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Research Article

MINIMUM INHIBITORY CONCENTRATION OF ANTIMICROBIAL SULFATED POLYSACCHARIDES OF *Sargassum cristaefolium* AGAINST *Bacillus subtilis*

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ABSTRACT. Bacterial resistance to antibiotics leads to multiple infections and a decline in curative abilities within the host. Furthermore, preliminary action is needed with natural-based products, as an alternative solution to prevent bacterial growth. The species *Sargassum cristaefolium* is a marine seaweed currently gaining attention for advanced applications, due to the various intrinsic biological activities, especially as an antimicrobial agent. The aim of this research, therefore, is to determine the minimum inhibitory concentration (MIC) and the selective toxicity of sulfated-polysaccharide extracts obtained from *S. cristaefolium*, against the gram positive bacteria, Bacillus *subtilis*. in addition, the efficacy was evaluated using tube dilution test and agar diffusion (disk plate test). The result showed an MIC of 200 ppm for sulfated-polysaccharides, and the least significance difference test demonstrated bacteriostatic selective toxicity. This yield is confirmed to be applicable as a natural and safe antibiotic product, needed for the remediation of numerous bacterial diseases.

Keywords *Sargassum cristaefolium, Bacillus subtilis, sulfated polysaccharide extracts, antibacteria activity*

INTRODUCTION

The increase in bacterial resistance to antibiotics has been identified as a major problem in recent years [1]. This phenomenon is caused by the microorganisms ability to produce hydrolytic enzymes needed to breakdown the target antibiotics agent. Consequently, it promoting the structural changes or gene mutation, reducing cell membrane permeability, and preventing the antibiotics enter their intracellullar cell [2]. Hence, there is an urgent demand for effective bacterial infection treatments [3].

Sulfated-polysaccharide extracted from seaweed are known to possess enormous beneficial properties, including antioxidant [4], antiviral [5], antiallergic [6], and antibacteria [7]. The cell wall particularly contains a high diversity of sulfate-binding-polysaccharide (carragenans, agarans, alginates, fucoidan, etc), proposed as a survival mechanism against pathogenic infections [8]. These unique substances were, therefore, extracted and the antibacteria activity were further studied. In addition, the sulfated-polysaccharides obtained from seaweed have the ability to provide health improvement benefits with no side effects on organisms, especially in humans [9].

Sargassum cristaefolium is a tropical seaweed, widely spread in western and central Pacific Ocean, including Indonesia [10]. This plant is characterized by slightly flattened

main branches, spatulate leaves, elliptical vesicles and androgynous receptacles [11]. Furthermore, *S. cristaefolium* also possesses medicinal values as raw material in pharmacy, cosmetics, healthy tea and functional food [12-14]. The aim of this study is to evaluate the antibacterial therapeutic potency of this seaweed by determining the minimum inhibitory concentration (MIC) and selective toxicity against *Bacillus subtilis.*

MATERIALS AND METHODS

Extraction of Sulfated-polysaccharides

Water extraction method was adopted[15-16] by washing the *S. cristaefolium* with running water and drying at temperatures between 40-55ºC. The samples were subsequently crushed and filtered using a 500 mesh sieve. Therefore, the formulated powder was macerated with 85% ethanol (1:6) for 24 hours, filtered using a filter cloth, and the residue was sequentially extracted with 85% ethanol over 10 hours in a water bath at 70ºC, and further filtered with a filter cloth. This residue was added with $CaCl_2$ 2% (1:6) at 70ºC for 9 hours, followed by centrifugation, and then HCI 0:01 M was added (1:6) for 9 hours under similar temperatures, and centrifuged at 3000 rpm for 15 min, before filtering with whatman filters (no 40, 8 µm). Furthermore, the sample was subjected to evaporation, using a rotary evaporator, under vacuum conditions (IKA®, HB 10 Digital).

Antibacterial Activity

Strain SP of *B. subtilis* was cultured from the Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya. This bacteria was then were cultivated in Nutrient Broth and incubated at 37ºC for 24 h under aerobic conditions to enable exponential growth. Therefore, the turbidity concentration (10^7 colony-forming unit (CFU/ml)) was estimated using 0.5 Mc. Farland Units.

The extracts were diluted with 10% dimethyl sulfoxide (DMSO) (v/v), to reach a final concentration of 40.000 ppm, and then a twofold serial medium from 40.000 to 200 ppm was placed in the culture (multiwell plates Corning, NY, USA). Therefore, screening for antibacterial activity was performed as described, with some modification [17]. Meanwhile, petri plates containing Trypticase Soy Agar (TSA) medium and 200 ppm ampicilin were used as a positive control and the inhibition zone of sulfated-polysaccharides were observed at 24, 36, 48, 60, and 72 h to obtain the selective toxicity activity. These measurements were performed in triplicate and expressed as mean ± standard deviation.

Statistical Analysis

The data collected were analysed statistically to determine possible differences between samples and control, and also to define the optimal dose, through one-way ANOVA and Least Significance Difference (LSD) tests. Furthermore, statistical significance was set at $\alpha < 0.05$ in all cases.

RESULTS

The Weight of Yield

The result showed a total sulfated-polysaccharides of *S. cristaefolium* yield reached 1.79%. The colour of extracts was a dark brown viscous and non-powdered, featuring residual solvents from the extraction process, which result from the separation from inherent water content. In addition, the solvents used include ethanol and HCl, with respective boiling points of 79ºC and 110ºC, and both were evaporated for >3 hours, using a *rotary vacuum evaporator,* at a maximum temperature of 40ºC. The procedure caused elevated retained quantities in the extracts, due to the high *S. cristaefolium* powder to solvent ratio during extraction [18]. This was also affiliated with the duration of maceration and extraction, which possibly softened the *Sargassum cristaefolium* cell wall, leading to easier water absorption [19, 20].

An Analysis of Anti-Bacterial Activity

Several doses of sulfated-polysaccharide from *S. cristaefolium* extracts ranging from 1.5625 to 800 ppm were selected to determine the minimum inhibitory concentration against *B. subtilis* bacteria, as seen in Figure 1. Furthermore, tube turbidity was reported from the least concentration to 100 ppm, while clearness ensued at 200 ppm, and up to the highest dose. Therefore, 200 ppm was chosen as the minimum inhibitory concentration (MIC) for further experiment.

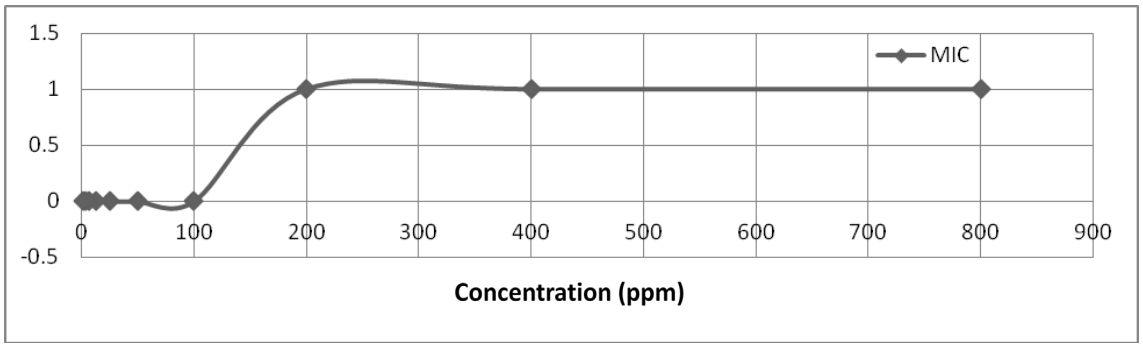

Fig. 1. *Minimum ınhibitory concentration of sulfated-polysaccharides S. cristaefolium against B. subtilis (score 0 indicated the tube turbidity; score 1 means the tube clearness)*

Tests to understand the antibacterial mechanism were conducted on serial twofold concentration from 40.000 to 200 ppm, as seen in Figure 2. Similar to the MIC result, the 200 ppm sulfated-polysaccharide extracts inhibited the bacteria growth, at a diameter of 115±0.85mm. In addition, the inhibition zone became larger with increasing concentrations. Furthermore, maximum inhibition was recorded at 40.000 ppm, with a diameter of 505±0.21mm, similar to the positive control ampicillin at 502.5±0.9mm.

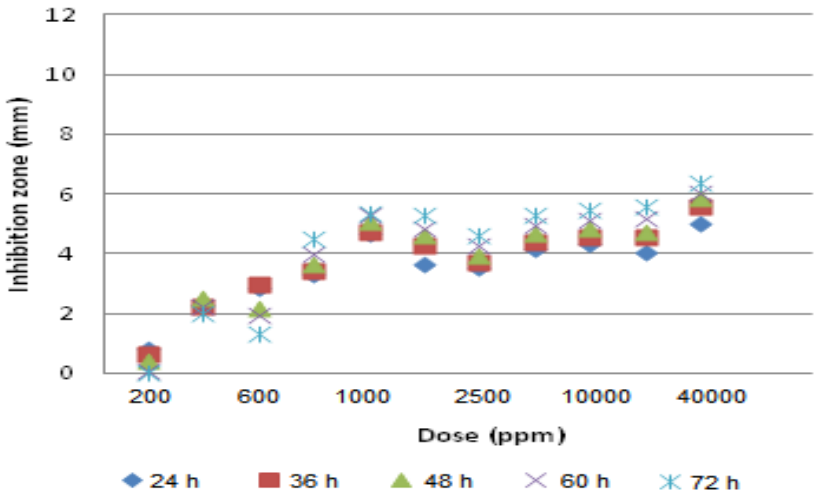

Fig. 2. *The inhibition concentration of sulfated-polysaccharides S. cristaefolium against B. subtilis in several concentration*

The antimicrobial activity against this bacteria showed reduced inhibitory zone, where the highest degradation of 70±1.35mm was detected in 600 ppm after 72 h, and 5±0.4 mm for 200 ppm, after 48 to 72 h. Conversely, ampicilin inhibition at 200 ppm was increase from 650±3.2 to 1085±5.4mm on 36 and 72h, respectively (Fig. 3).

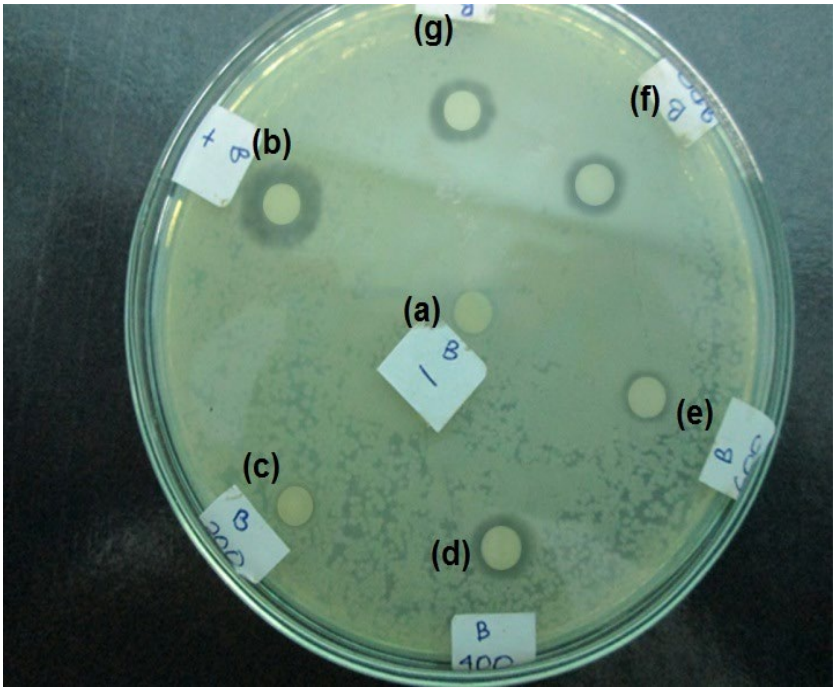

Fig. 3. *The inhibition zone of sulfated-polysaccharides S. cristaefolium against B. subtilis. (a) B. subtilis in DMSO 10%; (b) B. subtilis in Ampicillin 200 ppm; (c) B. subtilis in sulfated-polysaccharide extracts 200 ppm; (d) 400 ppm: (e) 600 ppm; (f) 800 ppm; (g) 1000 ppm.*

DISCUSSION

This study has provided MIC and selective toxicity activity of antimicrobials sulfated-polysaccharide *S. cristaefolium* for *B. subtilis.* The present study separated extracts into 6 fractions with the lowest concentration 200 ppm to 40.000 ppm. Limited data was provided by MIC values only for gram positive bacteria *B. subtilis*. After 72h, there is no indication of selective toxicity from this sulfated-poysaccharide extracts against *B. subtilis.*

In our study, *B. subtilis* experienced an adaptation phase on 0 to 5 hours, followed by a sustained logaritmic phase on the 6 hours. Furthermore, the stationary and the death phase ensued on 12 and 14 hours, respectively[21]. Nevertheless, after 24h, the bacteria was resuscitated and spread inside the inhibition zone, which indicate this extracts was bacteriostatic. According to Rao et al[22], The cell wall of gram positive bacteria generally contains 90% semi permiable peptidoglycan, needed for protection against foreign substances. Particularly, *B. subtilis* is spore-producing bacteria formed in parent cells, and is known to contain miniscule cytoplasm, genetic materials, ribosomes, and protein in the cell wall. In addition, spore is regarded as the resting form[23] which grows in to new cells under possible condition, including instances of parent cell death[24]. This spore germination and growth possibly multiplied after 36h observation time, featuring an increase in quantity.

This finding is congruent with the report by Yamashita[25], where several dietary polysaccharides obtained from seaweed demonstrated positive effect against several enterotoxigenic bacteria, including *Escerichia coli, Aeromonas hydrophila and B. subtilis.* Moreover, the carrageenans fraction (λ, γ and κ) had bacteriostatic effects against *B. subtilis* at 2500 ppm, which was absent at the high-level dose of 5000 ppm. Another research by Ambarwati et al [26] also gained similar results. The crude methanol extracts of *G. latissima* leaves inhibited *B.* subtilis in 10.000 ppm. Conversely, the most active fraction of *G. latissima* using the same solvent and bacteria was able to inhibit in 312.5 ppm.

The present assay in all doses demonstrated lower inhibition ability compared to the positive control ampicillin 200 ppm. This outcome was associated with the differences in mechanisms of action[27]. In addition, the sulfated-polysaccharides tend to trigger the osmosis process in cells, in the presence of high polysaccharide sugar and sulfate salts concentration[27]–[28]. These constituents facilitate water discharge from the vacuole, leading to continuous loss and bacteria weakness[29]. Therefore, the cytoplasmic membrane shrinks and detaches from the cell wall (plasmolysis), and consequently causing microbe death[30].

The antibacterial activity of *S. cristaefolium* sulfated-polysaccharides was less effective than ampicillin, proven by the narrow inhibition zone. This size differences was due to variations in mechanism of action, as Ampicillin treats infections by inhibiting cell wall synthesis[31], following the fusion with penicillin-binding proteins (PBPs) present in the bacteria. This leads to disruption in the transpeptidation of peptidoglycan chains[31]–[32], and the subsequent activation of intrinsic proteolic enzymes, therefore causing increased osmotic pressure and ultimately bacteria lysis (plasmoptysis)[33]. Furthermore, ampicilin at a dose of 10 mg showed the widest inhibition zone (88%) compared to other antibiotics (minocyline, streptomycin, tetracycline, etc) in inhibiting both gram positive and negative bacteria[34]. However, sustained application is implicated in the onset of resistance, resulting from the formation of betalactamase enzyme, which break and inactivate beta lactam's ring,

ultimately decreasing the ampicillin's effectiveness in any doses[35]–[36]. A research by Kamath[37] on the effect of several natural plant products against various bacterium, including *B. subtilis,* also affiliated the higher incidence of resistance to the characteristic cell wall impermeability. This phenomenon occurs relatively more with gram-positive over -negative bacteria.

Conversely, the inhibition method of sulfated polysaccharides was different, featuring cell wall and cytoplasmic membrane disruption. This results in the leakage of essential molecules after protein dissolution, and ultimately causing death[38]. Furthermore, the sulfated groups present further improved the antibacterial activity[39] by modifying the biotic surfaces[40]. A similar research with sulfated-polysaccharides from *S. sswartzii* showed various inhibitions, and *B. subtilis* was identified as the most susceptible[20]. In addition, natural products were proven to be potential therapeutic agents with no side effects[41], hence the need to consider using sulfated-polysaccharides extracted from *S. cristaefolium* as acceptable antibiotics against gram-positive bacteria *B. subtilis*.

CONCLUSION

Sulfated-polysaccharides from *S. cristaefolium* seaweed in this study were confirmed to confer inhibitory activities against the growth of *B. subtilis.* The smaller zones of inhibition observed after several hours indicated bacteriostatic selective toxicity. In addition, further study is required to understand the possible action mechanism of sulfated-polysaccharides against gram negative bacteria.

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