Malaysian Journal of Chemistry, 2024, Vol. 26(1), 168-179
(Special Issue: *International Conference on Natural Products, ICNP 2023*)

Preliminary Study on Physicochemical and Biological Properties of Chitosan Extracted from Mud Crab (*Scylla olivacea*) Shells

Norhaliza Ahad[1], Awang Ahmad Sallehin Awang Husaini[1], Sumiyyah Sabar[2], and Wan Roslina Wan Yusof[2,3]*

[1]Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS), 94300 Kota Samarahan, Sarawak, Malaysia
[2]Chemical Sciences Programme, School of Distance Education (SDE), Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[3]Centre for Pre-University Studies, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
*Corresponding author (e-mail: wywroslina@unimas.my)

Chitosan, a non-toxic and biodegradable biopolymer, has been immensely exploited due to its inherent features, such as its ability to inhibit bacterial growth and free radical scavenging. This biopolymer can be extracted from crustaceans, molluscs, insects, or fungi chitin. Interestingly, different sources of this biopolymer affect the physicochemical and biological activities. Thus, this study aims to characterise, compare the physicochemical and biological properties of extracted chitosan (E.ch) from mud crabs, *Scylla olivacea* (*S. olivacea)*, and commercial chitosan (C.ch). The E.ch was extracted via the chemical method, and their physicochemical and biological properties, such as degree of deacetylation (DD), viscosity, antibacterial, antifungal, as well as antioxidant activity, were characterised. The results revealed that E.ch with high DD and low viscosity showed better antibacterial activity against *Pseudomonas aeruginosa* (*P. aeruginosa*), better antifungal activity against *Aspergillus flavus* (*A. flavus*) and high antioxidant activity compared to C.ch. The higher DD of E.ch contributed to better antibacterial, antifungal, and antioxidant activity. In conclusion, the findings of this study on the physicochemical and biological properties of E.ch from mud crabs could be utilised in various fields such as biomedical, pharmaceutical, and agriculture applications.

Keywords: Antibacterial; antifungal; antioxidant; biopolymer; chitosan; mud crab

Received: September 2023 ; Accepted: November 2023

The mud crab, scientifically known as *Scylla olivacea* (*S. olivacea*), is a crab species that commonly inhabits burrows within densely populated mangrove areas in the Indo-West Pacific region [1]. Mud crab is one of the most delicious seafood delicacies owing to its tender meat and flavourful taste. However, hard exoskeletons are waste because they are not consumable. Improper seafood waste management can lead to environmental pollution. The current study used mud crab shell waste to extract chitosan and produce functional materials. Chitosan is a linear polysaccharide consisting of 2-amino-2-deoxy-(1-4)-β-D-glucopyranose residues (D-glucosamine units) [2]. Several methods have been used to extract chitosan, including chemical and biological extraction [2]. The chemical extraction method used in this study involved four main steps: demineralization, deproteinization, discolouration, and deacetylation. Acid treatment is applied in demineralization to remove calcium carbonate, whereas deproteinization involves alkali treatment to remove proteins from mud crab shells [3]. The discolouration step is applied to remove the colour pigment and obtain a white appearance on the sample [4]. Deacetylation uses alkali treatment to remove the acetyl group from the chitin backbone to produce chitosan [5]. Chitosan has many applications in various fields, such as agriculture, pharmaceuticals, cosmetics, and food processing [6].

Their physicochemical and biological properties were characterised to determine whether mud crab chitosan could be used in future applications. The physicochemical and biological properties tested in this study were chitosan yield percentage, degree of deacetylation (DD), viscosity, morphology, antibacterial, antifungal, and antioxidant properties. Determining DD and viscosity is crucial because they influence chitosan's biological properties and applications [6]. The higher DD of chitosan indicates its high purity and a high cationic effect in biological applications [7]. Chitosan extracted from crab (*Carcinus mediterraneus*) shells can inhibit *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) but more effectively on *E. coli* [8]. In addition, the antifungal activity of crab chitosan extracted by Hajji et al. [8] was able to inhibit *Aspergillus niger* (*A. niger*) and *Fusarium solani* (*F. solani*) with the same zone of inhibition (17 mm).

†Paper presented at the International Conference on Natural Products 2023 (ICNP 2023)

Aspergillus flavus (*A. flavus*) is a pathogenic fungus that causes crop spoilage due to aflatoxin production [9]. Meanwhile, *Marasmius cladophyllus* (*M. cladophyllus*) can be a pathogenic fungus once changes occur with the tree or plant host [10]. Besides, a previous study reported that *Marasmius palmivorus* (*M. palmivorus*), which is in the same genus as *M. cladophyllus*, can be applied as a bioherbicide against pathogen fungi, but *M. palmivorus* also can cause palm bunch and stem rot disease of coconut palms [11,12]. Thus, *M. cladopyhllus* can become pathogenic depending on the environmental conditions, its interaction with the host plant, or the presence of endobacteria and mycoviruses that can influence fungal behaviour [13]. There are still few reports on the antifungal activity of chitosan extracted from mud crab shells (*S. olivacea*) (E.ch) against *A. flavus* and *M. cladophyllus*, so it is essential to investigate the potential antifungal activity against these fungi for future application in agriculture. In this study, a commercial chitosan (C.ch) was used as a reference to confirm that chitosan extracted from mud crab shells was successful. E.ch can have different physicochemical properties than C.ch, which can cause variations in its biological properties. Thus, the current study aims to characterise and compare the physicochemical and biological properties of E.ch with C.ch.

EXPERIMENTAL

Chemicals and Materials

Commercial chitosan from crabs with medium molecular weight (Mw) and 2,2-diphenylpicrylhydrazyl (DPPH) were purchased from Sigma Aldrich Corporation. Mud crab shells were obtained from a wet market in Kota Samarahan, Sarawak, Malaysia, to extract chitosan. *E. coli, S. aureus, P. aeruginosa, A. flavus*, and *M. cladophyllus* strains were subcultured and provided by Molecular Genetic Lab of the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS). All chemical reagents used in this study were used directly without further purification.

Sample Collection and Preparation

Mud crab shells were washed under running water to remove contaminants and adhering proteins. The mud crab shells were dried in an oven at 70°C until thoroughly dried [14]. The dried shells were crushed and ground into powder using a mortar and pestle. The mud crab shell was stored in a freezer at 4°C for long-term storage and dried in an oven at 70°C before chitosan extraction.

Chitosan Extraction

Mud crab shells undergo four steps: demineralization, deproteinization, discolouration, and deacetylation. In the demineralization steps, mud crab shells were treated with 7 % (v/v) hydrochloric acid (HCl) at a ratio of 1:10 (w/v) for 3 hours at 60°C [15]. The demineralized sample was washed with distilled water until it reached a pH of 7. Next, 2 % (w/v) potassium hydroxide (KOH) was used in the deproteinization steps at 90°C for 2 hours with a 1:20 (w/v) ratio [16]. The extracted chitin was washed with distilled water until it reached pH 7. Then, in the discolouration steps, the sample was treated with acetone repeatedly to obtain a white colour and dried in an oven overnight at 50°C after air-drying for two hours. Finally, deacetylation was conducted using sodium hydroxide (NaOH) at a concentration of 45 % (w/v) in a 1:20 (w/v) ratio for two hours at 110°C. The extracted chitosan was washed with distilled water until it reached pH 7. The final product was dried overnight in an oven at 50°C.

Percentage Yield (%)

The percentage yield of E.ch was calculated according to Equation (1) [17].

$$\text{Yield (\%)} = \frac{\text{Dry weight of chitosan (g)}}{\text{Dry weight of raw crab shell (g)}} \times 100 \tag{1}$$

Degree of Deacetylation (DD)

The DD of E.ch and C.ch was estimated using Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy (Agilent Technologies, Cary 630 FTIR spectrometer). The sample was analysed with an attenuated reflectance accessory from 650-4000 cm^{-1}. The degree of acetylation (DA) and DD of the samples were estimated using Equation (2) and Equation (3) [18,19]. A_{1320} and A_{1420} are the absorbances of the peaks at wavenumbers 1320 and 1420 cm^{-1}, respectively.

$$\text{DA (\%)} = \frac{\left(\frac{A_{1320}}{A_{1420}}\right) - 0.3822}{0.03133} \tag{2}$$

$$\text{DD (\%)} = 100\ \% - \text{DA (\%)} \tag{3}$$

Viscosity

E.ch and C.ch were diluted in 1 % acetic acid (Acac). The viscosity was determined using a Brookfield viscometer equipped with spindle number two at a speed of 50 rotations per minute (rpm) and a temperature of 25°C [16]. The value was expressed in millipascal-second (mPa.S).

Morphology

The morphologies of E.ch and C.ch were determined using a JSM-IT200 InTouchScope™ Scanning Electron

Microscope. According to Zhang et al. [20], platinum samples were coated with an ion sputter. The accelerating potential was 10 kilovolts (kV), and the magnification power was 50 to 10,000 times for image acquisition.

Preparation of Bacterial and Fungal Inoculum

The antimicrobial properties of E.ch and C.ch were tested against Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. coli*), and (*P. aeruginosa*) as well as two fungi (*A. flavus*) and (*M. cladophyllus*). The Gram-positive and Gram-negative bacteria were pre-cultured in Mueller Hinton broth (MHB) overnight in a rotary shaker at 37°C. Afterward, each strain was adjusted to 10^8 cells/mL concentration using 0.5 McFarland standard [21]. The fungal inocula were prepared from fungal isolates cultured for 48 hours in potato dextrose broth (PDB). A spectrophotometer (A_{595} nm) was used to adjust the spore density of the fungus to a final concentration of 10^6 spores/mL.

Determination of Zone of Inhibition

Mueller-Hinton Agar (MHA) was sterilised using an autoclave machine and poured into sterile plates for solidification. *S. aureus, E. coli*, and *P. aeruginosa* (10^5 CFU/mL) were swabbed on each MHA. Then, three paper disks were loaded with 0.5 mg/mL of ampicillin, 20 mg/mL of E.ch and C.ch. The fourth empty paper disk was used as a negative control. The bacterial plates were incubated overnight at 37°C, and the inhibition zones were determined 24 hours later. Ampicillin was used as a positive control [21]. The larger the inhibitory zones, the more potent the anti-bacterial activity [22].

Minimal Inhibitory Concentration (MIC)

E. coli, S. aureus, and *P. aeruginosa* were inoculated into three 10 mL nutrient broth solutions and incubated at 37°C with continuous shaking at 180 rpm for 16 hours to produce the broth culture. After 16 hours, 100 µL of each broth culture was transferred to 10 mL of nutrient broth and incubated for 2 hours under the same conditions. The turbidity of bacterial growth was measured until it reached 0.08 or 5×10^5 CFU per McFarland standard at optical density, OD_{600}.

Bacterial cultures were prepared by dilution to 1:100 or 1:200 [23]. First, 150 µL of MHB was transferred to five wells of a first-row 96-well microtiter plate. Next, 150 µL of E.ch or C.ch at a 4 mg/mL concentration was transferred to the first well for serial dilution. A two-fold dilution was conducted by taking 150 µL of mixture from the first well and transferred to the next well. The procedure was repeated until the final well. Finally, 50 µL of diluted bacterial culture was added to each well, and the final volume was 200 µL. Each well contained a final concentration of 2.0, 1.0, 0.5, 0.25, or 0.125 mg/mL.

The wells were incubated at 37°C with agitation at 180 rpm for 16-24 hours. After 24 hours, bacterial growth and sensitivity were measured at a wavelength of 600 nm using a Biotek Epoch 2 microplate reader [23]. Ampicillin was used as a positive control with each well final concentration of 0.50, 0.25, 0.125, 0.63, and 0.031 mg/mL [21]. MIC was the lowest concentration of a tested sample at which no bacterial growth was observed (transparent or translucent).

Minimal Bactericidal Concentration (MBC)

A volume of 1/10 of the bacterial culture was inoculated into new MHA from the MIC sample concentration to the highest concentration sample. The mixture was incubated for 24 hours at 37°C. Ampicillin was used as a positive control in the MBC. MBC was defined as the lowest concentration at which bacteria did not grow [23].

Mycelium Growth Measurement

The antifungal properties of E.ch and C.ch were tested against two fungi: *A. flavus* and *M. cladophyllus.* E.ch and C.ch were dispersed in acetic acid and distilled water at 6 mg/mL concentrations as stock solutions [22]. Potato Dextrose Agar (PDA) was prepared by sterilisation in an autoclave. Both E.ch and C.ch with different concentrations (0.08, 0.4, and 0.8 mg/mL) were introduced to a PDA that was already prepared. The mixture was then poured into a sterile petri dish (9 cm). After cooling the mixture, a 5.0 mm diameter fungus mycelium was placed on the test plate and cultured at 27°C for two to three days. Mancozeb was used as a positive control [21]. Once the mycelium reached the margins of the control plate (no treatment), the antifungal index was calculated using Equation (4). D_a indicates the diameter of the growth zone in the test plate, and D_b indicates the diameter of the growth zone in the control plate.

$$Antifungal\ index\ (\%) = \left(1 - \frac{D_a}{D_b}\right) \times 100 \qquad (4)$$

Scavenging of 1,1-Diphenyl-2-picrylhydrazyl Radicals (DPPH)

The DPPH scavenging capacity of E.ch and C.ch were measured using the method described by Si Trung and Bao [24] and Sun et al. [25] with slight modification. DPPH solution was prepared at 0.4 mM with ethanol. The reaction mixtures were prepared with 3 mL of E.ch or C.ch dissolved in 1 % acetic acid at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) with 1 mL of DPPH ethanol solution to a final volume of 4 mL. The mixture was rapidly agitated and maintained at room temperature for 30 min in a dark room. The absorbance was determined using a UV-VIS spectrophotometer (Shimadzu UV-2600i UV-

Visible spectrophotometer). Ascorbic acid was used as positive control. The free radical scavenging activity was calculated using Equation (5). Reduced absorbance indicates increased DPPH radical-scavenging activity [26]. A_0 refers to the absorbance of the reaction mixture without a sample, and A_1 is the absorbance of the reaction mixture with the sample at 517 nm.

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \qquad (5)$$

Statistical Analysis

Each experiment was performed thrice and the final data are expressed as the mean ± standard deviation. Statistical differences ($p<0.05$) were compared using one-way, two-way analysis of variance (ANOVA), and Student's t-test (t-test).

RESULTS AND DISCUSSION

Percentage Yield (%)

The percentage yield of E.ch obtained in this study was 6 % (Table 1). The yield percentage of E.ch was higher than chitosan extracted from crabs (*Carcinus mediterraneus*) and freshwater crabs (*Potamon algeriense*) [27,28]. However, E.ch yield percentage was lower than chitosan extracted from mud crab shell (39 %), mud crab (*Scylla olivacea*) shell (44.57 %), and king mangrove crab (*Scylla serrata*) (14 %) [7,29,16]. A high chitosan yield (39 %) was obtained because of the high chitin yield in the previous step of study reported by Ali et al. [7]. A low yield percentage of E.ch can be affected by acetyl group loss during the deacetylation process due to high alkaline concentration and temperature usage, which leads to chain degradation [30,31]. Cadano et al. [29] and Öğretmen et al. [32] also stated that excessive washing during demineralization can reduce the chitosan yield because chitosan is still soluble in weak acids and lost during washing. The differences in chitosan yield are influenced by the efficacy of the amount of mineral and protein removed [32].

Viscosity

The viscosity of E.ch and C.ch are listed in (Table 1). The viscosity of E.ch was 3.70 ± 0.09 mPa.S was found to be lower compared to C.ch with 469.33 ± 6.03 mPa.S. Van Den Broek and Boeriu [33] stated that the higher the molecular weight, the higher the viscosity. Nurhayati et al. [34] stated that an increase in the period of demineralization and deacetylation leads to a decrease in the viscosity of chitosan. Avelelas et al. [35] also reported that the viscosity of chitosan decreased due to prolonged exposure to NaOH for 120 min. The E.ch exposure period to NaOH was the same as Avelelas et al. [35], which explains the low viscosity. The viscosity of E.ch was low compared to the viscosity of chitosan extracted from snow crab leg (*Chionoecetes opilio*) shell (14.77 ± 0.12 mPa.S), blue crab (*Callinectes sapidus*) shell (362 ± 6.27 cP), and mud crab (*S. olivacea*) shell (383.9 cP) [30,36,16].

Fourier Transform Infra-Red (FTIR) Spectra and Degree of Deacetylation (DD)

The FTIR spectrum of E.ch were identical to the C.ch spectrum, as shown in (Figure 1), indicating that chitosan was successfully extracted from mud crab shells. C.ch and E.ch spectrum showed peaks at specific wavelengths, indicating chitosan: 3335-3350 cm^{-1} (O-H, stretching), 2873 cm^{-1} (C-H, stretching), 1641-1643 cm^{-1} (N-H, bending, amine), 1579 cm^{-1} (N-H, bending, amine), 1419 cm^{-1} (C-H, bending), and 1151 cm^{-1} (C-O, stretching). The FTIR spectrum of E.ch is similar to the FTIR spectrum of *S. olivacea* reported by Sarbon et al. [16] and Mulyani et al. [37].

Table 1. Physicochemical properties of E.ch and C.ch.

Characteristic	E.ch	C.ch
Yield (%)	6.00±1.73	-
DD (%)	81.00±0.58	80.00±0.00
Viscosity (mPa.S)	3.70±0.09	469.33±6.03

Values are presented as mean ± standard deviation in triplicate.
(-) indicates no data.

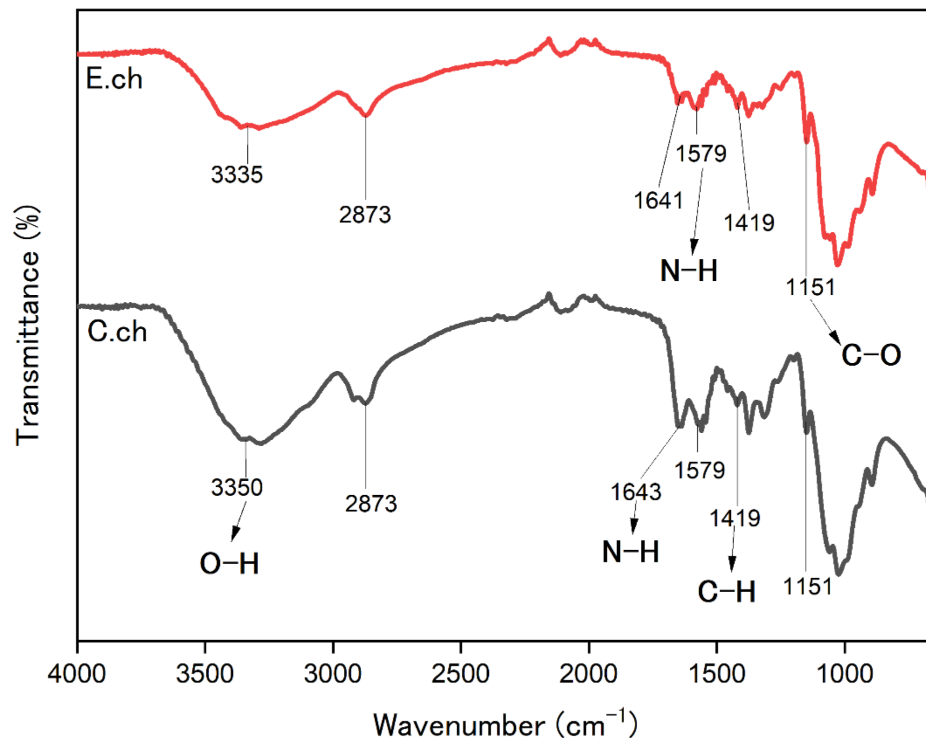


Figure 1. FTIR spectra of E.ch and C.ch.

The degree of deacetylation (DD) of E.ch (81 %) was higher than DD of C.ch (80 %), as shown in (Table 1). Interestingly, the DD of E.ch was identical to the DD of chitosan extracted from *S. olivacea* [37]. However, the DD of E.ch (81 %) was higher than DD of chitosan extracted from mud crab shells (53.4 %) by Sarbon et al. [16] but lower than DD of chitosan extracted from mud crab (92 %) by Ali et al. [7]. The difference in DD was caused by the different parameters used for extracting chitosan in the present study compared with the previous study, such as solvent concentration, temperature, and reaction period. The NaOH concentration and temperature used for deacetylation in the present study were 45 % and 110°C, respectively, compared with the 40 % NaOH and 105°C used by Sarbon et al. [16]. A high NaOH concentration and temperature are required to remove the acetyl groups [35,38]. In addition, demineralization in the present study involves heating, while Sarbon et al. [16] used only room temperature. The heating used in the demineralization process facilitates the diffusion of the solvent into the chitosan matrix and efficiently removes the mineral [39]. DD of chitosan extracted from mud crab by Ali et al. [7] was higher than DD of E.ch because of the usage of a high NaOH concentration (55 %). DD determination is vital because it influences chitosan's antibacterial, antifungal, and antioxidant properties.

Morphology

The Scanning electron microscopy technique (SEM) was used to observe the surface morphology of E.ch and C.ch. The SEM results are shown in (Figure 2) at different magnifications. At 50 times magnification, some surfaces of C.ch were smooth, with no pores or flaky particles. E.ch showed an irregular shape (50×, Figure 2a), rough surface (500×, Figure 2a), and tiny pores (2000×, Figure 2a). E.ch irregular shape was observed because of the flake forms [40,41]. E.ch results were supported by Arasukumar et al. [42], who reported the same morphology on chitosan extracted from lobster (*Thenus unimaculatus*) shells. Chitosan extracted from red snapper fish (*Lutjanus* sp.) scales and crab (*Portunus trituberculatus*) shells also showed irregular and rough layers [43,44]. However, chitosan extracted from insects such as cicada slough, silkworm chrysalis, and grasshoppers reported no porosity, with a compact surface structure different from E.ch [45]. It is hypothesised that the different morphologies of chitosan occur because of the different sources used to extract chitosan.

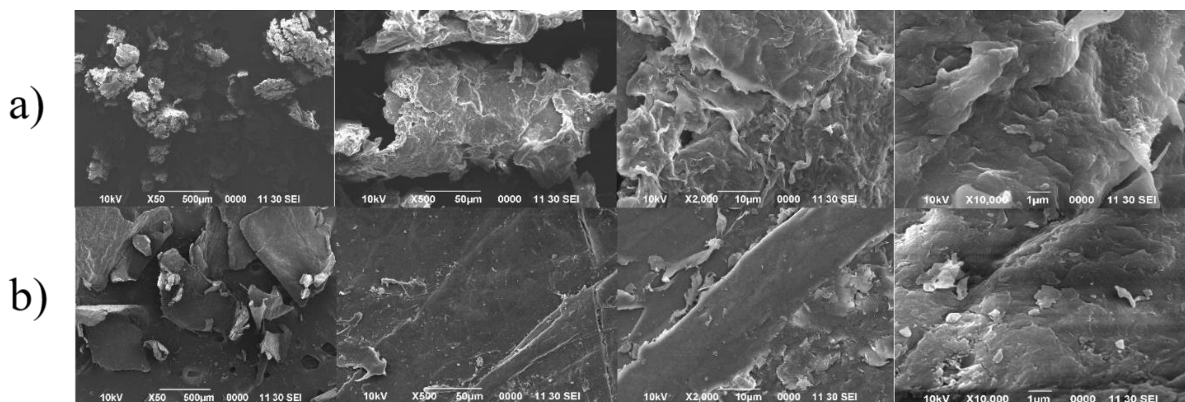


Figure 2. SEM analysis with magnifications 50×, 500×, 2000×, and 10000× from left to right for a) E.ch and b) C.ch.

Antibacterial Activity

E.ch showed low antibacterial activity against *E. coli, S. aureus,* and *P. aeruginosa* compared to ampicillin. However, E.ch displayed high antibacterial activity against *E. coli, S. aureus,* and *P. aeruginosa* compared to C.ch. The zones of inhibition (mm) of E.ch and C.ch are recorded in (Table 2) as the mean and standard deviation. The range of zone inhibition of 0.5 mg/mL ampicillin was measured from 23.3 ± 4.93 to 30.7 ± 3.21 (mm), while 20 mg/mL of E.ch zone of inhibition range in between 9.7 ± 0.58 and 10.0 ± 0.0 (mm). A 20 mg/mL of C.ch showed a zone of inhibition range from 8.00 ± 1.00 to 9.7 ± 0.58 (mm). The p-value was less than 0.05, indicating significant differences between the average zone inhibition by E.ch, C.ch, and ampicillin for those three types of bacteria.

Determination of MIC and MBC

The MIC values for E.ch and C.ch were the same (2 mg/mL) for all bacteria except for C.ch, which had no MIC value against *P. aeruginosa*. MIC value for ampicillin was 0.031 mg/mL against all bacteria. However, the MBC values for E.ch and C.ch against *E. coli, S. aureus*, and *P. aeruginosa* could not be determined in the 0.125 to 2 mg/mL range. This finding reveals that the MBC value of E.ch against *P. aeruginosa* might be higher than 2 mg/mL. Thus, the concentrations of E.ch and C.ch should be increased in future studies to determine the MBC of E.ch. Meanwhile, the MBC value of ampicillin against *E. coli* and *S. aureus* (0.032 mg/mL) were identical except for MBC value of ampicillin against *P. aeruginosa* (0.25 mg/mL). The MIC and MBC data are shown in (Table 3). From this result, E.ch and C.ch can only inhibit the bacteria but cannot kill them. Ampicillin still had the highest bactericidal effect against all those three types of bacteria. E.ch had better antibacterial activity against *P. aeruginosa* than C.ch because C.ch did not have an MIC value. In addition, the results from the disk diffusion test showed that E.ch had better antibacterial activity than C.ch, which could be attributed to the higher DD of E.ch compared to C.ch. Ke et al. [46] mentioned that the high DD of chitosan has a greater positive charge than chitosan with low DD. With chitosan's more significant positive charge, the electrostatic forces between chitosan and the negatively charged bacterial cell wall became stronger.

Other than that, Gram-negative bacteria (*P. aeruginosa*) are much more susceptible to E.ch than Gram-positive bacteria (*S. aureus*). Findings of the present study support the suggestion made by Ke et al. [46] that Gram-negative bacteria are more sensitive to chitosan compared to Gram-positive bacteria. The different effectiveness of chitosan against Gram-negative and Gram-positive bacteria is caused by their different cell membrane compositions, which leads to different antibacterial mechanisms.

Table 2. Antibacterial activity of E.ch and C.ch against *E. coli, S. aureus,* and *P. aeruginosa*.

Bacteria	Zone of inhibition (mm)		
	Ampicillin (0.5 mg/mL)	E.ch (20 mg/mL)	C.ch (20 mg/mL)
E. coli	23.3 ± 4.93^{a}	9.7 ± 0.58^{b}	8.0 ± 1.0^{c}
S. aureus	30.7 ± 3.21^{a}	9.7 ± 2.08^{b}	8.3 ± 1.53^{c}
P. aeruginosa	28.7 ± 6.11^{a}	10.0 ± 0.00^{b}	9.7 ± 0.58^{c}

Values are presented as mean ± standard deviation. Means with different letters within the row indicate ($p<0.05$) significant differences between the samples for each bacterium.

Table 3. MIC and MBC of E.ch, C.ch and ampicillin (mg/mL) against *E. coli, S. aureus,* and *P. aeruginosa.*

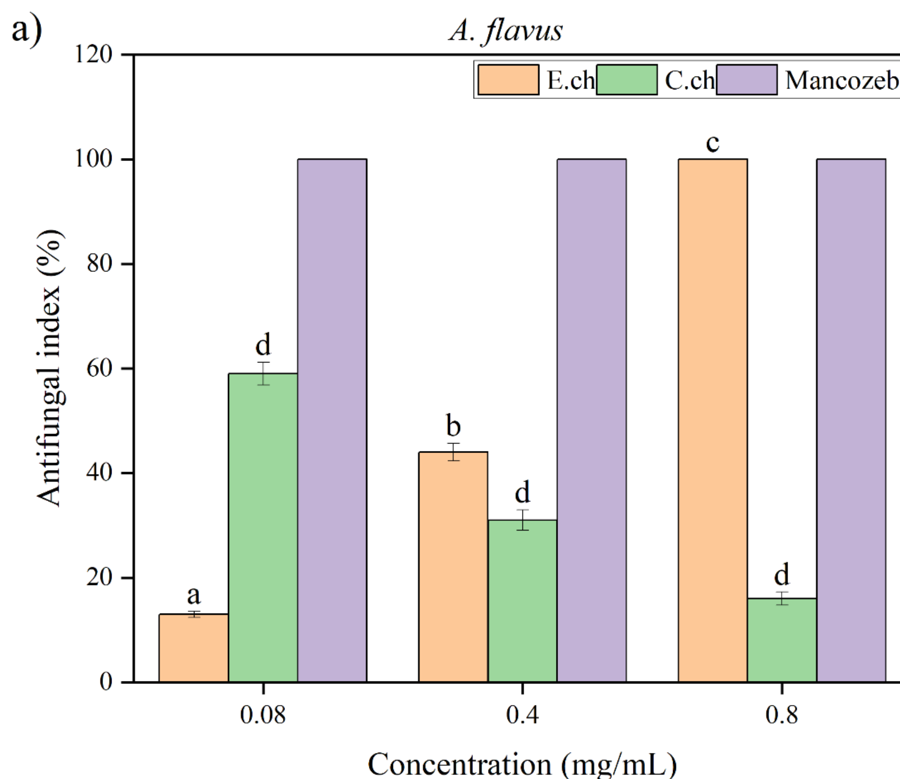
Bacteria	E.ch (mg/mL)		C.ch (mg/mL)		Ampicillin (mg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	2	-	2	-	0.031	0.031
S. aureus	2	-	2	-	0.031	0.031
P. aeruginosa	2	-	-	-	0.031	0.25

(-) indicates no MBC activity

When chitosan interacts with the negatively charged teichoic acid in the peptidoglycan of Gram-positive bacteria, the cell membrane can be damaged, and their intracellular leakage allows chitosan to invade the bacterial cell [47]. In Gram-negative bacteria, positively charged chitosan neutralises the negative charge of lipopolysaccharides, which can disrupt the outer membrane, allowing chitosan to enter the cell membrane and lead to cell death [47].

Antifungal Activity

The antifungal indices of E.ch and C.ch against *A. flavus* and *M. cladophyllus* are shown in (Figure 3). Mancozeb was used as a positive control, and an agar plate without any sample was used as a negative control. (Figure 3a) showed an increasing trend in E.ch antifungal index from 0.08 to 0.8 mg/mL, while C.ch showed a decreasing trend in the antifungal index from 0.08 to 0.8 mg/mL. In 0.8 mg/mL of E.ch, no growth of *A. flavus* was observed, indicating it has the highest antifungal index compared to 0.8 mg/mL of C.ch, which only showed a 16 % antifungal index. However, Mancozeb still had the highest antifungal activity due to the absence of *A. flavus* growth observed at all concentrations. Furthermore, there was a significant difference ($p<0.05$) between E.ch and C.ch at different concentrations against *A. flavus*. Based on (Figure 3b), E.ch, C.ch, and Mancozeb antifungal index against *M. cladophyllus* increased with increasing concentration. C.ch showed a higher antifungal index at all concentrations than E.ch. However, Mancozeb still possessed the highest antifungal index compared with E.ch and C.ch. Thus, there was a significant difference ($p<0.05$) between E.ch, C.ch, and Mancozeb against *M. cladophyllus*.



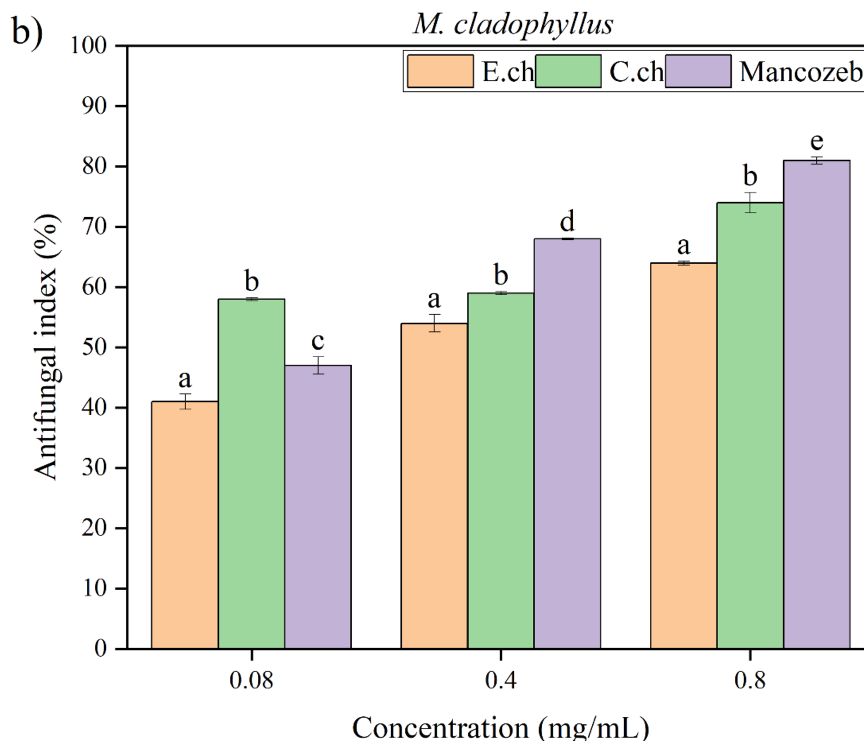


Figure 3. The effect of E.ch, C.ch, and Mancozeb with concentrations ranging from 0.08 to 0.8 mg/mL towards the growth of a) *A. flavus* and b) *M. cladophyllus.* Different letters indicate ($p<0.05$), where significant differences exist while same letters represent no significant differences.

The antifungal activity of chitosan occurs via two mechanisms. First, polycationic chitosan attracts and attaches to the anionic fungal cell membrane, disrupting cell permeability [40]. Changes in cell permeability cause leakage of intracellular content [8]. Second, chitosan can enter fungal cells and inhibit mRNA and protein synthesis by adsorbing all essential nutrients [8]. However, each fungus has different defence mechanisms against foreign substances; therefore, the antifungal mechanism of chitosan can differ based on the fungus species. Alves et al. [48] reported that the antifungal mechanism of chitosan against *A. flavus* disturbed the spore and conidiophore populations by electrostatic interaction between chitosan and the *A. flavus* cell membrane. Other than fungus species, DD, Mw, and sample concentration can influence the antifungal activity of chitosan [9,49]. In addition, Kulawik et al. [50] reported that high DD and low Mw chitosan had high antifungal activity against *Candida albicans*. Chitosan with a high DD possessed many free amino groups that were positively charged, so it can increase the antifungal activity, which explains the high antifungal index of E.ch against *A. flavus* compared to C.ch because E.ch has a higher DD than C.ch [35]. The present result, supported by Alves et al. [48], showed that 97 % of DD of chitosan has high antifungal activity compared to 79 % of DD of commercial chitosan. However, the antifungal activity of E.ch was lower than C.ch against *M. cladophyllus,* which explains why chitosan's antifungal activity depends on the fungal species.

DPPH Radical Scavenging Activity

DPPH was used to estimate the testing antioxidant capacity (TAC) of C.ch and E.ch. Antioxidants are defined as the ability of a compound to inhibit free radical chain reactions by complexing with them [51]. In addition, the antioxidant compound must also be able to scavenge reactive radicals and produce a new stable radical, which changes colour from violet to pale yellow [51,52]. The results of the DPPH radical scavenging activities of ascorbic acid, C.ch, and E.ch are shown in (Figure 4). E.ch showed a higher scavenging activity than C.ch. However, the ascorbic acid still had the highest scavenging activity, from 92.12 % to 95.3 % at 0.1 to 1.0 mg/mL, compared to C.ch and E.ch. At 1.0 mg/mL, E.ch (30.78 %) had the highest scavenging activity compared to C.ch (25.58 %). Sarbon et al. [16] mud crab chitosan also showed 30 % DPPH scavenging activity at 10 mg/mL. E.ch had high scavenging activity due to its high DD compared to C.ch, in which more amine in E.ch reacts with DPPH to form stable molecules. In addition, C.ch has a higher viscosity and medium Mw than E.ch. Thus, C.ch has a robust intramolecular hydrogen bond, which restricts the reaction of amines with DPPH [53]. Muthu et al. [54] also stated that chitosan scavenging ability depends on DD and Mw, which supports these results.

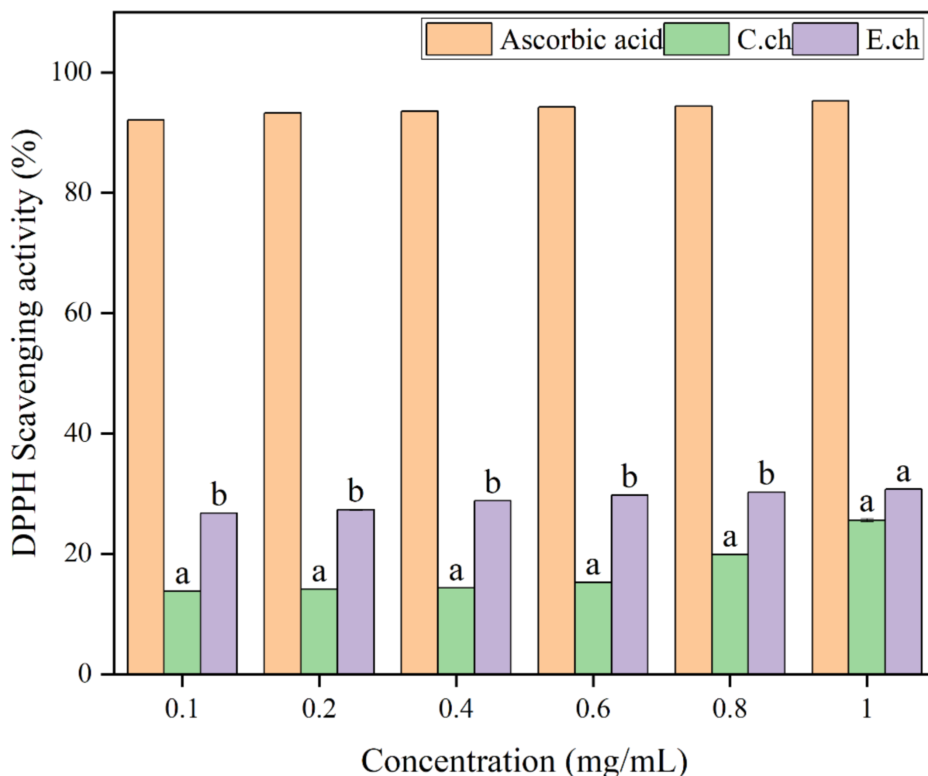


Figure 4. DPPH scavenging activity of ascorbic acid, C.ch, and E.ch. Means with different letters indicate ($p<0.05$), significant differences exist between the samples type at same concentration. Means with same letters indicate no significant differences between the samples type with similar concentration.

CONCLUSION

In conclusion, the physicochemical and biological properties of E.ch were characterized. E.ch obtained in this study possessed a higher DD than C.ch but had a lower viscosity than C.ch. There was a significant difference in the antibacterial activity between E.ch and C.ch, with E.ch showed much better antibacterial activity than C.ch. The similarity of MIC value (2 mg/mL) of E.ch and C.ch against *E. coli, S. aureus* was revealed in this study except for MIC value of C.ch against *P. aeruginosa*. However, the MBC value of E.ch and C.ch could not be determined at concentrations ranging from 0.125 to 2 mg/mL against *E. coli, S. aureus*, and *P. aeruginosa*. This research shows that 2 mg/mL of E.ch can only inhibit the bacteria growth but not as bactericidal agent. Therefore, the concentrations of E.ch and C.ch should be increased in future studies to determine their MBC values.

Furthermore, there was a significant difference between the antifungal activities of E.ch and C.ch against *A. flavus*. E.ch showed better antifungal activity against *A. flavus* than C.ch. However, C.ch had a higher antifungal index against *M. cladophyllus* than E.ch. The antifungal indices of E.ch and C.ch increased when their concentrations were increased. However, the antifungal activity of E.ch was found to be dependent on the fungal species. Besides, E.ch showed better antioxidant activity than C.ch. E.ch had better antibacterial, antifungal, and antioxidant activities than C.ch because of its high DD. Thus, E.ch from mud crab shells has the potential for further modification to enhance its functionality for future agricultural and pharmaceutical applications.

ACKNOWLEDGEMENTS

We acknowledge financial support from the Tun Ahmad Zaidi Chair research grant (C09/TZC/2158/2021). In addition, we would like to acknowledge the Excellent Student Programme (PPC), sponsored by the Public Service Department (PSD), for financial support in completing this research. Furthermore, we would like to thank the Centre of Pre-University Studies and the Faculty of Research Science and Technology, Universiti Malaysia Sarawak (UNIMAS), for providing excellent facilities for this research.

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