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Comparative antibacterial activity of commercial chitosan and chitosan extracted from *Auricularia* sp.

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ABSTRACT

The threat of antimicrobial resistance continues to rise, hence the need for the discovery of new antibiotics, especially from alternative sources like plants, fungi and other organisms, also increases. In this study, chitosan is derived from *Auricularia* sp., and its antibacterial property, together with commercial chitosan, were evaluated against *Escherichia coli* and *Staphylococcus aureus*. Chitinous material from *Auricularia* sp. were deproteinized and deacetylated using different concentrations of NaOH at varying temperature for hours. Extracted and commercial chitosan were verified through FT-IR Analysis, and deacetylation degrees of the two were also determined. FT—IR Analysis showed that both chitosan have similar trends in the FT-IR spectrum but differ in deacetylation degrees. The results also showed concentration-dependent antibacterial activities of both extracted and commercial chitosan against *E. coli* and *S. aureus* wherein as the concentration increases, the activity also increases. Moreover, both chitosan exhibited greater activities against *S. aureus* than in *E. coli,* and it was revealed that they exhibit significantly higher activities against Streptomycin sulfate against *S. aureus* and comparable activities against *E. coli*. Overall, the results have showed that the chitosan extracted from *Auricularia* sp. exhibited greater antibacterial activities on both Gram-positive and Gram-negative bacteria compared with the commercial chitosan.

1. Introduction

The rising threat of antibiotic resistance has alerted many people, most especially scientists, since not only humans will be negatively affected by this crisis but other organisms as well, such as algae, plants, and animals. This has prompted experts to look for other possible solution for this dire turning point. Many have resorted to study about biochemical substances from living organisms due to adaptive nature of life forms to create ways how to combat infections or pathogenic attacks.

As the first natural antibiotics were derived from fungi, these organisms were now closely examined for antibiotics. In fact, about 20% of ~400 known antibiotics were of fungi origin (Donovick, Jong, 1989). Fungi produce many bioactive substances which may be antibacterial, antifungal or antiviral. Thus, many scientists are now considering mushrooms as potential source of antibiotics (Alves et al., 2009; Janeš et al., 2007). One of which is *Auricularia* sp., commonly known as wood ear or *tainga ng daga* in the Philippines. This mushroom is being utilized by many people around the globe for many of its uses, but mainly for food and medicine (Kirby, 2003).

As the mentioned crisis continues to rise, scientists are setting their sights on other potential biochemical substances to solve such predicament. One of the most promising biochemical substances that are studied extensively by scientists is chitosan. Chitosan is a derivative of chitin, a fundamental component of cell walls of fungi and can be found on exoskeleton of many crustaceans, arthropods and mollusks. Many studies that conducted have revealed that chitosan from crustaceans exhibit antimicrobial activity (Goy et al., 2009, Kong et al., 2010). Furthermore, has numbers of applications in various industries. It sparked the interest of many scientists because of its many applications in medicine, biology, chemistry, and many industries and businesses. Therefore, this study aimed to evaluate the antibacterial activity of extracted chitosan from *Auricularia* sp. and commercial chitosan against selected bacteria.

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2. Materials & methods

2.1. Bacterial strains

The test organisms *Staphylococcus aureus* ITDI 0004 and *Escherichia coli* ITDI 0078 were obtained from the Industrial Technology Development Institute (ITDI) Culture Collection of the Department of Science and Technology.

2.2. Collection and culture preparation of Auricularia sp

Fresh samples of *Auricularia* sp. fruiting bodies were collected at the oval of the Polytechnic University of the Philippines, Manila Campus. Identification of mushroom samples were verified by the Bureau of Plant Industry, Manila. The cap of the mushroom undergone surface sterilization and was cut into small pieces. The tissues were inoculated on Potato Dextrose Agar and incubated for 5 days at room temperature. The cultured mycelia were then inoculated in 100 mL Potato Dextrose Yeast Broth (PDYB) and were incubated at 30 °C for 7 days at 150 rpm in an orbital shaking incubator (Orbital Shaker Yih Der LM-590R).

2.3. Extraction and purification of chitosan

The fungal mycelia were filtered, washed twice with sterile distilled water, and then dried in oven at 60 °C for 5 h. The dried mycelia were deproteinized using 4 M NaOH (1:20 w/v) with constant stirring at 100 °C for 2 h. After heating, the supernatant was discarded, and the chitin were filtered, and washed four times using sterile distilled water. The deproteinization process was done twice and the material was finally dried in a dry oven at 50 °C for 3 h. For the deacetylation process, the chitin was placed in 50% NaOH solution (1:10 w/v) at 90 °C, with constant stirring for 3 h. It was filtered, and the supernatant was removed. It was once again stirred in 50% NaOH solution (1:15 w/v) at 120 °C for 2 h. It was filtered and washed with sterile distilled water until pH 7.0 is reached. The extracted chitosan was now dried in an oven at 50 °C for 2 h (Ospina et al., 2015).

Extracted chitosan was purified by dissolving in 1% acetic acid. Insoluble materials were removed through filtration. The pH of the filtered solution was then adjusted into pH 10 for the dissolved chitosan to precipitate and undergone centrifugation afterwards. The precipitated chitosan was collected, and supernatant was discarded. The pellets were rinsed with sterile distilled water until pH 7.0 was achieved. The chitosan was oven-dried and was put in a clean and dry container. The purified chitosan was then dissolved in 1% acetic acid to obtain a starting concentration of 1 mg/mL.

Commercial chitosan used in this study was purchased from Qingdao Greentech Biochemicals Trading Co. Ltd., China. It was purified through the procedure mentioned above.

2.4. Solubility testing

The solubility of both commercial and extracted chitosan from *Auricularia* sp. was tested at different concentrations of Acetic Acid: 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.25%, 1.5%, 2%, 5%. One gram of chitosan was dissolved in 100 mL of acetic acid solution using vortex (Wu et al., 2005).

2.5. Percentage yield

The percentage yield of chitosan extracted from *Auricularia* sp. was obtained using this formula:

$$\% \quad Yield = \frac{Cf}{Ci} x100$$

Where, Cf = dry weight of extracted chitosan, Ci = dry weight of mycelia.

2.6. Fourier Transform Infrared (FT-IR) analysis

The FT-IR analyses of both extracted chitosan and commercial chitosan were done to verify whether the both chitosan possess similar chemical components. The analysis was conducted in De La Salle University, Manila. The results of the extracted chitosan and commercial chitosan were compared, and the degrees of deacetylation (DD) were determined using the equation by Zhong et al. (2007):

$$DD(\%) = \left(\frac{1-(A1655/A3450)}{1.33}\right) x100$$

Where A1655 = mean % absorbance before and after wavenumber 1655 (i.e. 1650, 1660). A3450 = mean % absorbance of wavenumber A3450.

2.7. Minimum Inhibitory Concentration (MIC)

One hundred microliters of prepared concentrations of extracted and commercial chitosan were dispensed in the well of microtiter plates. Afterwards, 100 μL of standardized bacterial suspension was inoculated on each well. Streptomycin sulfate was used as a positive control, 1% acetic acid served as the negative control, and Mueller-Hinton broth with only inoculum for blank control. The plates were incubated at 37 °C for 24 h. Presence of pellets on the bottom of the wells indicates bacterial growth. The lowest concentration that showed growth inhibition was the MIC (Guzman and Padilla, 2017).

2.8. Minimum Bactericidal Concentration (MBC)

To avoid bias, the wells used in MIC were used to determine the MBC. Each well was homogenized and streaked on MHA. Afterwards, the plates were incubated at 37 °C for 24 h. The presence of at least a single colony confirms the results of the MIC. The lowest concentration where no growth was formed was the MBC (Guzman and Padilla, 2017).

2.9. Agar well diffusion assay

A uniform amount of inoculum was prepared using 24-h old cultures inoculated in Mueller-Hinton Broth (MHB). The turbidities of the inoculum were compared using 0.5 McFarland Standard and was adjusted accordingly to obtain a concentration of 1.5×10^8 CFU/mL bacterial suspension. Test bacteria were inoculated onto Mueller Hinton Agar (MHA) plates and wells were created using a 6 mm sterile cork borer.

Different chitosan concentrations were prepared through two-fold serial dilution. One hundred microliters of each chitosan concentrations were dispensed in the wells. The plates were incubated at 37 °C for 24 h. Similar procedure was done to test the antibacterial activity of commercial chitosan. Streptomycin sulfate and 1% acetic acid served as the positive and negative control, respectively. The zones of inhibition (ZOI) were measured in millimeters (mm) using Vernier caliper.

3. Results and discussion

3.1. Percentage yield of extracted chitosan

A percentage yield of 5.81% was obtained from the chitosan extracted from the fruiting bodies of *Auricularia* sp. This is higher than the yield obtained from previous studies using the mushroom species *Lentinus edodes, and Pleurotus sajo-caju,* with percentage yields of 3.3% and 1.2%, respectively. The percentage yield obtained from *Auricularia* sp. was also higher than the pathogenic yeast *Candida albicans*. However, the results in tis study was inferior with the yield obtained from the pathogenic molds, *Rhizopus oryzae* with 14% yield, and *Aspergillus niger* with 11% yield (Pochanavanich, 2002).

Table 1
Solubility of Chitosan in Varying Concentrations of Acetic Acid.

Concentration	Commercial chitosan	Extracted chitosan
5%	+	+
3%	+	+
2%	+	+
1.5%	+	+
1.25%	+	+
1%	+	+
0.9%	–	–
0.8%	–	–
0.7%	–	–
0.6%	–	–
0.5%	–	–

(+) Soluble; (-) Insoluble.

These results show that *Auricularia* sp. yielded a higher amount of extracted chitosan than the other mushroom species, signifying its higher efficiency as a source of chitosan. Moreover, despite the lower percentage yield in comparison with the other fungal sources, the non-pathogenicity and edibility of *Auricularia* sp. makes it a preferable source of natural products such as chitosan.

3.2. Solubility testing for commercial and extracted chitosan

Table 1 shows the solubility of both commercial and extracted chitosan on acetic acid with different concentrations. The results show that both chitosan were soluble from 5% to 1% acetic acid concentrations, but were insoluble at concentrations lower than 1%. Hence, the chitosan used in the succeeding assays were dissolved in 1% acetic acid. Previous studies used 1% acetic acid despite its antibacterial activity (No et al., 2002; Wu et al., 2005; Romanazzi et al., 2009).

3.3. FT-IR analyses of commercial and extracted chitosan

Fig. 2 shows the spectra of both extracted and commercial chitosan using the FT-IR analysis, which indicates the noticeable similarities in peaks between the two. This verifies that the extracted chitosan is similar to the commercial chitosan.

On Figs. 3 and 4, the broad and wide wavelength on commercial and extracted chitosan (with a peak of 3421.28 and 3418.60, respectively) indicate the strong presence of hydroxyl group in both samples. Meanwhile, sharp peaks at 2917.97 $cm^{-1}$and 2875.39 cm^{-1}, and 2919.75 cm^{-1} and 2856.27 cm^{-1} of both commercial and extracted chitosan indicate strong presence of alkanes in the said samples which support the correct chemical structure of chitosan (Fig. 1) that is mostly made of C-C single bonds. It is supported by the C-H stretching that corresponds high alkane signal. Meanwhile, on the sharp peak 1601.01 cm^{-1}, it shows the N-H bend. This medium signal implies the presence of primary amines. In addition, the N-H rocking on the fingerprint region of the IR graph that is found on peaks 833.11 cm^{-1} and 723.05 cm^{-1} of commercial and extracted chitosan signifies the presence of primary or secondary amines. There is also a sharp but weak presence of C-N stretch in the peaks 1256.95 cm^{-1} and 1252.20 cm^{-1}

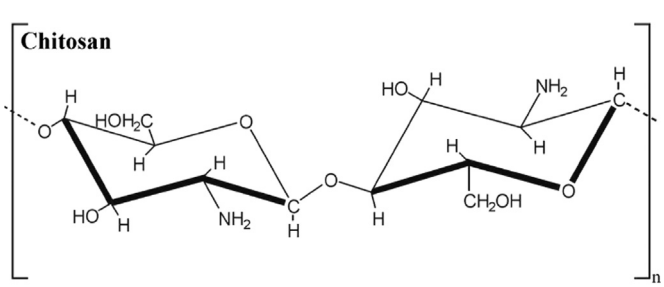

Fig. 1. Chemical Structure of Chitosan (López-García et al., 2014).

of commercial and extracted chitosan, respectively. It indicates that there is a C-N bond. Lastly, the strong presence of ether part in the chitosan is seen in sharp peaks 1079.09 cm^{-1} in commercial and 1078.72 cm^{-1} in extracted chitosan.

Table 2 shows the tabulated summary of the IR of both Commercial and Extracted Chitosan.

Meanwhile, computations showed that the degree of deacetylation (DD) of commercial chitosan is 83.66%, and 86.81% on extracted chitosan, indicating that the extracted chitosan is more deacetylated than the commercial chitosan.

3.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The results of the two-fold dilution assay in *E. coli* showed that the 250 μg/mL of extracted chitosan and 500 μg/mL concentration of commercial chitosan are the MIC because of the absence of white pellets at the bottom of the wells, an indication of growth inhibition. On the other hand, 125 μg/mL of extracted chitosan and 250 μg/mL of commercial chitosan in *S. aureus* do not show indication of growth, making them the MIC (Table 3).

The results show that the MBC for the extracted chitosan in E. coli is 500 μg/mL and 1000 μg/mL for the commercial chitosan since these concentrations did not exhibit any bacterial growth. Moreover, the extracted chitosan was able to yield no growth at 250 μg/mL and the commercial chitosan at 500 μg/mL for S. aureus. (Table 4)

Extracted chitosan was able to eradicate E. coli and S. aureus at lower concentrations than the commercial chitosan, which signifies its greater bactericidal activities.

3.5. Agar well diffusion assay

The results of the agar well diffusion assay confirmed the observations made from the preceding antibacterial assays that both extracted and commercial chitosan were able to inhibit *E. coli* and *S. aureus*. Zones of inhibition (ZOI) observed also imply the concentration-dependent antibacterial activities exhibited by both chitosan against the test organisms, wherein the diameters of the zones of inhibition is directly proportional to the concentration of the chitosan (Fig. 5).

Fig. 6 shows that the concentration that yielded the highest activity (45.83 mm) for extracted chitosan against *E. coli* was 1000 μg/mL. However, there was no significant difference between its activity and that of the 500 μg/mL with 40.83 mm ($p = 0.057$). On the other hand, the highest concentration of 1000 μg/mL of commercial chitosan yielded the highest antibacterial activity among the tested concentrations with 45 mm ($p = 0.006$). These results show that for extracted chitosan, the best concentration was 500 μg/mL, whereas 1000 μg/mL was the best concentration for commercial chitosan. These indicate that the extracted chitosan exhibits greater antibacterial activity than the commercial chitosan since it was able to yield a significant inhibitory activity at a lower concentration.

Moreover, the results of agar well diffusion assay using *S. aureus* showed that the highest concentrations of both extracted and commercial chitosan were the most effective with zones of inhibition of 66.17 mm ($p = 0.022$) and 62.83 mm ($p = 0.002$), respectively (Fig. 7). However, the extracted chitosan was able to yield a greater antibacterial activity against *S. aureus* as compared with commercial chitosan.

The observed greater zones of inhibition of extracted chitosan compared with commercial chitosan against both *E. coli* and *S. aureus* implies that the extracted chitosan is more effective than the commercial chitosan in inhibiting Gram-positive and Gram-negative bacteria. This may be due to the higher deacetylation degree of the extracted chitosan (86.81%) than the commercial one (83.66%) (Kong et al., 2010; Younes et al., 2014). The antibacterial property of chitosan has been implied to be dependent on the interaction of positive charge

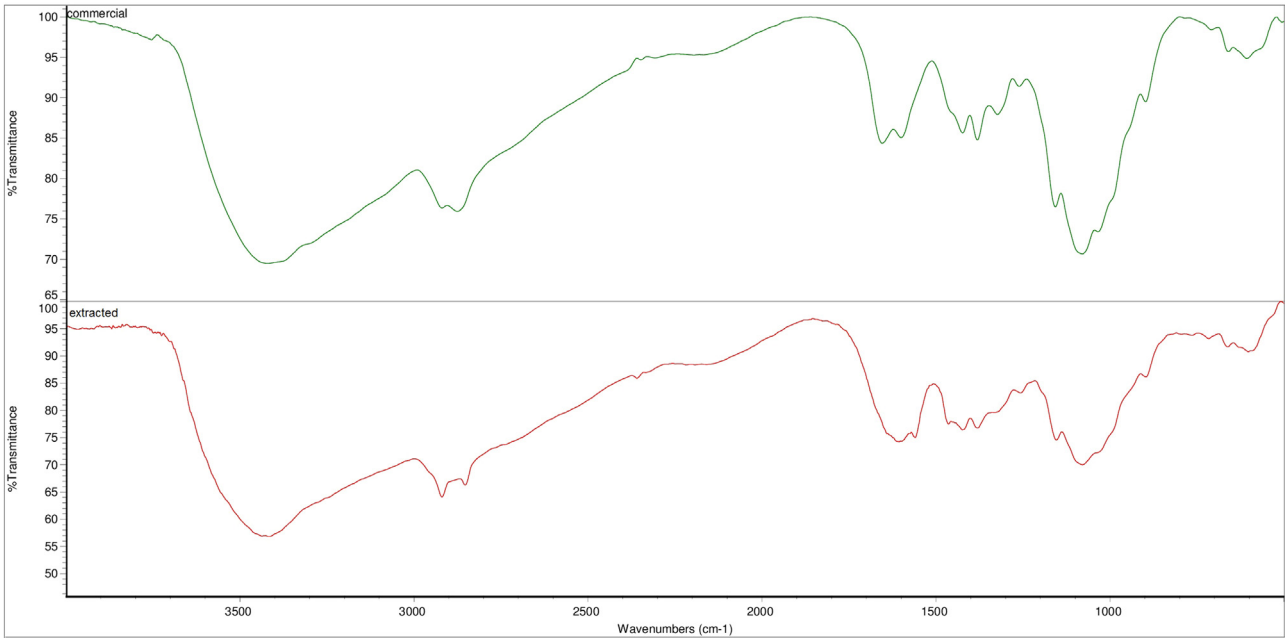

Fig. 2. Stack Spectra of Commercial and Extracted Chitosan.

with negatively charged membranes, making them dependent on the degree of deacetylation. Chitosan with higher DD are higher in positive charges and is expected to strongly inhibit both Gram-positive and Gram-negative bacteria.

Furthermore, it was observed that *S. aureus* is more susceptible to both chitosan than *E. coli* (Fig. 6 and 7). The degree of susceptibility of *S. aureus* and *E. coli* on both chitosan has something to do with the composition of their cell wall and electrostatic interaction between the

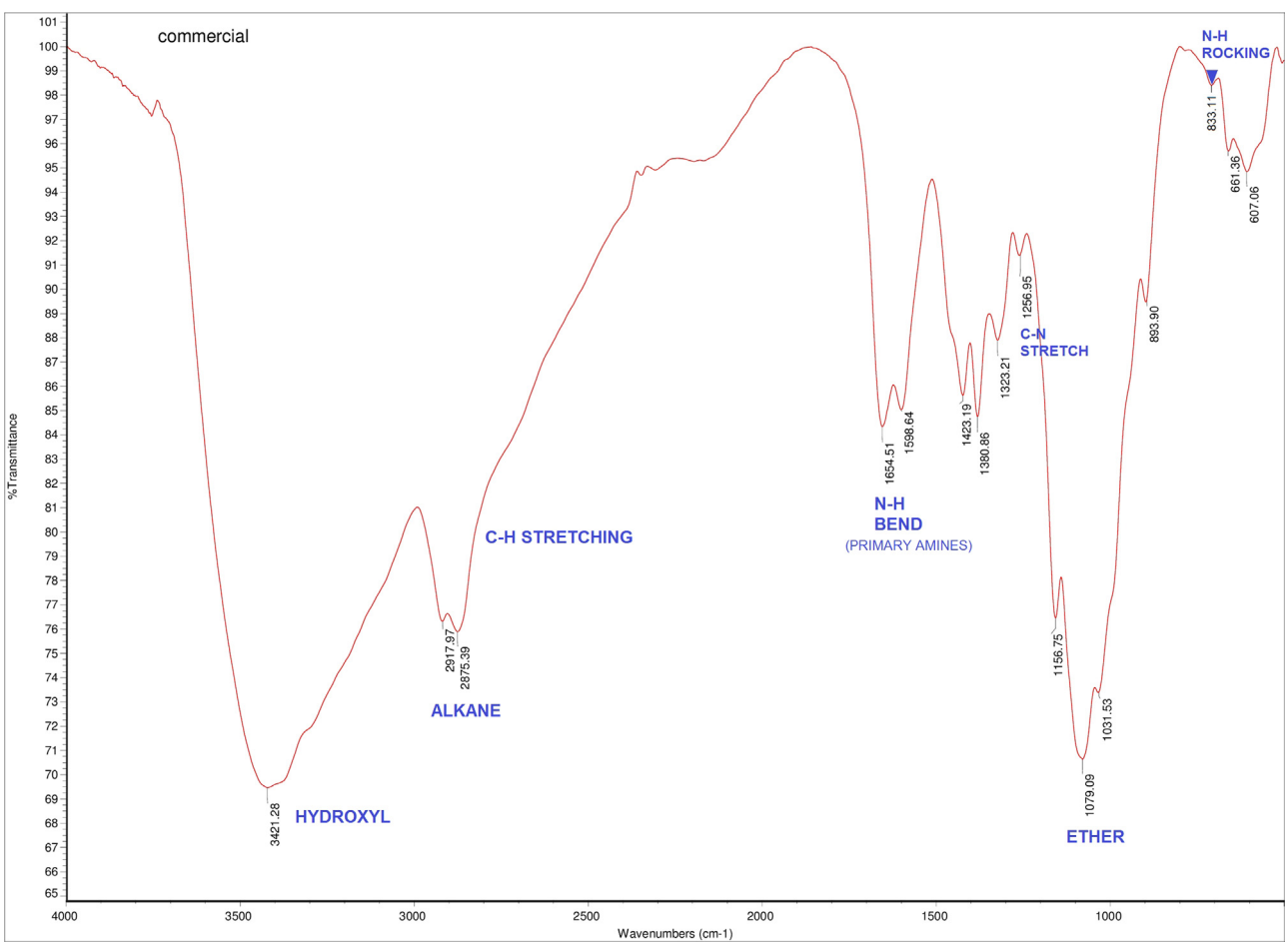

Fig. 3. IR Spectra of Commercial Chitosan.

Fig. 4. IR Spectra of Extracted Chitosan.

Table 2
Functional groups, peaks, intensity and shape present in chitosan.

IR wavelength peak (Commercial)	IR wavelength peak (Extracted)	Functional group	Intensity and shape
3421.28	3418.60	-OH (Hydroxyl)	Broad, wide, strong
2917.97, 2875.39	2919.75, 2856.27	C-C (Alkane)	Sharp, strong
1659.51	1601.01	N-H (Primary amine)	Sharp, medium
1256.95	1252.20	C-N Stretch	Sharp, weak
1079.09	1078.72	C-O-C (Ether)	Sharp, strong

Table 3
Minimum Inhibitory Concentrations of Commercial and extracted chitosan.

Concentration	Commercial chitosan		Extracted chitosan	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
125 µg/mL	+	+	+	–
250 µg/mL	+	–	–	–
500 µg/mL	–	–	–	–
1000 µg/mL	–	–	–	–
Positive	–			
Negative	+			

(+) With growth; (-) No growth.

Table 4
Minimum Bactericidal Concentrations of Commercial and Extracted Chitosan.

Concentration	Commercial Chitosan		Extracted Chitosan	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
125 µg/mL	+	+	+	+
250 µg/mL	+	+	+	–
500 µg/mL	+	–	–	–
1000 µg/mL	–	–	–	–
(+) Control	–			
(-) Control	+			

(+) With growth; (-) No growth.

polycationic structure and anionic components of their cell surface. Gram-positive bacteria contain teichoic acids on their cell surface which are covalently attached to the peptidoglycan responsible for making the whole cell surface negatively-charged. It is essential for cell division and other fundamental aspects of the Gram-positive bacterial physiology. Chitosan, a natural cationic polymer, binds non-covalently with teichoic acids incorporated in the peptidoglycan layer which disrupts the teichoic acids as well as altering the function of the peptidoglycan, despite of its thickness. On the study made by Raafat et al. (2008), they compared the antibacterial activity of chitosan against wild type *S. aureus* and teichoic acid deletion mutant *S. aureus*. Mutant *S. aureus* did not undergo cell wall lysis because of the less negatively

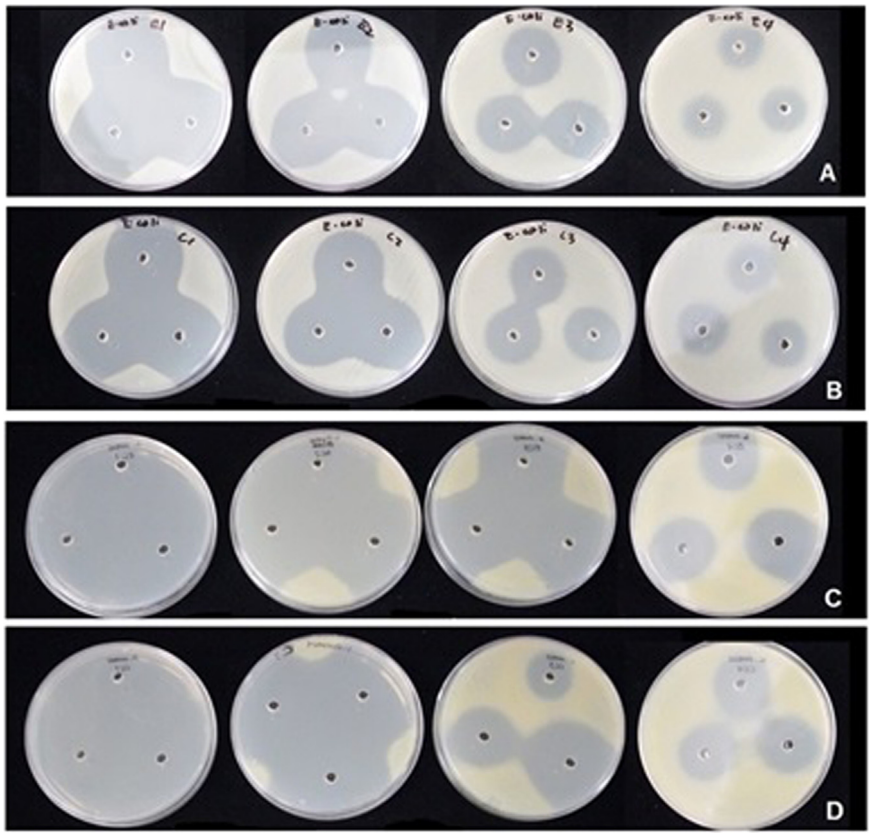

Fig. 5. Agar well diffusion assay of extracted (A, C) and commercial (B, D) chitosan against *E. coli* (A, B) and *S. aureus* (C, D).

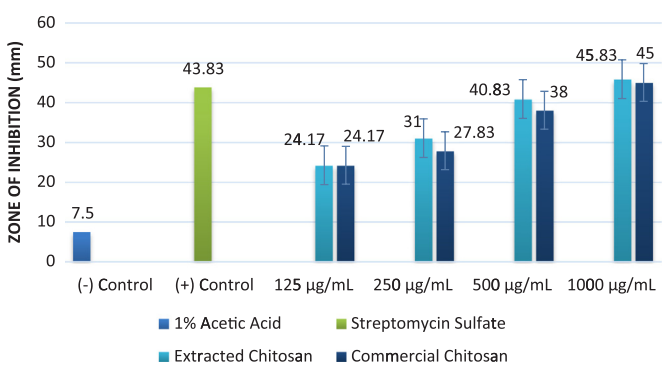

Fig. 6. Antibacterial activity of the positive and negative controls, and extracted and commercial chitosan against *E. coli*.

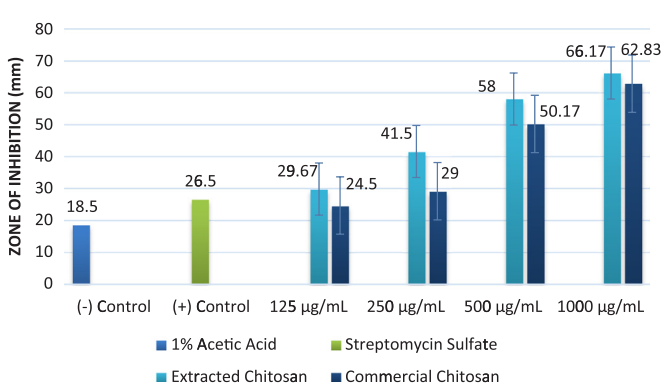

Fig. 7. Antibacterial Activity of the Positive and Negative Controls, and Extracted and Commercial Chitosan against *S. aureus*.

charged cell walls, making the polycationic nature of chitosan a major factor contributing to its antibacterial action (Tachaboonyakitat, 2017), thus, indicating that the primary mode of action is related to electrostatic interactions of chitosan with teichoic acids that could lead to disruption different functions, resulting in cell death (Verlee et al., 2017). The cell wall of Gram-negative bacteria, on the other hand, is more complex yet thinner than Gram-positive bacteria. It contains a semi-permeable outer membrane lying over a peptidoglycan layer that inhibits the entry of some antibiotics into the cell. It is an asymmetric lipid bilayer that contains lipopolysaccharide (LPS) with glycolipid as its main component. Chitosan also interacts with Gram-negative bacteria through electrostatic interaction with the negative charges of LPS that result in changes in permeability. The uptake of 1-N-phenylnaphthylamine then increases, indicating that the membrane is already damaged. Hence, further binding of chitosan on outer membrane makes the barrier lose its function. However, electrostatic interactions of chitosan with LPS on Gram-negative bacteria do not significantly affect the dynamics of cytoplasmic membrane since chitosan is bounded to the outer membrane, making the peptidoglycan and periplasmic membrane less unaffected and the whole envelope intact. Therefore, Gram-positive bacteria are more sensitive and susceptible to the antibacterial mechanism of chitosan than Gram-negative bacteria (Tachaboonyakitat, 2017) as observed with the results of this study showing the greater antibacterial activities from both extracted and commercial chitosan against Gram-positive *S. aureus* than the Gram-negative *E. coli*.

The activities of both chitosan against both test organisms were also compared with the activity of the positive control, Streptomycin sulfate (Figs. 6 and 7). It was revealed that the activities of both extracted ($p = 0.315$) and commercial chitosan ($p = 0.550$) are significantly similar with the activity of Streptomycin sulfate against *E. coli*, with a mean diameter of 43.83 mm. This implies that the activities of both extracted and commercial chitosan are comparable with Streptomycin

sulfate against *E. coli*. However, there is a significant difference between the activities of extracted ($p = 0.000$) and commercial chitosan ($p = 0.000$), and the positive control against *S. aureus*, with a mean diameter of 26.5 mm. The results suggest that the extracted and commercial chitosan are more effective in inhibiting the growth of *S. aureus* than Streptomycin sulfate. Results also showed that the negative control, 1% acetic acid, exhibited antibacterial activities against *E. coli* and *S. aureus*. Nonetheless, the activity of the negative control is significantly different compared with the activities of both extracted ($p = 0.000$) and commercial chitosan ($p = 0.000$) on both test bacteria. Therefore, it is safe to assume that the chitosan extracted from *Auricularia* sp. has better antibacterial activity than the commercial chitosan and Streptomycin.

The results signify the potential use of chitosan extracted from *Auricularia* sp. in inhibiting the growth of *E. coli* and *S. aureus*. The greater antibacterial activities that were observed as compared to the commercial chitosan as well as with the controls indicate the possibility of developing this chitosan as a more effective antibacterial substance sourced from mushroom species.

4. Conclusion

Both the chitosan extracted from *Auricularia* sp. and commercial chitosan exhibit concentration-dependent antibacterial activities against both *S. aureus* and *E. coli.* Both extracted and commercial chitosan are also more effective than the positive control which is Streptomycin sulfate. Furthermore, it was observed that both chitosan exhibit greater activities against *S. aureus* than *E. coli* due to the composition of cell walls of the organisms. Moreover, the results showed that the extracted chitosan is more effective in inhibiting the growth of both Gram-positive and Gram-negative bacteria than the commercially available chitosan due to its higher deacetylation degree.

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Declaration of interest

None.

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