Malays. Appl. Biol. (2022) 51(4): 103-108
https://doi.org/10.55230/mabjournal.v51i4.17

ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF CUTTLEBONE CHITOSAN AGAINST *Escherichia coli, Staphylococcus aureus* AND *Candida albicans*

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Accepted 17 October 2022, Published online 31 October 2022

ABSTRACT

The discovery of new antibiotics for infectious diseases has become challenging due to the rise of antimicrobial resistance. Meanwhile, chitosan has been considerably used in many branches of research. It has been discovered to have some good benefits in medicals, pharmaceuticals, and food technologies. In this study, chitosan was prepared from the cuttlebone of *Sepia* sp. by chemical method and analyzed by using FT-IR spectrophotometer for the confirmed presence of its functional groups. There are three types of reactive functional groups in the chitosan which are the amino group and primary and secondary hydroxyl group attached to the C-2, C-3, and C-6 positions respectively. Chitosan has a high cationic property due to the presence of its amino group. The bacteriostatic activity of chitosan occurs due to its positive charge in acidic concentration that interacts with the negatively charged residue of carbohydrates, lipids, and proteins located on the cell surface of bacteria. The antioxidant activity was conducted using DPPH radical scavenging assay with a chitosan concentration ranging from 0.1 to 10 mg/mL and a hydrogen peroxide scavenging assay with a chitosan concentration ranging from 0.1 to 1.6 mg/mL. The antimicrobial activity of chitosan from cuttlebone was analyzed against two different bacterial strains (*Escherichia coli* & *Staphylococcus aureus*) and a fungal strain, *Candida albicans* by disc diffusion and minimum inhibitory concentration (MIC) method. The results show that through the DPPH radical assay, the scavenging activity was 59.7% at the concentration of chitosan at 10mg/mL, while through the hydrogen peroxide assay the scavenging activity was 56% at the concentration of chitosan at 1.6 mg/mL. Besides, this chitosan from *Sepia* sp. has concentration-dependent antimicrobial activity with higher antifungal activity compared to antibacterial activity against all tested organisms and may become a potential agent for antibiotic discovery.

Key words: Antimicrobial activity, antioxidant, chitosan, cuttlefish

INTRODUCTION

Antibiotic has been used to treat many types of infectious diseases. The most concerning issue is involving the skin pathogen. Many antibiotics have been prescribed to patients with skin concerns to treat their skin issues. However, the prolonged usage of those antibiotics has led to the emergence of antibiotic resistance or antimicrobial resistance (AMR) (Haddad *et al*., 2018; Steffy *et al*., 2018). This phenomenon has encouraged researchers around the world to explore new possible sources of antibiotics which have less effect to be consumed or used by humans. In recent years, the marine environment has been looked up as a possible source of products for human or animal medicine (Biswas *et al*., 2016). Studies on antimicrobial compounds of marine invertebrates may give valuable information for new antibiotic discoveries and give new insights into bioactive compounds in mollusks. Among marine invertebrates, cephalopods are considerably important as a food resource as well as a source being considered in the discovery of new substances for drug development. Cuttlefish bone which has been commercially used as a dietary supplement for pet birds might become a new source of antibiotics. Besides, this cuttlefish bone has been traditionally used to treat some skin problems by folks. This bone has been identified to have a structural element of polysaccharide known as chitin. Chitin can be simplified to chitosan which is readily soluble in some inorganic and organic acids like acetic acid and hydrochloric acid. From previous research, chitosan has been identified to have an antimicrobial effect against some bacteria.

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However, there are few studies on the chitosan that has been prepared from cuttlefish bone in Malaysia. The purpose of this project is to prepare chitosan from cuttlebone chitin collected from Malaysia seashore by chemical method. Cuttlebone will be used as it has been identified to have the ability in the cut and bone healing process by previous research. Besides, it also can be found easily along the seaside and at restaurants as a waste. The chitosan will also be tested for its antioxidant, antimicrobial, and antifungal ability by disc diffusion and minimum inhibitory concentration method.

MATERIALS AND METHODS

Preparation and identification of chitosan

Cuttlefish, Sepia sp. was collected from Kuala Terengganu seashore. Cuttlebone was dissected out, weighed, and dried before being pulverized to be stored in powder form for preparation of chitosan (Khazri *et al*., 2016). The preparation of chitosan involved three main chemical methods which are deproteinization, demineralization, and deacetylation processes. Cuttlebone powder's sample was deproteinized with 2.0 M NaOH (1:16 w/v ratio) at room temperature (25 °C) for 48 h with pH ranging from 11-13. The solution was filtered and the sample residue was washed with distilled water until neutral pH (pH 6.5-8.0) is achieved. The sample residue (deproteinized product) was dried overnight at 40 °C. The deproteinized product was demineralized with 1.0 M HCl (1:16 w/v ratio) for 24 h at room temperature (25 °C) with pH ranging from 1.0-2.5. The solution was filtered and the deproteinized product was washed with distilled water until neutral pH (pH 6.5-8.0) is achieved. The washed deproteinized product was oven-dried at 80 °C until constant weight is obtained (Mahdy *et al*., 2013; Ahing *et al*., 2016). The obtained product (designated as cuttlebone powder chitin, PC) proceeded with the deacetylation process by using a concentrated base. The PC was treated in 50% (w/w) of NaOH (1:10 w/v solid: solvent ratio) at 90 °C for 2 h. The deacetylated PC was washed with distilled water and air-dried at room temperature. The product (later assumed as chitosan) was kept in a sterile and sealed container at 4 °C until further use. The chitosan was analyzed by using FT-IR spectrophotometer for the presence of its functional groups at specific wavenumber.

DPPH scavenging assay

To determine the antioxidant properties of chitosan, The DPPH scavenging assay was carried out. The sample ranging from 0.1 – 10 mg/mL in 0.2% acetic acid solution (4 mL) was mixed with 1 mL of DPPH-containing methanol solution becoming 10 mg/mL of DPPH solution. The solutions were mixed using a vortex-mixture and kept in a dim room for 30 min. The absorbance was measured at 517 nm against black with ascorbic acid (Vitamin C) as control. The DPPH radical fixation was calculated as the method used by Ramasamy *et al*. (2014) in Equation 1:

$$\text{Scavenging effect (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Hydrogen peroxide (H_2O_2) scavenging assay

By using a spectrophotometer, The H_2O_2 scavenging activity was determined at 230 nm. The samples in 0.2% ascorbic acid were added into an H_2O_2 solution that was prepared in a phosphate buffer of pH 7.4. The absorbance was measured at 230 nm against a blank solution with phosphate buffer without H_2O_2 and the standard compound butylated hydroxytoluene (BHT) at 230 nm. The percentage of hydrogen peroxide scavenging assay was calculated using Equation 2 based on Luan *et al*. (2018):

$$\text{Scavenging effect (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Antimicrobial assay

The antimicrobial activity of the chitosan was tested by using the disc diffusion method. The stock solution of chitosan was prepared in two different concentrations at 100 mg/mL and 50 mg/mL. A sterile antimicrobial disc was immersed in each concentration of a stock solution of chitosan. Tetracycline and fluconazole discs are used for both Nutrient agar and Potato Dextrose agar as positive control respectively. DMSO solution disc with 1% percentage was used as the negative control. The media with discs was incubated at 37 °C for 24 h and 72 h for bacterial and fungi plates respectively (Jackson *et al*. 2018). Nutrient broth dilution was used for the MIC assay. A concentration of 50 µL of nutrient broth was added into every well of 96 micro wells. The chitosan was diluted with 1% of DMSO at various concentrations of 100, 80, 60, 40 and 20 mg/mL. The first well was filled with each 50 µL of chitosan with different concentrations. A concentration of 50 µL of broth nutrients containing *S. aureus*, *E. coli,* and *C. albican* was filled to the next wells. The plate was then incubated for 3 days and the turbidity of the solution was observed (Wan *et al*., 2018).

RESULTS AND DISCUSSION

FTIR analysis

The yield percentage of chitosan extracted from cuttlebone was 20%. The sample may be overly washed off during the wash-off process of chemical residue from the sample. The chitosan is analyzed by using an FT-IR spectrophotometer and the occurrence of the functional group related to the chitosan is labeled in Figure 1.

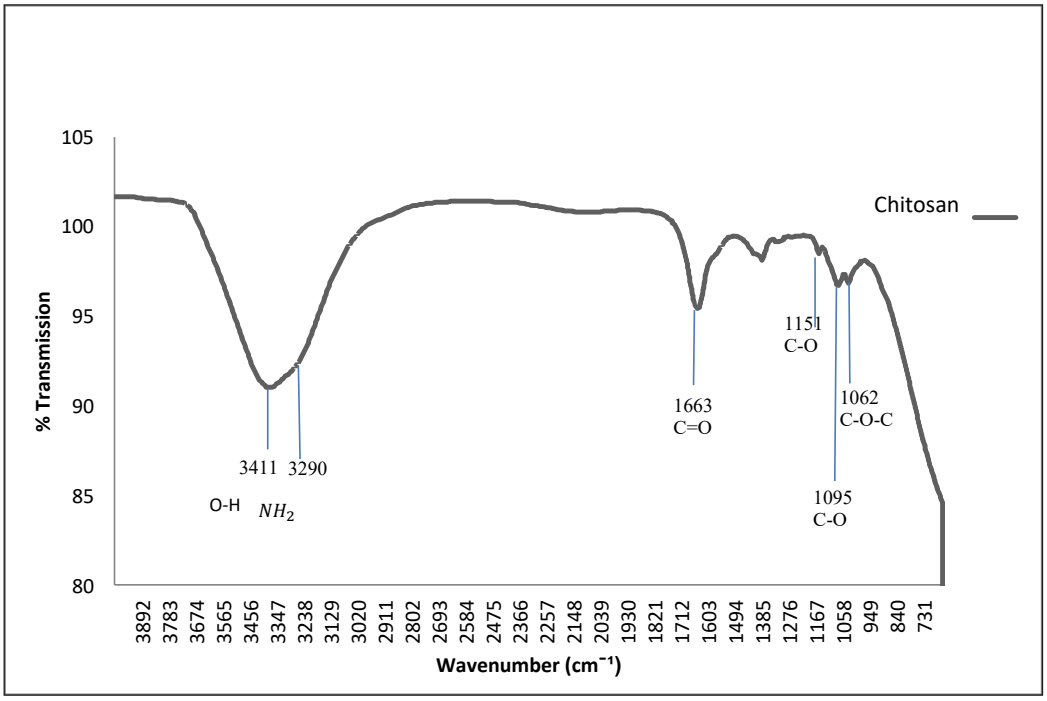

Fig. 1. FT-IR analysis of chitosan.

Based on the chemical structure of chitosan in Figure 1, The peaks between 3200 cm^{-1} and 3600 cm^{-1} are due to the O-H and N-H bonding (Vijayalakshmi *et al*., 2016; Nandana *et al*., 2021). This indicates the presence of alcohol and the doublet peaks are due to the –NH2-containing amide group. The peak at 1663 cm^{-1} is due to C=O, while the absorption peaks between 1200 - 800 cm^{-1} are due to the presence of C-O-C and C-O bonding (Fernandes *et al*., 2014; Vijayalakshmi *et al*., 2016; Yang *et al*., 2021).

DPPH scavenging assay

The scavenging activity (%) of different concentrations of chitosan and ascorbic acid was presented in Table 1. The scavenging activities of chitosan at different concentrations were ranging from 16.14% to 59.65% as the concentration increased. Although the percentage of the scavenging activity is not too high, there are close to the scavenging activity of control ascorbic acid which showed that this chitosan does have some antioxidant activity.

Hydrogen peroxide scavenging assay

Through a Hydrogen peroxide scavenging assay, we run the test with standard BHT. We could postulate that both the scavenging activity of chitosan and BHT increases as the concentration increases. The full results were portrayed in Table 2. However, our results are not too consistent as the scavenging activities of chitosan for the fourth concentration are higher than BHT but at the fifth concentration which is the highest concentration, 1.6 mg/mL, the percentage of scavenging activity of chitosan is slightly lower than the percentage of scavenging activity of BHT. This might be occurred due to some error in our preparation. However, we can conclude that our tested sample, chitosan, does have antioxidant activity when compared to the standard. The antioxidant activity of this chitosan might be present due to the existence of the amine group, NH_2, in the properties of chitosan. This important group encapsulates the free radicals substances around them (Kim, 2018).

Antimicrobial assay

As for the antimicrobial assay, the results showed that the chitosan does have a great antimicrobial and antifungal reaction against all tested microorganisms. The results are displayed in Table 3 and Table 4.

Table 3 and Table 4 showed that the antifungal effect of the chitosan against *C.albicans* is higher compared to the antibacterial effect of the chitosan against *S. aureus* and *E. coli*. Chitosan consists of an amine group which is responsible for its cationic property. When the positively charged chitosan in acidic conditions interacted with the negatively charged residues of carbohydrates, lipids, and proteins of the microorganisms, it will be resulting in the interference with bacterial energy metabolism and the electron transfer chain which will inhibit the growth of bacteria (Shih *et al*., 2021). Theoretically, gram-negative bacteria should be more susceptible to chitosan compared to gram-positive bacteria as gram-negative bacteria possess a more negative charge compared to gram-positive bacteria as it is

Table 1. The scavenging activity (%) of different concentrations of chitosan and ascorbic acid. Data presented as mean ± SEM. Different letters within the same column indicate significant differences (p<0.05)

Concentration (mg/mL)	Scavenging activity (%)	
	Chitosan	Ascorbic acid
0.1	16.14 ± 0.423^{a}	30.36 ± 3.083^{b}
0.5	30.79 ± 4.312^{b}	31.21 ± 2.871^{b}
1	36.52 ± 1.484^{b}	39.92 ± 1.813^{bc}
5	44.60 ± 0.979^{b}	45.01 ± 2.503^{c}
10	59.65 ± 4.988^{d}	76.10 ± 1.125^{a}

Table 2. The scavenging activity (%) of chitosan and BHT at different concentrations. Data are presented as mean ± SEM. Different letters within the same column indicate significant differences (p<0.05)

Concentration (mg/mL)	Scavenging activity (%)	
	Chitosan	BHT
0.1	38.03 ± 0.982^{a}	30.77 ± 1.726^{b}
0.2	34.71 ± 0.718^{a}	33.48 ± 1.267^{bd}
0.4	47.01 ± 1.481^{b}	44.44 ± 2.716^{c}
0.8	54.27 ± 2.219^{b}	41.45 ± 1.726^{cd}
1.6	55.98 ± 2.714^{b}	69.23 ± 1.236^{a}

Table 3. Antimicrobial and antifungal activity of various concentrations of chitosan prepared from cuttlebone

Species	Inhibition zone of chitosan prepared from cuttlebone (mm ± SD)		Positive control (Tetracyclin/ Fluconazole)	Negative control (1% DMSO)
	100 mg/mL	50 mg/mL		
S. aureus	13 ± 0.57	11 ± 0.57	23 ± 0.57	-
E. coli	12 ± 0.57	10 ± 0.57	21 ± 0.57	-
*C. albican*s	14 ± 0.57	12 ± 1.53	17 ± 0.57	-

mm=millimeter
SD=standard deviation

Table 4. MIC of chitosan prepared from cuttlebone against tested microorganism

Microorganism	Chitosan extract (%)				
	100	80	60	40	20
S. aureus	-	-	++	++	+++
E. coli	-	++	++	+++	+++
C. albicans	-	-	-	+	++

*MIC concentration; - No growth; + Cloudy solution (slightly growth); ++Turbid solution (strong growth); +++ Highly turbid (dense growth)

more enriched with lipopolysaccharides that are often attached to the phosphorylated group (Ke *et al*., 2021). However, from this study, the chitosan was more susceptible to *S. aureus* which is a gram-positive bacterium compared to *E. coli,* a gram-negative bacterium.

Data analysis

Both *S. aureus* and *E. coli* showed significance different at a concentration of 100 mg/mL and 50 mg/mL. However, there is no significant difference between 100 mg/mL and 50 mg/mL of concentration for *C. albican*. The data was tabulated in Table 5.

CONCLUSION

In a conclusion, chitosan isolated from cuttlebone was proven to have antioxidant activity and antifungal activity. Based on the results, the cuttlebone's chitosan showed antioxidant activity when conducted with a DPPH scavenging assay and hydrogen peroxide

Table 5. Mean values results

Species	Concentration	
	100 mg/mL	50 mg/mL
S. aureus	12.67 ± 0.577a	9.67 ± 0.577b
E. coli	12.00 ± 0.000a	9.67 ± 0.577b
C. albican	13.67 ± 0.577a	11.67 ± 1.528ab

scavenging assay. As the concentration of chitosan increased, the antioxidant activity was also improved. For the antifungal activity toward *S. aureus, E. coli,* and *C. albicans*, it was concluded that the concentration of chitosan was directly proportional to the antifungal activity. The cuttlebone's chitosan showed antioxidant activity when conducted with a DPPH scavenging assay and hydrogen peroxide scavenging assay. As the concentration of chitosan increased, the antioxidant activity was also improved. The antimicrobial and antioxidant ability of chitosan from cuttlebone could make it a new possible skin product in the future.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Fakulti Sains Gunaan Kampus Kuala Pilah for the facilities and equipment provided and big gratitude to the author's families for the financial support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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