RESEARCH ARTICLE | MAY 17 2017

Antibacterial activities of β-glucan (laminaran) against gram-negative and gram-positive bacteria FREE

A. Chamidah; Hardoko; A. A. Prihanto

AIP Conf. Proc. 1844, 020011 (2017)
https://doi.org/10.1063/1.4983422

Antibacterial Activities of β-Glucan (Laminaran) against Gram-Negative and Gram-Positive Bacteria

A. Chamidah[1, a)], Hardoko[1] and A. A. Prihanto[1]

[1] *Brawijaya University, Jl.Veteran No. 1, Malang 65145, East Java, Indonesia*

[a)] Corresponding author: achamidah@yahoo.co.id

Abstract. This study aimed to determine the antibacterial activity of β-Glucan (laminaran) of LAE and LME extracts from brown algae Sargassum crassifolium using HPMS and Ultrasonication against Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus) and Gram-negative bacteria (Salmonella typhimurium and Escherichia coli). The highest antibacterial activities of LME extract obtained using the HPMS method against Gram-positive bacteria (B. subtilis and S. aureus) were at 18:10 and 18.80 mm. The ultrasonication method showed a lower inhibition trend than the HPMS method, with MIC and MBC values of 250 mg/ml and 2-8 CFU/ml, respectively, in all Gram-negative and Gram-positive bacteria. The results showed that LME extract at a concentration of 250 mg/mL is bacteriostatic against Gram-positive and –negative bacteria.

INTRODUCTION

Currently, antibiotic use increases significantly due to severe infections and bacterial pathogen resistance against drugs due to the uncontrolled use of antibiotics.[1] The treatment of resistant pathogenic bacteria becomes more difficult ,[2] requires high costs and causes adverse effects on the host (the occurrence of hypersensitivity and reduced profitable microbes in the gut).[3] Decreased efficiency and pathogen resistance to antibiotics has necessitated the development of new alternative antibiotics.[4]

The use of plants for healing a disease is based on the experience inherited from ancestors. However, today, the selection of natural ingredients for treatment is based on research evidence. Besides being more economical, the side effects of medicinal plants are relatively small compared to synthetic drugs, as the use of medicinal plants with proper formulations is very important and certainly more secure and effective[5].

Marine algae are a source of raw materials that are widely used in the pharmaceutical, medical, food and cosmetic industries.[6] Antimicrobial compounds derived from algae consist of a diverse group of chemical compounds.[7] Many substances, including polysaccharides (alginate, carrageen, and agar), are derived from algae containing bioactive compounds and are used in medicine and pharmacy.

Beta glucan is a homopolysaccharide which can be synthesized by algae, fungi, yeasts, and bacteria.[8] Beta glucan is a compound distributed among brown algae, fungi, lichen, and barley, serving as a component of the fungal cell wall or a substance of food reserves in plants.[9] Depending on their origin, these polysaccharides are known by a number of common designations, i.e. laminaran, callose, curdlan, scleroglucan, pachyman, lentinan, laricinan, and paramylon.[10]

Various studies have revealed that the β-glucan consumed could provide a medicinal effect, acting as an antioxidant, anti-cholesterol, anti-radiation, anti-aging, and anti-tumor compound. [11-12] Krestin is a protein-bound polysaccharide isolated from mushrooms which function as an anti-bacteria.[13] Saskiawan and Hasanah (2015) examined the antimicrobial activity of the polysaccharide compounds extracted from white oyster mushroom against *B. subtilis*.[14] Beta-1,3-glucan of the *Agrobacterium* sp. is found to be anti-infective against certain types of

The 7th International Conference on Global Resource Conservation
AIP Conf. Proc. 1844, 020011-1–020011-7; doi: 10.1063/1.4983422
Published by AIP Publishing. 978-0-7354-1516-4/$30.00

microorganisms (*Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Pneumocystis carinii*, *Listeria monocytogenes*, *Leishmania donovani*, and *Herpes simplex*).[15] Generally, the exploration of brown algae, *Sargassum* sp., as an antimicrobial, anti-infective, anti-cholesterol, anti-inflammatory, antioxidant, anti-tumor, and anti-aging has focused on its secondary metabolites, i.e. its content of polyphenols, particularly phlorotanin; however, information of its primary metabolites, in particular, β-glucan (laminaran), is barely known. However, β-glucans from different areas and varied genera have different structures and contents, [16-17]including their bioactive capabilities as an antimicrobial.

EXPERIMENTAL DETAILS

Research Period and Location

This study was conducted during May-August 2015. The extraction was carried out at the Laboraratorium of THP Department, Faculty of Food Technology, University of Brawijaya, Malang, Indonesia. The LSIH microbiological analysis was conducted at the UB and Quality Testing Laboratory Development and Fishery Products, Surabaya.

Preparation of β-Glucan (Laminaran)

The brown algae, *Sargassum* sp. was indirectly dried under the sun. The dried samples (simplicial) were smoothened into powder. The algae were extracted using a method modified from one previously developed by Yvin (1999) and Mohsen *et al*. (2007).[18-19] Using two different pieces of extraction equipment, i.e. a Hot Plate Magnetic Stirrer (HPMS) and ultrasonication, the β-glucan (laminaran) generated from the first extraction using sulfuric acid solution was called LAE, whereas that from the second extraction (resulted from re-extraction of the residue with distilled water) was called LME. This finally led to the production of LAEm, LMEm, LAEs, and LMEs extracts.

Preparation of Media

Mueller Hinton agar (MHA; Difco™, France) and Mueller-Hinton broth (MHB; BBL™, France) media were prepared according to the instructions specified on the label. Next, the media were sterilized at 121˚C for 15 minutes and then stored under a sterile environment at room temperature for subsequent use.

Preparation of Bacteria

Microbial cultures used were obtained from the Food and Nutrition Culture Collection (FNCC) of Pusat Studi Pangan dan Gizi Universitas Gadjah Mada consisting of Gram-negative bacteria (*Salmonella typhimurium* FNCC 0135 and *Escherichia coli* FNCC 0091) and Gram-positive bacteria (*Bacillus subtilis* FNCC 0059 and *Staphylococcus aureus* FNCC 0047). Up to 20 mL samples of bacteria were taken from the bacterial stock and subsequently cultured in NA medium. The culture was incubated at 37°C for 24-48 hours. Once rejuvenated, the pure colonies of bacteria were subjected to antibacterial tests.

Preparation of McFarland 0.5 Standard

Three to five isolates of bacteria colonies were selected from pure isolates using a sterile inoculation loop and inoculated into 3 ml of Mueller-Hinton broth (MHB) in a sterile tube and mixed well. Turbidity levels of bacterial suspension were equated in accordance with the turbidity of 0.5 McFarland turbidity standards, which were prepared commercially (bioMerieux® SA, France). The accuracy of bacterial suspension was confirmed using a spectrophotometer. Sterile MHB was used and measured at a wavelength of 625 nm as a blanko solution for comparison. Absorbance values were used in the range of 0.08 to 0.10, which were associated with 10^7 to 10^8 number of bacteria per ml of MHB.

Disc Diffusion Susceptibility Test

Each of the β(1,3)-glucan (laminaran) extracts, LAE and LME, were dissolved in 60% methanol until a concentration of 10 mg/ml was reached, and then the solution was homogenized with a vortex at high speed. Bacterial inoculum was set according to the McFarland 0.5 standard, and then 0.1 ml of suspension was taken using a micropipette from each bacterial inoculum to be overlaid on the MHA plate. Soluble extract (20 mL) at a concentration of 10 mg/ml was transferred into a commercial blank disc (Oxoid, UK) and then measured with a diameter of 6 mm where the disc was placed in the bacterial area using sterile forceps. Blank discs were later impregnated with 20 mL of 60% methanol, acting as a negative control, while the commercial antibiotic amoxicillin disc was impregnated with 10 mg gentamycin, acting as a positive control. Plates were incubated at 37°C and the inhibition zones were measured after 24 hours. Each test set was repeated 3 times (in triplicate).

MIC (Minimum Inhibitory Concentration) Test

MIC Testing of β-glucan (laminaran), LAE and LME, was conducted using the dilution technique. One full loop of the exponential phase of bacterial cultures was associated with 0.5 MacFarlands Standard inoculated into MHB medium with different concentrations of LAE and LME extracts: 0, 0.25 and 0.375 mg/ml. After that, the tube was incubated at 37°C for 24 hours. Turbidity was then measured after the incubation period. MIC is defined as the lowest concentration of the extract which completely inhibited the growth of the microorganisms tested.

MBC (Minimum Bactericidal Concentrations) Test

The solution showed no bacterial growth in the MIC test when 20 mL of MIC was pipetted and poured into the new MHA medium. The test plate was subsequently incubated for 18 to 24 h at a temperature of 37°C. After incubation, the number of bacteria (CFU) was counted. MBC is defined as the concentration resulting from the reduction of 99.9% of the initial bacterial inoculum, thus allowing only 0.1% of bacteria to stay alive when re-cultured in a new MHA medium.[20] The number of bacterial colonies related to 0.1% of the previous bacterial inoculum in the broth microdilution test was calculated, where 0.1% of approximately 8 CFU showed less than or equal growth of bacterial colonies on media MHA known as MBC values (mg/ml). The lowest concentration of the extract showed no bacterial growth on agar plates referring to the MBC of each extract.

Data Analysis

Results of measurement of the inhibitory zone on the antimicrobial test were compared to each other. Components of the most active compound in the antimicrobial testing were those having the largest inhibitory zone diameter.

Determination of MIC and MBC was performed on the fraction results having the greatest antimicrobial activity. MIC and MBC values were determined after further testing. If after the advanced test there were bacterial growth, then the concentration would be expressed as MIC value. Conversely, if after further test there were no bacterial growth, then the concentration would be expressed as the value of the MBC.

RESULT AND DISCUSSION

Disc Diffusion Susceptibility Test

The antimicrobial activity of β-Glucan (laminaran) extracts of against Gram-negative and Gram-positive bacteria was determined based on Disc diffusion susceptibility test results conducted by using the Kirby-Bauer disc methods (1966). Based on the results of previous studies, it was known that the LAEs sample Laes contained no β-Glucan (laminaran) compounds for not showing the absorption characteristic (fingerprint) for β-Glucan (laminaran) at wave

number 887 cm-1,[21] thus, for subsequent studies, only three samples were used, ie LAEm; LMEm and LMEs. Based on the results of the measurement of the diameter of clear zone in Table 1, the inhibitory activity of the tested extract was observed.

TABLE 1. Zone of Inhibition of Gram Negative and Gram Positive Bacteria

Sample (mg/ml)	Diameter of Inhibition Zone (mm)			
	Salmonella typhimurium	***Escherichia coli***	***Streptococcus aureus***	***Bacillus substilis***
Kontrol (-)	0.00±0.00 a	0.00±0.00 a	0.00± 0.00 a	0.00±0.00 a
LMEs (250)	9.90±0.02 b	8.20±0.01 b	7.10±0.15 b	6.80±0.03 b
LMEs (375)	11.30±0.05 c	9.30±0.02 c	10.80±0.14 c	11.80±0.04 c
LAEm (250)	11.50±0.03 c	12.40±0.02 d	15.70±0.07 d	12.00±0.04 c
LMEm (250)	12.50±0.02 d	13.10±0.03 e	16.90±0.03 d	14.40±0.02 d
LAEm (375)	15.70±0.05 e	16.40±0.02 f	18.00±0.02 e	15.00±0.05 e
LMEm (375)	16.50±0.08 f	17.60±0.03 g	18.70±0.07 e	18.80±0.05 f
Kontrol (+)	31.00±0.01 g	29.00±0.05 g	35.50±0.01 f	39.40±0.04 h

Note : K (-) : sterile distilled water
K (+) : amoxicillin

From Table 1, it can be seen that there was no clear zone in the negative control, indicating the absence of bacterial activity. Monks et al. (2002) stated that the range of inhibition zone <7 mm refers to no inhibitory activity. [22] This occurs because there are inhibiting factors in paper discs, such as antibiotics or other antibacterial compounds, meaning that the growth of all tested bacteria, both Gram-negative and Gram-positive, is very well characterized by the absence of an inhibition zone.

Almost all LME extracts obtained from sonication methods, from low (250 mg/ml) to high (375 mg/ml) concentrations, had a relatively weak inhibition (6.80 to 10.80 mm), except for *E. coli* and *S. aureus* test samples at a concentration of 375 mg/ml, which was equal to 11.30 and 11.80 mm. Monks et al. (2002) stated that the inhibition zone range of 7-11 mm is considered relatively weak activity. [22]

The HPMS method shows very good inhibitory ability by forming the largest inhibition zone obtained in LAE and LME extracts at the concentration of 375 mg/ml, which is equal to 18.00-18.80 mm on *Salmonella typhimurium* and *Staphylococcus aureus* in LME extract with a concentration of 375 mg/ml. According to Monks *et al*. (2002), the inhibition zone >16 mm is considered relatively strong activity.[22]

This study showed that the higher the concentration of β-glucan (laminaran) extract (250 mg/ml <375 mg/ml), the larger the diameter of inhibition against Gram-positive and -negative bacteria, so it could be assumed that the higher the concentration of the extract, the greater a number of antibacterial compounds released, making the penetration of the compound into the bacterial cells through their own mechanism easier.

The ultrasonication method showed a tendency towards lower inhibition than the HPMS method, probably due to the degradation of bioactive compounds in the presence of vibrations during the ultrasonication process. This condition is evidenced when LAE samples from Ultrasonication method were tested for their functional groups by FT-IR; they appeared to lose their functional group synonymously with β-glucan (laminaran) at a wavelength of 887cm^{-1}. [21] Likewise, LME extract obtained from the HPMS method appeared to have a higher inhibitory zone than the LAE extract from the same method. Such higher antibacterial activity of LME extracts is likely due to the degree of purity or concentrations of β-glucan (laminaran) at the same weight (per gram) being higher when compared to LAE containing other components.[21]

The inhibition ability obtained in LME extracts from the HPMS method against Gram-positive bacteria (*B. subtilis* and *S. aureus*) was the highest compared to that against Gram-negative bacteria (*E. coli* and *S. typhimurium*). The magnitude of this inhibition zone, according to Kusmiyati and Agustini (2007), is because Gram-positive bacteria generally have a higher sensitivity to the antimicrobial compounds in comparison to Gram-negative bacteria.[8] Differences in sensitivity to antimicrobials are due to the different structure of the cell wall in both groups of bacteria. It was confirmed by Sudjaswadi (2006) that the effectiveness of the antimicrobial compound is influenced by the character of the wall or cell membrane of the bacteria.[23] The cell wall of Gram-positive bacteria consists of peptidoglycans, which are very thick, giving rigidity to the cells. The administration of antimicrobial compounds to Gram-positive bacteria could inhibit cell wall assembly and result in the incorporation of glycan

chains to prevent the formation of cross-links in the peptidoglycan cell wall, resulting in a weak cell wall structure and causing the death of bacteri.[24] Damage to the cell wall or obstacles to its formation could result in cell lysis, [25] thereby causing a greater inhibition zone.

The antibiotic amoxicillin is able to inhibit all kinds of bacteria; both Gram-positive and -negative bacteria, with inhibitory activity, were classified as very strong. Amoxicillin was chosen because it is a broad-spectrum antibiotic, against including gram-positive and -negative and aerobic and anaerobic species, and can dissolve in water [26] making it easier to use. The antimicrobial activity of β-glucan extract (laminaran) compared to commercial antibiotics with known inhibitory mechanisms, namely amoxicillin (inhibit cell wall synthesis), was lower. However, today, there is a tendency to return to nature; therefore, even though β-glucan (laminaran) has a strong inhibitory activity, it remains selected for further investigation.

Results of a study by [14] showed that the inhibitory zone of a polysaccharide compound oyster mushroom extract with *B. subtilis* produced was 9.57 mm whereas for *E.coli* was 8.55 mm, which means a relatively weaker activity compared to that obtained in this study. Thus, the obtained β-Glucan (laminaran) has high antibacterial activity. The difference is probably due to the structure of β-Glucan (laminaran) is a reserve component of the polysaccharide of algae while the β-Glucan is a structural component of fungal cell walls. It is confirmed by El Boshy *et al*. (2010) that the β-glucan from different sources also differs in structure.[28] But variation may also be due to antibacterial activity extraction method, the solvent used in the extraction and the season in which the samples were collected that were also different in addition to the raw material.

Test MIC and MBC

The determination of MIC and MBC values is important as a benchmark for the selection of appropriate and effective concentrations of compounds to be used for medical purposes.[28] It is also important to determine whether the antimicrobial compound β-glucan (laminaran) is bacteriostatic or bactericidal, and to determine the MIC and MBC values of β-glucan (laminaran) against Gram-positive bacteria (*B. subtilis* and *S. aureus*) and Gram-negative species (*S. typhimurium* and *E. coli*). Determination of the MIC values of β-glucan (laminaran) was performed at concentrations of 50 mg/ml to 375 mg/ml (w/v). The MIC values of β-glucan (laminaran) are shown in Table 2.

TABLE 2. MIC Value of Gram Negative and Gram Positive Bacteria

Experiments	Concentration (mg/ml)	*Salmonella thypimurium*	*Escherichia coli*	*Streptococcus Aureus*	*Bacillus substilis*
LMEs	50	+	+	+	+
	100	+	+	+	+
	250	-	-	-	-
	375	-	-	-	-
LAEm	50	+	+	+	+
	100	+	+	+	+
	250	-	-	-	-
	375	-	-	-	-
LMEm	50	+	+	+	+
	100	+	+	+	+
	250	-	-	-	-
	375	-	-	-	-

Note : (+) growth detected
(-) growth undetected

MIC value was expressed by the smallest concentration that could still give 100% inhibition of bacterial growth and is characterized by the absence of the test bacteria to grow. Observation after 24-h incubation at a concentration of 250-375 mg/ mL suggested that solution color in a test tube was clear. Table 2 shows that the concentration of 250 mg/ ml was the lowest concentration of the β-Glucan (laminaran) extract, which could produce inhibition zone on all Gram-positive and Gram-negative bacteria, so it was used as the MIC value. Thus, the MIC value of LMEs, Laem and LMEm extracts against Gram-positive and Gram-negative bacteria was 250 mg/ mL. When referring to

the classification of the strength of inhibition, according to Monks *et al.* (2002), the ability to β-Glucan (laminaran) extract in inhibiting growth at a concentration of 250 mg/ml were classified as a weak activity.[22]

MBC is defined as the concentration resulting from the reduction of 99.9% mg/ml of the initial bacterial inoculum, meaning that only 0.1% of bacteria will survive when cultures in a new MHA medium.[20] The number of bacterial colonies connecting it to 0.1% in the initial bacterial inoculum-broth microdilution test, where 0.1% is roughly equivalent to a number of bacteria of 8 CFU. The MBC value of β-glucan extract (laminaran) is shown in Table 3.

TABLE 3. MIC Value of Gram Nagative and Gram Positive Bacteria

Sample	Bacteria			
	Salmonella thypimurium	***Escherichia coli***	***Streptococcus Aureus***	***Bacillus substilis***
LMEs (250)	17.0 c	17.0 c	11.0 b	9.0 b
LAEm (250)	8.0 b	6.0 b	4.0 a	3.0 a
LMEm (250)	4.0 a	3.0 a	2.0 a	3.0 a

From Table 3 it can be seen that the MBC value of β-glucan (laminaran) extract at a concentration of 250 mg/ml was 2-8 CFU/ml in LAE and LME extracts obtained using the HPMS method for all Gram-negative and Gram-positive bacteria tested, with the growth of bacteria in media being characterized by the formation of bacterial colonies on blood agar medium, in the amount of ≤ 8 according to the 0.5 McFarland standard. Thus, LMEs extract at a concentration of 250 mg/mL is considered bactericidal against Gram-positive and Gram-negative bacteria, with its MBC value of 250 mg/ml. As described by [27], the antibacterial effect could be inferred from its MIC and MBC values against the growth of bacteria matching a number of bacteria in accordance with the 0.5 McFarland Standard. The 0.5 McFarland standard suspension is a suspension showing the bacterial turbidity concentration equal to 108 CFU/ml.

On the LMEs extract obtained from the ultrasonication method, the MBC value for all Gram-negative and Gram-positive bacteria was > 8 CFU. This means that the bacteria were taken from a concentration of 250 mg/ mL still showed bacterial growth. This means that the LMEs extract at a concentration of 250 mg/ mL was only bacteriostatic against Gram-positive and Gram-negative. This is probably due ultrasonication weakens the bond in compounds of β-Glucan (laminaran), thereby reducing its ability to inhibit bacterial growth. From table 3, it is also known that the inhibition of Gram-positive bacteria tends to be higher than that of Gram-negative. Gram-positive bacteria more easily inhibited than Gram-negative ones.[29] This is because Gram-positive bacteria are more sensitive than Gram-negative bacteria. Among Gram-positive bacteria, *S.aureus* are more susceptible to algae extract, followed by *B.subtil*is. Similar with results from, *S.aureus* was susceptible to all algae extract used in this study.[30]

SUMMARY

The highest antibacterial activities of LME extract obtained from the HPMS method against Gram-positive bacteria (*B. subtilis* and *S. aureus*) were at 18.10-18.80 mm, which were higher than those against Gram-negative bacteria (*E. coli* and *S. typhimurium*) i.e. 16.50-17.60 mm. The MIC value of β-glucan (laminaran) extracts, the LMEs, LAEm and LMEm, against Gram-positive and Gram-negative bacteria, were 250 mg/ml. MBC values of β-glucan extract (laminaran), the LAEm and LMEm extracts at a concentration of 250 mg/ml were 2-8 CFU/ml in Gram-negative and Gram-positive bacteria and is bactericidal. The resulting LME extract at a concentration of 250 mg/mL is bacteriostatic against Gram-positive and –negative species.

ACKNOWLEDGMENT

Authors would like to thank Brawijaya university for facilitate this study

REFERENCES

1. R. Lavanya and N. Veerappan, *Advances in Biological Research* **5**(1), 38-44 (2011)
2. K. Sieradzki, R. B. Robert, S. W. Haber and A. Tomasz, *N. Engl. J. Med*. **340**,. 517-523 (1999)
3. O. Idose, T. Guther, R. Willeox and A. L. Deweck, *Bull. WHO* **38**, . 159-158 (1968)
4. P. Smith, M. P. Hiney and O. B. Samuelsen, *Annu. Rev. Fish Dis.* **4,** Pp. 273-313 (1994)
5. W. Bauer, W. M. Kirby, J. C. Sherris and M. Turck, *American J. Clin. Pathol.* **45**, 493–496 (1966)
6. I. Tuney, B. H. Cadirci, D. Unal, and A. Sukatar, *Tur. J. Biol*. **30**, 1-5 (2006)
7. K. Siddhanta, K.H. Mody, B. K. Ramavat, V. D. Chauhan, H. S. Garg, A. K. Goel, M. J. Doss, M. N. Srivastava, G. K. Patnaik, and V. P. Kamboj, Indian J. Exp. Biol. 35(6), 638-643 (1997).
8. Kusmiyati and N.W.S. Agustini, *Biodiversitas* **8**(1)**,** 48-53 (2007)
9. O. Chizhov, A. Dell, H. R. Morris, A. J. Reason, S. M. Haslam and R. A. McDowell, *Carbohydr. Res.* **310**(3), 203–210.
10. T. N. Zvyagintseva, N. M. Shevchenko, I. B. Popivnich, V. V. Isakov, A. S. Scobun, E. V. Sundukova and L. A. Elyakova, *Carbohydr Res.* **322**, 32–39 (1999)
11. E. Taskin, M. Ozturk, and O. Kurt, *Afr. J. Biotechnol*. **6,** 2746-2751 (2001)
12. Y. K. Lee and H. Shen, "Basic Culturing Techniques", in *Handbook of Microalgae Culture : Biotechnology and Applied Phycology* (Blackwell Publishing Company, United Kingdom, 2004).
13. S. P. A. Wahyuningsih, A. N. Masitha, and D. Winarni, *Berk. Penel. Hayati* **17**(2), 177–183 (2012)
14. I. Saskiawan and N. Hasanah, *Pros Sem Nas Masy Biodiv Indon*. **1**(5), 1105-1109 (2015)
15. G. Hetalnd, N. Ohno, I. S. Aaberge and M. Lovik, *FEMS Immunol. Med. Microbiol*. **27**(2)**,** 111-116 (2000)
16. S. Hornsey and D. Hide, *Br. Phycol. J.* **20**(1), 21-25 (1985)
17. M. Kandhasamy and K. D. Arunachalam, *African Journal of Biotechnology* **7**(12), 1958-1961 (2008)
18. J. C. Yvin, F. LeVasseur, and F. Hud'Homme, U. S. Patent No. 59 80 916 (1999)
19. M. S. Mohsen, S. F. Mohamed, F. M. Ali and O. H. El-Sayed, *J Appl Sci Res.* **3**(10), 1178-1185 (2007)
20. P. C. Taylor, F. D. Schoenknecht, J. C. Sherris and E. C. Linner, *Antimicrob. Agents Chemother*. **23**(1), 142–150 (1983)
21. Chamidah, "Identifikasi, Potensi, Fraksinasi Dan Sifat Fisiologis Serat Pangan Alga Coklat Sargassum Duplicatum," Phd Thesis, Universitas Gadjah Mada Yogyakarta, 2013.
22. N. R. Monks, C. Lerner, A. T. Henriques, M. Farias, E. E. S. Schapoval, E. S. Suyenaga, B. Rocha, G. Schwartsmann, and B. Mothes, *Journal Of Experimental Marine Biology And Ecology* **281**(1-2), 1-12 (2002)
23. R. Sudjaswadi, Farmasi Indonesia, **17**(2), 98–103 (2006)
24. A. M. M. Leven, *J Infect Dis.* **155**(3), 377-389 (1987)
25. E. Jawetz, J. L. Melnick and E. A. Adelberg. *Mikrobiologi untuk Profesi Kesehatan,* translated by H. Tomang (Penerbit EGC, Jakarta, 2001).
26. A. Syarif, A. Setiawati, A. Muchtar, A. Arif, B. Bahry, B. Suharto, D. Tirza, F. D. Suyatna, H. R. Dewoto, H. Utama, I. Darmansjah, L. S. Kunardi, M. S. S. Wiria, Nafrialdi, P. F. Wilmana, Z. S. Bustami, P. Ascobat, R. Setiabudy, S. O. Santoso, S. K. Suherman, S. Sukarban, R. Sunaryo, S. Wardhini, S. G. Ganiswara, T. Handoko, U. Sjamsudin, V. H. S. Ganiswarna, Y. Mariana, Y.H. Istiantoro dan J. Zubaidi, *Farmakologi dan Terapi edisi 4*. (Gaya Baru, Jakarta, 2001) .
27. Siregar, "Daya Antibakteri Ekstrak Buah Mahkota Dewa (Phaleria macrocarpa [Scheff.]Boerl) terhadap Pertumbuhan Streptococcus mutans (in vitro)", Bachelor Thesis, Universitas Sumatera Utara, 2011
28. El Boshy *et al*., *Majalah Farmasi Indonesia* **17**(2), 98-103 (2010)
29. E. A. Paz, R. N. Lacy and M. Bakhtian. *The B-Lactum antibiotics penicillin and cephalosporin in perspective* (Hodder Strong, London, 1995), Pp. 324
30. M. J. Pelczar and E.C.S. Chan, *Dasar-dasar Mikrobiologi 2* (UI Press, Jakarta, 1988)