellum* and *S. thunbergii* showed antipyretic, analgesic, and anti-inflammatory activities in mice [7]. *S. echinocarpum, S. duplicatUM* and *S. polycystum* performed potential antioxidant [8]. *S. aquifolium, S. ilicifolium* and *S. polycystum* [9]. showed antibacterial activity against *Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli* [9]. *S. pallidum* can serve as antioxidant and anti-hemolysis agents exhibiting potential for further exploration as functional food or complementary

medicine [10]. *S. tenerrimum* from India is reported to have antibacterial activity against *Klebsiella pneumonia*, *Escherichia coli*, *Staphylococcus aureus*, *Proteus sp.*, *Streptococcus sp.*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Salmonella sp.*, *Shewanella sp.*, *Vibrio fluvialis*, *Vibrio splendidus*, *Vibrio cholera*, *Shigella flaxneri*, *Staphylococcus epidermidis*, *Aeromonas liquefaciens* and *Bacillus subtilus*. Furthermore, *Sargassum sp*. also have antifungal activity against *Aspergillus niger, A. flavus, A stetreus*, *Candida albicans* and *Penicillum* sp. [11]. This study aims to evaluate the inhibition ability of *Sargassum sp.* extracts against pathogenic bacteria categorized as multidrug resistant bacteria. Using conservation and biotechnological approach, the study is expected to resolve the resistance of pathogenic bacteria towards synthetic drugs and to prevent more infections.

2. Materials And Methods

The samples *Sargassum sp.*were collected from five different locations, i.e. Teluk Awur (6° 7' 36.48" S, 110° 24' 0" E), Panjang Island (06°34"30' LS, 110° 37"44' BT), Bandengan (°07'36"LS 110°24'00"BT / 6,1268°LS 110,4°BT), Ujung Piring (6° 30' 40.91" S, 110° 40' 06.42" E) and Bondo (6° 28' 26.79" S, 110° 42' 33.13" E) at Jepara, Central Java.

The solvents used : n-hexane, ethyl acetate, and methanol as solvents to extract the bioactive compounds in *Sargassum sp*. Antibacterial activity against *S. aureus, E. coli,* and *S. epidermis* was evaluated through the Agar Diffusion method by using Zobell as media. The media used consisted of peptone, yeast, and agar [12].

2.1 Samples preparation

The collected seaweeds were washed with tap water to remove the epiphytes and the remaining debris. A whole thallus of seaweed was taken for taxonomic identification based on morphology study. Before natural drying under the shade, seaweeds were cut to pieces (± 5 cm). Further, the dried seaweeds were ground with a multi use blender to obtain powder material.

2.2 Extraction of Sargassum sp.

Dry seaweed of 25 gr was extracted gradually by using 100 mL n-hexane, ethyl acetate, and methanol, respectively. The extraction was performed for 24 h at room temperature in obscurity. The extracted seaweed was filtered by Whatman Filter Paper to separate the solute from the residues. The remaining residues of dry seaweed material were re-extracted using the same process and solvents. Filtrates obtained from the extraction process were pooled. N-hexane, ethyl acetate, and methanol filtrates were evaporated to dryness with vacuum rotary evaporator at 40 °C in 500 mmHg. n-hexane, ethyl acetate and methanol extracts of *Sargassum sp*. were dried using N_2 before storage in -20 °C [13]. Extraction yield was determined with following formula :

$$Ce = \left(\frac{W_2}{W_1}\right) \times 100\%$$

Where :
Ce = Extraction yield (%)
W_2 = Weight of extract (gr)
W_1 = Weight of initial sample (gr)

2.3 Resistance analysis of Multidrug-Resistant (MDR) bacteria

Staphylococcus aureus, *Escherichia coli,* and *S. epidermidis* were analyzed against five commercial antibiotics. The antibiotics were chloramphenicol, ampicillin, erythromycin, amoxicillin, and tetracycline.

2.4 Antibacterial activity analysis

Bacterial culture in liquid media was centrifuged at 5000 rpm for 10 min and was washed with PBS.

0.1 mL of bacterial solution was read with a spectrophotometer at 600 nm to obtain optical density between 0.6-0.8 [14].
MDR bacterial solution of 0.1 mL previously cultured in liquid media was spread evenly on to solid media in the petri dish using spread technique [14]. The inoculated bacterial solution was left for 30 min to diffuse on the media. Extract and chloramphenicol served as a positive control was prepared in distilled water at 2500 ppm. The sterile paper disc was impregnated with 20 µL of antibiotics solution at 50 µg/disc [13]. And 20 µL of solvents without extracts as a negative control. Paper discs were placed on the solid media in Petri dish previously inoculated with MDR bacteria. All Petri dishes were incubated at 37 °C for 24-48 h.

2.5 Artemia salina Brine Shrimp Lethality Test (BSLT)
BSLT was performed to evaluate the toxic effect and cytotoxicity of chemical compounds against the larvae of shrimp *Artemia salina.* The test was based on [15], [16], [17]. *A. salina* was immerged in fresh water for 15-30 min to clean the attached feces. 0.25 gr of eggs were put in a box filled with 500 mL seawater at 25-30 °C with pH at 7-8. Irradiation from TL 40 watt light was placed above the box to light it during the hatching for 48 h. After 48 h, the eggs hatched to nauplii instar III/IV and were ready to use for BSLT test [18].

Ten larvae of *A. salina* were introduced into a test tube filled with extract showing the best antibacterial activity. Concentrations of extracts applied for the BLST were: 1000, 500, 100, 50 and 10 ppm with ethanol 2% [15]. Negative control used was methanol concentrated at 6, 4, 2 and 1%. The observation was made at 1, 3, 6, 12, 18, 24 and 36 h after the introduction of larvae into extract by using a loop. Determination of time interval was according to the lethal concentration of a compound whether it was acute or chronic lethal. LC_{50} was calculated from the linear regression equation based on the observation made. All tests were performed in triplicate.

2.6 Phytochemical profiling of Sargassum sp. extracts
This analysis was meant to examine the presence of certain chemical compounds in the tested samples, in this case, *Sargassum sp*. By determining a presence of certain compounds in plant matrix, a correlation can be made between the compounds and the biological activities performed. Thus it facilitates the phytopharmacological procedures [12]. Phytochemical profiling of *Sargassum sp*. extracts was carried out to evaluate the presence of alkaloid, flavonoid, saponin, steroid, and triterpenoid. *Sargassum sp*. dry material of 0.05 gr was dissolved in 10 drops of sulfuric acid 2 N, and three drops of this solution was introduced to Marquis, Dragendorff, Meyer and Wagner reagent. These reagents, previously prepared in separated test tubes, were designated to test the alkaloid content in a sample. For flavonoid, 0.05 g of dry seaweed material was added with 0.1 mg magnesium and 0.4 mL amyl alcohol, a mixture of HCl 37% and ethanol 95% in the same volume. Further, four mL of alcohol was added to the solution. To detect the presence of saponin, 0.05 g of dry seaweed material was dissolved in hot water. This test was also called as foam test due to the foam established on the surface of the solution. For the steroid/triterpenoid test, 0.05 g of dry seaweed material was dissolved in 2 mL of chloroform in test tube added with Lieberman Burchard reagent.

2.7 Statistical analysis
Data from resistance and antibacterial analysis were statistically analyzed using SPSS 16. Test of normality by Shapiro-Walk and homogeneity by Levene test with the significant level at 0.05 were performed. Multivariable analysis of variance (MANOVA) was used to analyze the interaction between variables, *i.e.,* sampling locations, *Sargassum sp*., type of solvents and bacteria. Post-hoc test of Tuckey was applied to acknowledge the significant difference between tested variables ($p<0.05$).

3. Results And Discussion

Use of different solvents in extracting the bioactive compound from *Sargassum sp*. yielded dried extract differently. N-hexane resulted the lowest yield of extraction with 0.23 ± 0.05% of dry extract compared to ethyl acetate and methanol with 0.56 ± 0.12% and 2.18 ± 1.23% of dry extract, respectively. It indicates that the bioactive contained in *Sargassum sp*. tend to dissolve in polar solvents than in semi or no-polar one, implying that the extracted compounds are polar. Basic principle of extraction is the appropriate selection of solvents used to extract the solute. Non-polar solvents dissolve non-polar solutes while polar solvents dissolve polar solutes [19]. Bactericidal activity of *Sargassum sp*. extracts showed a diverse activity based on different locations. It might be suggested that there is a geographical variation in biological activity of *Sargassum sp*. Inhibition zone of extracts from *Sargassum sp*. collected at Bondo, Ujung Piring, and Bandengan were significantly different one to each other (p<0.05). Also, these same samples were also significantly different to those from Panjang Island and Teluk Awur (p<0.05). It is possible that these two stations, Panjang Island and Teluk Awur, have similarity in their water qualities. Furthermore, extracts of *Sargassum sp*. from Panjang Island and Teluk Awur showed the most active against the MDR bacteria than the other extracts. It was based on the biggest inhibition zone shown by the extracts from these two locations. It leads to an assumption that the environmental conditions in Panjang Island and Teluk Awur are favorable for the production of bioactive compounds.

Table 1 shows the statistical analysis of the antibacterial activity of *Sargassum sp*. based on different stations, species, and solvents. As can be seen from Table 1, antibacterial activity was best performed by *S. crassifolium* extracted with ethyl acetate. Moreover, samples from Panjang Island showed the best activity.

Table 1. Analysis of variance from each variable analyzed

Variance	*df*	*F*	*p*	*Tukey p <0.05*
Station	4	493.4	0	Bondo^{a}< Ujung piring^{b}< Bandengan^{c}< Teluk awur^{d} < Pulau panjang^{d}
Species	6	99.65	0	Bin^{a}< Echi^{b}< Plag^{b}< Cine^{b}< Poly^{c}< Dupli^{cd} < Cras^{d}
Solvent	2	4602	0	n-hexana^{a}< Metanol ^{b}< Etil asetat^{c}

Df: degree of freedom; *F*: F value; *p*: probability; Bin: *S. binder*; Cine: *S. cinereum*; Cras: *S crassifolium;* Dupli: *S. duplicatum;* Echi: *S. echinocarpum;*Plag: *S .plagyophyllum;* Poly: *S. polycystum;* Sa: *S. aureus.* The order of variables was based on the smallest of the biggest average value (<); different letters indicate significant difference (p> 0.05).

Factors influencing the difference in inhibition zone from the antibacterial assay are the sensitivity of organisms, the media of culture, the condition of incubation, and the agar diffusion rate. Meanwhile, the agar diffusion rate depends on the concentration of tested samples, the composition of media, temperature and period of incubation [20]. Aside from these factors, it can also be suggested that different type of bioactive compounds extracted from *Sargassum sp*. play an important role in their bactericidal effects towards the MDR bacteria.

Type of solvents also contributes to the antibacterial effect of *Sargassum sp*. extract. Ethyl acetate and methanol extracts exhibited the highest inhibition zone; meanwhile, the n-hexane extracts had the smallest inhibition zone. Such difference might be due to different polarity of bioactive compounds from *Sargassum sp*. As non-polar solvents, n-hexane attracts non-polar compounds. As for semi-polar to polar compounds, they prefer to dissolve in ethyl acetate and methanol. Ethyl acetate is an aromatic compound characterized by its semipolar with $CH_3CH_2OC(O)CH_3$ as its chemical structure. This

compound can dissolve analytes tending to be semipolar and polar [21]. It indicates that the bioactive compounds extracted from *Sargassum sp*. are semipolar.

From 42 extracts tested, one best extract with a wide spectrum of antibacterial effect and the biggest inhibition zone was chosen. It was *S. duplicatum* from Teluk Awur extracted with ethyl acetate. This extract was further tested for its toxic effect using BSLT and results are presented in Table 2.

Table 2. BSLT represented by the value of LC_{50} of *S. duplicatum* in different time interval

ta; *S. duplicatum*; ea							
Time (hours)	y = a + b*x	R^2	R	Correlation (%)	LC_{50} Concentration (ppm)	Criteria	
1	-	-	-	-	-	-	-
3	y = 0.017 x - 1.051	0.989	0.9945	99.45	3003	Not Toxic	-
6	y = 0.025 x + 2.130	0.904	0.9508	95.08	1915	Not Toxic	-
12	y = 0.034 x + 5.311	0.891	0.9439	94.39	1314	Not Toxic	-
18	y = 0.036 x + 13.27	0.906	0.9518	95.18	1020	Not Toxic	-
24	y = 0.082 x + 12.04	0.946	0.9726	97.26	463	Toxic	Chronic
30	y = 0.076 x + 28.47	0.930	0.9644	96.44	283	Toxic	Chronic
36	y = 0.143 x + 30.42	0.915	0.9566	95.66	137	Toxic	Chronic

Toxicity of *S. duplicatum* extracted in ethyl acetate was evaluated towards the larvae of *A. salina* through the BSLT method. It seems that the toxicity of this extract started 24 h after introducing the larvae of *A. salina* to ethyl acetate extract of *S. duplicatum.* Value of LC_{50} after 24 h was 463 ppm implying that this concentration could cause lethal effect towards 50% of the larvae population.

Table 2 displays that ethyl acetate extract *S. duplicatum* is considered as active or toxic because of its ability to cause 50% of mortality to the tested organisms with less than 1000 ppm [23]. Based on the category of toxicity from a compound (15). It generates toxic effect during the time interval 18 h.
BSLT method is widely used as a method to evaluate a toxicity of natural product as mentioned by several studies [22]; [23]. The advantages of this method are the simplicity. The high reproductivity (24 h). cheap and can be used to determine the intensity of cytotoxicity.

Table 3. Phytochemical profiling of *S. duplicatum* extracted with ethyl acetate

Phytochemical profile	*S. duplicatum*	Standard (color)
Alkaloid		
Dragendorf	+	Red to orange
Meyer	+	Murky white
Flavonoid	+	Layer of amyl alcohol is red/yellow/green

Steroid	-	Green/blue
Phenolic	+	Green or greenish blue
Triterpenoid	-	Violet or purple
Saponin	-	Foaming on top of the layer
Tanin	-	Greenish brown or dark blue (bluish black)

Table 3 shows that the ethyl acetate extract of *S. duplicatum* contained alkaloid. Flavonoid and phenolic compounds. These compounds are assumed to be responsible for the antibacterial effect of *S. duplicatum* against *S.aureus*. *E. coli* and *S. epidermidis.* Inhibition of bacterial growth in the presence of antibacterial agents is provoked by the combination of these agents with bioactive compounds. These bioactive compounds might be from the secondary metabolites such as alkaloids. Peptides. Terpenes. Pigments and sterols [24]. Mechanisms of antibacterial from a compound can take place in several ways, which are : 1) the destruction of bacteria cell wall; 2) the change of permeability in a membrane cell and 3) prevention of protein and nucleic acid synthesis. Furthermore, concentration of extracts and bacteria species of bacteria the availability of organic material, temperature, and pH have been suggested as factors affecting the antibacterial effect of a compound [25].

Alkaloid serves as antibacterial agents and provokes the death of bacteria cell due to the presence of alkali groups containing nitrogen in its structure [26]. It leads to a change in amino acid structure and the genetic balance experiences a lysis. Moreover, flavonoid could obstruct the growth of bacteria by destroying the permeability of bacteria cell wall. Flavonoid can release transduction energy to the cytoplasmic membrane of bacteria and to inhibit the motility of bacteria. Hydroxyl group in flavonoid contributes to the alteration of the organic component and transport of nutrition generating a toxic effect towards the bacteria. Phenolic compounds also have a hydroxyl group in their chemical structure. The mechanism to eliminate the bacteria is mainly based on the difference of polarity between lipid and hydroxyl group. If the bacteria contain more lipids, then higher concentration is needed to rupture the bacteria cell. The mechanism of phenolic activity as an antimicrobial is the ability of phenolic to cause shrinkage. also known as astringent [27]. Phenols can form a complex with a microbial enzyme to reach the membrane cell of bacteria through the cell wall. Bacteria cell wall consists of different polysaccharides and proteins; thus. it allows part of phenols to penetrate the cell wall.

4. Conclusion

The present study reveals that *Sargassum* sp. extract can inhibit the growth MDR bacteria. It implies that *Sargassum* sp. contains bioactive compounds having the property as an antibacterial agent. of this extract. Results obtained unlocks the potential of *Sargassum* to replace commercial antibiotic against the MDR bacteria. Nevertheless. it is important to further consider the type of solvent used to extract the target compounds that are active against the MDR bacteria. Apparently. certain solvents might have a negative impact towards the living cell. as presented in this study. Eventhough ethyl acetate extract of *Sargassum* sp. showed to be the most effective against MDR bacteria, this extract was also toxic. As a consequence. it is crucial to perform further research related to the application of a more eco-friendly method to extract the target compound from *Sargassum* sp.

5. Acknowledgment

Authors would like to express gratitude to DRPM-SIMLITABMAS RISTEK DIKTI- Jakarta period 2016/2017 for the financial supports for this study.

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