ANTIBACTERIAL ACTIVITY OF ETHANOL EXTRACT OF *SARGASSUM POLYCYSTUM* AGAINST *STREPTOCOCCUS MUTANS* AND *LACTOBACILLUS CASEI*: IN VITRO

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ABSTRACT

Introduction: *Sargassum polycystum* (brown seaweeds) has been recognized as a potential source of natural antibacterial and antimicrobial compounds. It has been used in many applications and products as they possess interesting biological activities that give benefit to the dental and medical area. **Aims:** The objectives of this study were to determine the growth curve of *Streptococcus mutans* and Lactobacillus casei, and also to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) value of *Sargassum polycystum* extract against *Streptococcus mutans* and *Lactobacillus casei*. **Materials and methods:** The growth of both bacteria were studied by growth curve analysis. Besides that, the antibacterial activity of *Sargassum polycystum* ethanol extracts against two oral cariogenic bacteria were examined using broth dilution method. The bioactivity of the seaweed extract was studied by finding the MIC and MBC value. **Results:** The time taken for the *Streptococcus mutans and Lactobacillus casei* to achieve exponential phase during growth is 4 and 5 hours respectively. MIC values of ethanol extracts of *Sargassum polycystum* in different concentration against *Streptococcus mutans* and *Lactobacillus casei* were unreliable because the presence of fungus (white-precipitate) at all 96-well plates including the positive and negative control. As there was no MIC value, partial inhibition was determined by calculating the optical density. Therefore, MBC analysis was unable to carry out because the MIC values were unreliable. **Conclusion:** The ethanol extract of *Sargassum polycystum* used in this study show partial bacterial inhibition against pathogens used. Therefore, the same study by using different type of solvent are recommended to be carried out in the future.

Keywords: *Sargassum polycystum*, brown seaweed, antibacterial activity, *Streptococcus mutans*, *Lactobacillus casei*, growth curve

INTRODUCTION

Oral cavity is colonized by 700 to 1000 bacteria species (Palmer, 2014) but not all species causing dental caries. *Streptococcus mutans (S. mutans)* is the main cause of dental caries while *lactobacilli* group are associated with the progression of lesion. Both *S. mutans* and *Lactobacillus casei (L. casei)* are facultative anaerobic, gram positive coccus that are commonly found in oral cavity.

Dental caries is the most common dental public health problem, which affecting 44% of the world population in 2010, (Jin et al., 2015). This problem has significant impact on the quality of life individuals and socio-economic that makes dental caries is a significant public health concern (Talib et al., 2011). Hence, many studies have been conducted to explore the alternative treatment options and one of it is by using natural resources such as seaweed. Seaweed or macro-algae has gained popularity recently especially in the field of medical and biochemical applications (Leary, Vierros, Hamon, Arico, & Monagle, 2009). It compounds have demonstrated various biological properties such as antibacterial, antimicrobial, antifungal, antiinflammation and anticancer (Thirumaran, Vijayabaskar, & Anantharaman, 2006).

Malaysia has an extensive coastline fringed by numerous islands, providing various habitats for the growth of seaweeds (Lian, Sim, Fauzi, & Moi, 2008). The taxa recorded from Malaysia (including Singapore) reported that 20 species of Cynophyta, 85 species of Chrolophyta, 87 species of Rhodophyta and 55 species of Phaeophyta (Phang, 2006). *Sargassum polycystum* is one of the species in Phaeophyta. It has been studied, and they showed promising antibacterial, antipyretic, analgesic, and anti-inflammatory, cytotoxicity, and antitumor activity (Kim & Lee, 2008). Thus, among all types of the seaweeds, *Sargassum* is proven to be a good source for the phytochemical analysis to identify the effect of the content as compared to the other types of seaweed (Balachandran, Maroky, Ajay Kumar, & Parthasarathy, 2016). Furthermore, the antibacterial effect needs to be studied in details against oral bacteria which are *S. mutans* and *L. casei*. To date, only limited studies investigated the antibacterial effect of *Sargassum* properties from brown seaweed species towards the oral cariogenic bacteria.

MATERIALS AND METHODS

Preparation of Seaweed's Extract

Sargassum polycystum were collected from coastal areas of Sabah. Initial collections of *Sargassum polycystum* were washed with distilled water to remove debris and to standardize the moisture content of the seaweed. Excess water was drained by blotting them on the filter papers, and shaded dried for 5 days and dried in an oven at 37°C. Then, the seaweed was ground into a fine powder and sieved to get a uniform powder size. The powder seaweeds were stored in a -20°C freezer.

Ethanol extraction method

25g of seaweeds powder was dissolved in 250 mL of 100% of ethanol at room temperature. The extract was kept at room temperature for a day while they were shook in orbital shaker under 100rpm, and then the solution was dried with an evaporator. The crude extracts were stored at -20° until further use.

Microbial strains

The oral cariogenic bacteria strains namely *S. mutans (*ATCC 25175), and *L. casei (*ATCC 4646) from Axon Scientific®, United State of America, were cultured and stored in glycerol stock in -80ºC.

Media preparation (broth)

Brain Heart Infusion Broth (BHIB), and Mar, Rogoso, Sharpe Broth (MRSB) were used in the anticariogenic assay for culturing the bacteria. The BHIB and MRSB powder was suspended to 1 liter of distilled water and mixed thoroughly until completely dissolved. All the mixtures were sterilized at 121 °C for 15 minutes, using an autoclave machine. Finally, the broths were stored at 4 °C until further used.

Growth curve

5 ml of broth from Brain Heart Infusion (BHI) flask was transferred to a small test tube and mixed with *S. mutans* inoculum. The wavelength was adjusted to 600 nm on the spectrophotometer and calibrates the machine to 0.0 O.D. or 100% transmittance. The optical density (O.D) of BHI broth was measured and calibrated to be 0.000 Abs. Then, the BHI broth was mixed with inoculum and the O.D was measured until the reading between 0.08Abs to 0.10Abs was achieved. The inoculum was incubated in incubator orbital shaker for 30 minutes. After first 30 minutes, the O.D. of the sample was measured in triplicate readings. At 30-minute intervals for up to 210 minutes, the culture was shake vigorously and transferred into sample tube. The same steps were repeated for 2-hour intervals for up to 8 hours and 4-hour intervals for up to 8 hours, with total of 24 hours. The optical density (y-axis) versus time (x-axis) for each growth condition was plotted. The same steps were repeated for Mar, Rogoso, Sharpe Broth (MRSB) and *L. casei* inoculum.

Minimum inhibitory concentration assay

The minimum inhibitory concentration (MIC) was determined by the broth dilution method and was repeated three times. MIC was used to determine the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. A 96-well plate was used for this assay whereby each of the wells plate was loaded with the 50 µL of broth respected to their bacteria. After that, 50 µL of the 20 mg/mL stock of seaweed extracts were transferred into the well (100 µL). Then, two-fold serial dilution was performed by transferring 50 µL from the highest concentration of treated culture (20 mg/mL) into the next well so that the final volume of each well was 50 µL. Then, each well plate was loaded with the 50 µL inoculums that grown to an exponential phase containing 10^7 and 10^4 CFU/mL of both bacteria respectively making final volume of all well 100 µL. The tested plate was incubated for 24 hours at 37 °C. The streptomycin was tested in the same manner. The concentration that gave zero optical growth was considered as MIC. All of the tests were done in triplicates and illustrated as shown in the Figure 1.

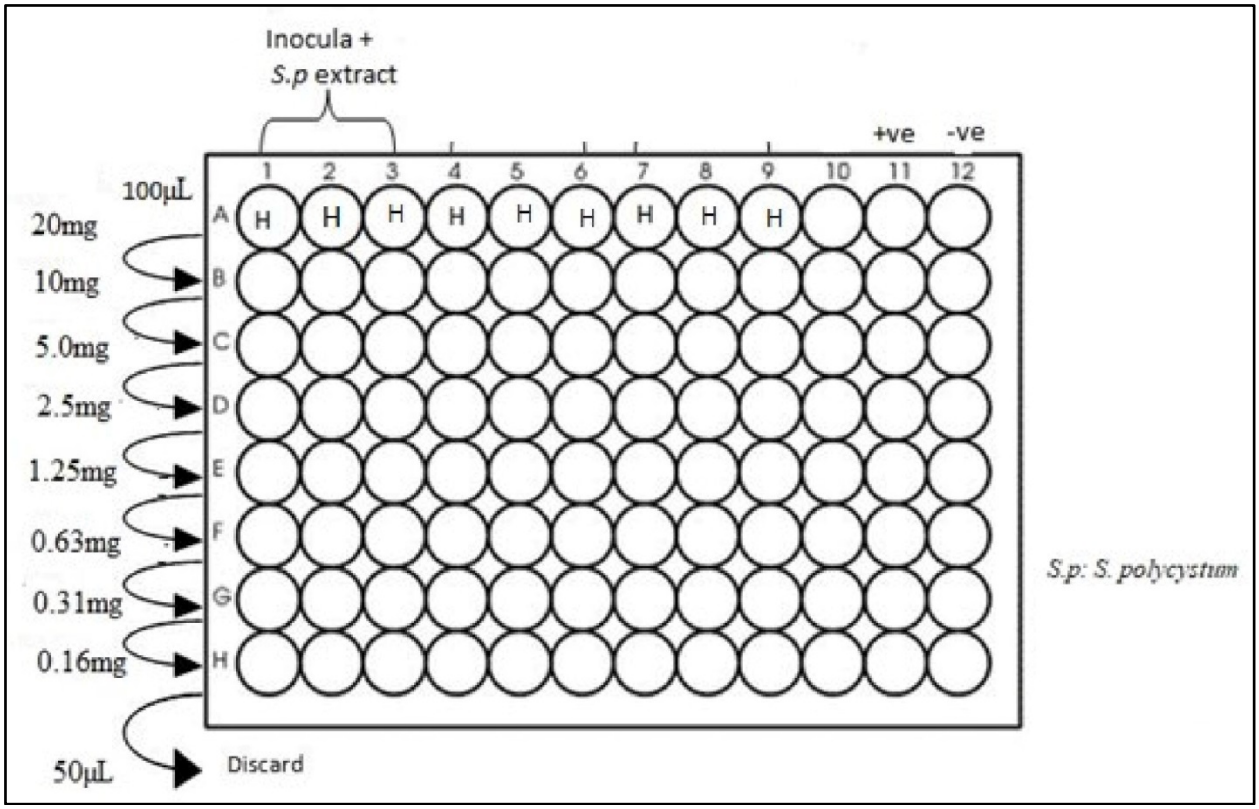

Figure 1. 96-well plate was used to determine the MIC concentration value.

RESULTS

Figure 2 shows the growth curve of *S. mutans* within 24 hours period. The strain of these bacteria took about 2.5 hour to complete the lag phase before it entered and started the exponential phase. The time taken for the *S. mutans* to achieve exponential phase during growth was 4 hours. Lastly, they entered the decline phase at 11.5 hours. No stationary phase can be recorded.

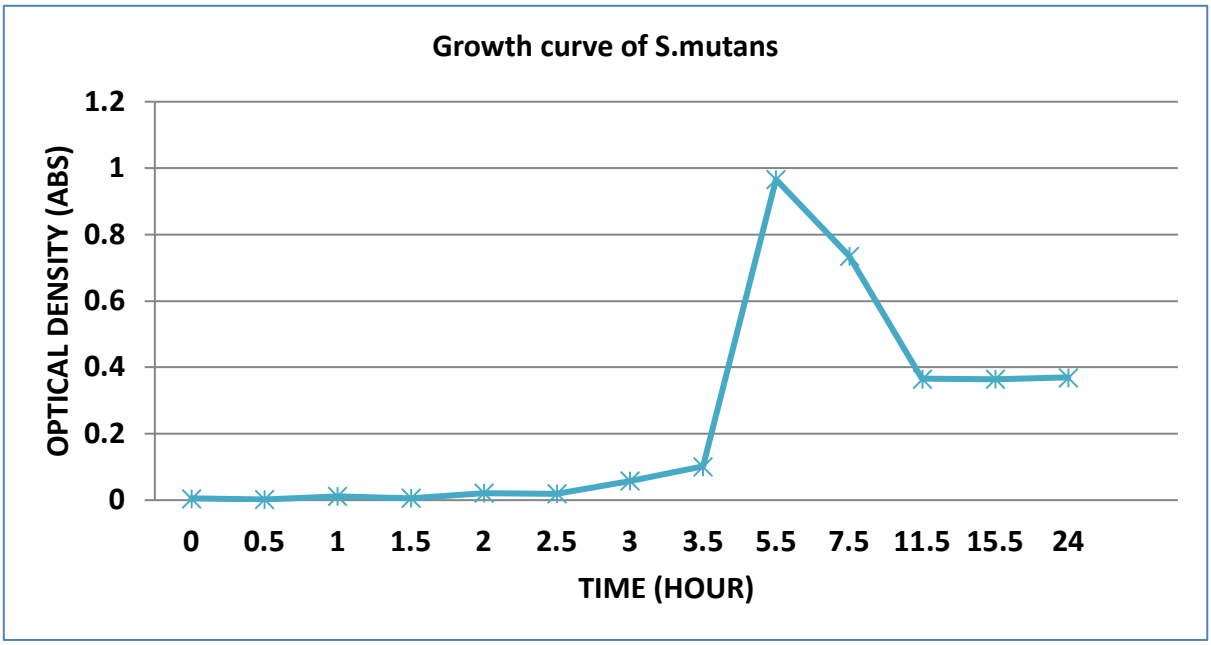

Figure 2. Growth curve of *S. mutans* within 24 hours period

Figure 3 shows the growth curve of *L. casei* within 24 hours period. The strain of these bacteria took about one and half (1.5) hour to complete the lag phase before it entered and started the exponential phase. The time taken for the *L. casei* to achieve exponential phase during growth was 5 hours. Lastly, they entered the decline phase at 7.5 hours. No stationary phase recorded.

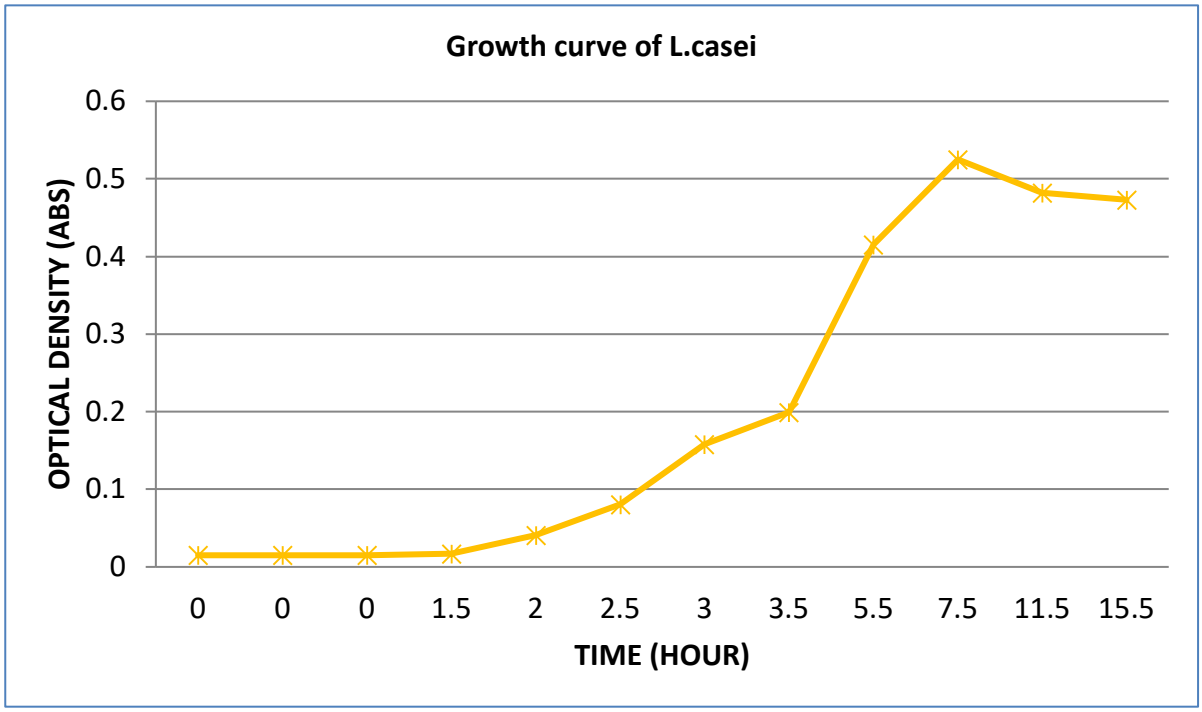

Figure 3. Growth curve of *L. casei* within 24 hours period

Table 1 shows the inhibition percentage of *S. mutans* and *L.casei* in different concentration of *Sargassum polycystum* extract. As there was no MIC value, partial inhibition was determined by calculating the optical density. It was found that the higher the concentration, the higher the percentage of inhibition.

Table 1 Inhibition percentage of *S. mutans* and *L.casei* in different concentration of *Sargassum polycystum* extract.

Seaweed extracts concentration (mg/ml)	**Inhibition (%)**	
	S. mutans	***L. casei***
100	95.5	86.9
50	91	53.8
30	93.4	49.1
15	36.1	44.3
7.5	22.3	32.2
3.25	22.2	25.6
1.625	14	25.8
0.8125	15.4	26.9

MIC analysis for *Sargassum polycystum* against *S. mutans* and *L. casei* were conducted by using 96 wells plate. Streptomycin was used as positive control while BHI and MRS broth were used as negative control. The experiment was repeated in three times.

Only streptomycin column which used as positive control showed inhibition. In contrast, no MIC values can be recorded due to unclear appearance for the rest of 88 wells. There was partial inhibition in some of the wells showing that *Sargassum polycystum* still had some antibacterial effect against *S. mutans* and *L. casei*.

The MIC value still unable to be obtained after the experiment was repeated for three times. It might be due to the contamination of the incubator or the seaweed itself. MBC analysis was not carried out since the result of MIC value unable to be obtained.

DISCUSSION

Growth curve showing four different phases, lag phase, exponential phase, stationary and death phase. In the lag phase, it is the period where the individual bacteria are maturing and not yet able to divide. During the lag phase cells change very little because the cells do not immediately reproduce in a new medium. The log phase or also known as exponential phase can be detected when the number of population doubled at a regular interval. The next phase of growth curve was the stationary phase which shown as a horizontal linear part of the curve, resulted from a situation in which growth rate and death rate are equal. At death phase (decline phase), the bacteria died due to lack of nutrients, environmental temperature above or below the tolerance band for the species, or other injurious conditions. Unfortunately, in this study, only log phase was emphasized and there was no stationary and death phase recorded.

As stated by Rolfe et al., (2012), the exponential phase represents the process of cell division involving multiple factors. This is the time for the bacteria to multiply the most. Study by Wilkins et al (2002) showed that the bacteria achieved log phase at about 5 hours, however this present study showed shorter time needed, 4 hours. According to Korbekandi et al., (2012), the optimum harvest time for *L.casei* cultured in MRS broth could be about 12 hours. It is contradicted with the present study which the *L.casei*

harvested only in 5 hours. This result is important in order to know the optimum time needed for the bacteria to be incubated. The longer incubation time might lead to bacteria samples deterioration.

The antibacterial activity of *Sargassum polycystum* had been widely studied but on different bacteria and solvents. Chiao-Wei and colleagues (2011) studied on antibacterial activity of *Sargassum polycystum* in methanol, dichloromethane and n-hexane, against *E. coli, P. aeruginosa, S. aureus*, and *B. cereus*. However, this research studied on ethanol extract of *Sargassum polycystum* against oral cariogenic bacteria which are *S. mutans* and *L. casei.* A lot of studies had used other types of solvents for example methanol, aqueous, dichloromethane and n-hexane. However, there was lack of study on ethanol extract in seaweed even though ethanol was the second most important solvent after water and the least toxic alcohol. Ethanol was suggested to attract both polar and non-polar molecules which contains in the samples. According to the study by Sánchez-Camargo et al., (2016), the highest content of phenolics in *Sargassum muticum* was detected in ethanol extracts, while the lowest was found in water extracts.

Regarding the study of antibacterial effects, the difference result of the present study and the other studies might be due to production of bioactive compounds, seasons, methods, organic solvents used for extraction of bioactive compounds and differences in assay method (Kandhasamy & Arunachalam, 2008). In this present study, the MIC value unable to be obtained for both bacteria strains throughout the three trials due to the presence of white precipitate at the base of all 96-well plates. The white precipitate indicated the absence of antibacterial activity of ethanol extract of *Sargassum polycystum* against the two cariogenic bacteria. The white precipitates might also indicate the presence of fungal contamination due to poor infection control in the incubator. However, partial inhibition of bacteria can be seen and proved by the reading of OD in certain wells of the plate. From this present study, we found that the higher the concentration of extract, the higher the percentage of inhibition.

The use of plant extract with known antimicrobial properties can be of great significance in therapeutic treatment but several studies had also reported various types of contamination of herbal medicine which includes microorganisms and toxins produce by the microorganisms, pesticides and toxic heavy metals (Talalay & Talalay, 2001).

CONCLUSIONS

In conclusion, ethanol extract of *Sargassum polycystum* used in this study showed partial inhibition of *S.mutans* and *L.casei*. There are potential sources of bioactive compounds and antibacterial activity of *Sargassum polycystum*. Therefore, further research need to be carried out in order to identify the biochemical compound of *Sargassum polycystum* against oral cariogenic bacteria.

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