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Characterization of Aquatic Beetles Shells (*Hydraenidae family*) derived chitosan and its application in order to eliminate the environmental pollutant bacteria

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Abstract: Chitosan is one of the natural biopolymer. Despite its importance, the characterization and extraction processes of this essential biopolymer are yet enigmatic. In this study, we developed an analytical procedure for the extraction of chitin and chitosan from the aquatic beetles' shells (Hydraenidae family). The commercial chitosan and isolated chitosan, obtained during our experiment were analyzed by XRD, EDX, SEM, FT-IR, TGA, and UV-vis DRS techniques. Our results indicate 38% chitin and 23.5% chitosan segregated from the aquatic beetles' shells. The ratio of protein residues to the wet weight (RWW) of the segregated chitosan was about 24%. FT-IR analysis attested that the isolated chitosan from the aquatic beetles' shells was identical to the commercial chitosan. The EDX analysis does not reveal any impurities or the used precursor's characterized peaks, confirming high purity percentage of the segregated chitosan. Furthermore, according to the SEM images the segregated chitosan has smooth surface with amorphous property. Ultimately, the antibacterial activities of isolated chitosan were investigated against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) bacteria to assess their potential antibacterial applications. The results showed that the isolated chitosan from the aquatic beetles represented an outstanding performance in the studied bacteria.

Keywords: Antibacterial activities, Characterization, Chitosan, Hydraenidae

1. Introduction

Among the various reported biopolymer, chitosan has unique properties such as magnetic, biodegradability, biocompatibility, anti-oxidant, anti-fungal, and anti-bacterial effects (Zou et al., 2016; Hamed, et al., 2016; Tamer et al., 2016) and have been extensively used for skin wound healing, homeostatic property, and for analgesic function (Tranquilan et al., 2016; Bano, et al., 2017; Dang et al., 2018). This natural polysaccharide is non-toxic and is widely used in the food processing, cosmetics, waste management, water clarification, tissue repair, drug and gene delivery (Al-Naamani et al., 2017; Yemisci, et al., 2018).

Chitosan is derived from the deacetylation of chitin. The commercial chitosan is usually manufactured from the exoskeletons of krill, squid, shrimp, and crab (Kucukgulmez et al., 2011; Cao, et al., 2018). Insects are recently considered as an alternative source for the generation of chitin and chitosan (Kaya et al., 2014). Extraction and characterization of chitin and chitosan from the cuticle of insects are common, but only few species were candidate so far to generate these materials (Sajomsang et al., 2010; Liu, et al., 2012; Ma et al., 2015; Kaya et al., 2018).

Furthermore, the chitosan isolated from various aquatic invertebrate species, for example the aquatic beetles shell (*Hydrophilus piceus*) has displayed the highest dry weight and chitosan (Shin et al., 2019).

Hydraenidae "the aquatic beetles" are cosmopolitan and belong to the coleoptera order. Few Hydraenidae usually live at the margins of rivers and in the vicinity of lotic biotopes. Hydraenidae include > 42 genera and ~1600 species and ~ 941 species live in the Palearctic region. Worldwide distribution of beetles makes the Hydraenidae as an alternative source of chitin and chitosan (Slipinski et al., 2011; Jäch, et al., 2015; Jäch et al., 2016).

Recent study has addressed the characterization and extraction processes of the chitosan extracted from Hydraenidae shells. We used x-ray diffraction (XRD), energy-dispersive X-ray spectroscopy (EDX), scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR), thermo gravimetric analysis (TGA), and UV-vis diffuse reflectance spectroscopy (DRS) analyses to understand the physio-chemical signatures of the extracted chitosan. Moreover, the chitosan extracted from Hydraenidae is compared to the commercial chitosan. The experimental results demonstrate that the extracted chitosan achieved during our experiments are quite identical to the commercial chitosan. Furthermore, the antibacterial activity of isolated chitosan from the aquatic beetles was assessed against *E. coli* and *S. aureus* bacteria to investigate their potential antibacterial applications (Nooshin et al., 2019).

2.Experimental procedures

2.1. Materials

In this research, commercial chitosan (from shrimp), procured from Sigma Aldrich, sodium hydroxide (NaOH), hydrochloric acid (HCL), and absolute ethanol with high quality were provided from Merck. *E. coli* (PTCC 1047) and *S. aureus* (PTCC 1189) were purchased from Iranian Research Organization for Science and Technology. Nutrient broth and nutrient agar were provided from Pronadisa (Spain). Distilled water was used all over the reaction procedure.

2.2. Sample collection and identification

The aquatic beetles (Hydraenidae) were collected from Baliqlu River, NW Iran (Ardabil) (Fig. 1A). The samples were collected from 17 stations, across the different parts of the river, The GPS position of the samples has been shown in Fig. 1B. The samples stored in plastic vials with ~95% ethanol, and authenticated by the Entomology lab of University of Atatürk Erzurum, Turkey (Fig. 2). We used distilled water to wash the samples repeatedly in order to remove the impurities. Then, the samples were dried at room temperature for 48 h.

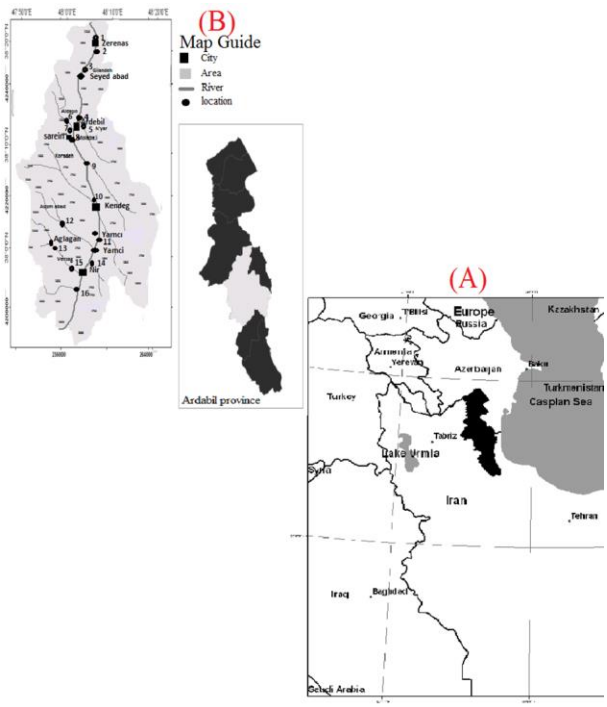

Fig. 1. A: Simplified map shows the target region. B: Map showing the selected stations and GPS locations of the samples.

Fig. 2. Hydraenidae family A) dorsal view B) ventral view.

2.3. Chitin and chitosan extraction process

We pulverized the aquatic beetles' shells using conventional mill. Powders (5 g) were dried in a drying oven at 60 °C for 2 h prior to extraction. The prepared powders were demineralized using 100 mL of 2 M HCl at 70 °C for 3 h, then filtered using filter paper and washed with distilled water. After that, for deproteinization process were using 100 mL of 2 M sodium hydroxide at 85 °C for 12 h. Then filtered again

and washed with distilled water. The resulted mixture for decolourization, were kept in solutions chloroform, methanol and distilled water for 1 h (in a ratio of 1:2:4) then filtered and washed. The resulted materials were dried in an oven at 65 °C for 24 h, and were used to calculate the percentage of chitin. One g resulted chitin were deacetylated to procure chitosan by treating them with 50% sodium hydroxide (w/v 1:20) at 120 °C for 2 h. Then resulted chitosan were washed with distilled water until reaching pH 7. Finally, the chitosan samples were dried in an oven at 65 °C for 24 h (Kaya et al., 2014; Kaya, et al., 2015).

2.4. Characterization techniques

The detailed characterization instruments are in the supporting materials.

2.5. Antibacterial activities assessment

The isolated chitosan from Hydraenidae shells were evaluated for antibacterial activity against E. coli and S. aureus bacteria, using colony forming units (CFU) enumeration method in concentration range of 0.1– 0.8 mg/mL and control (Fernandes et al., 2013). In this method, the different concentrations of isolated chitosan were prepared by dissolving in 1% acetic acid. Then, the bacterial cells concentration was adjusted to 108 CFU mL−1 in the reaction solution (Omrani and Fataei, 2018). Different concentrations in the stated range were prepared in the nutrient broth medium in a final volume of 3 mL. The tubes holding culture media in a shaker incubator for 12 h at 37 °C were incubated. The optical density was measured at 600 nm. Afterwards, the culture media were inoculated consuming 100 µL of the overnight culture of the bacteria in nutrient agar, and the plates were incubated for 24 hours at 37 °C. The number of viable colonies was counted for each isolate in triplicate and viability of them in the treated culture was expressed as percent of the control.

3.Results and discussion

The results show after demineralization, de-proteinization, and de-colorization, the chitin (1.807 g) was obtained from the residue (4.68 g). Moreover, through deacetylation chitosan (1.10 g) was obtained from chitin (1.7 g). Additionally, the weight of aquatic beetles' shells residue was more than 60% of the raw material. The yield of chitin and chitosan from the aquatic beetles' shells were 38 and 23.5%, respectively. Furthermore, the average ash content for the isolated chitosan were calculated 0.34%. The purity of chitosan can be determined with the percentage protein residues. The protein residues of isolated chitosan were used the Laurie's method at concentrations of 1-300 µg/L and due to the ratio to wet weight (RWW) were calculated to be 24% (Heu et al., 2003).

The commercial chitosan and chitosan extracted from the aquatic beetles' shells were examined with XRD analysis and displayed in Fig. 3. According to the Fig. 3, chitosan has two different crystal forms (2θ ≈ 10° and 2 θ ≈ 20°) belonging to the monoclinic system. Due to the more intermolecular and intermolecular hydrogen bonds and higher degree of deacetylation, chitosan from aquatic beetles shells the diffraction peaks at in the 10° and 20◦ are sharper with wider and stronger diffuse scattering peaks and then that of the commercial chitosan (Sajomsang et al., 2010).

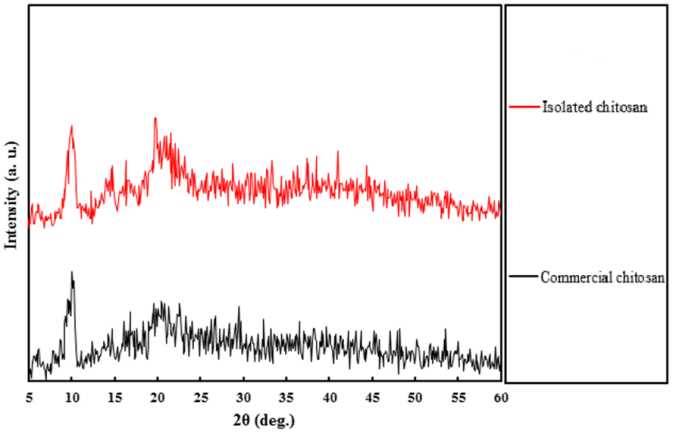

Fig. 3. XRD patterns for the isolated chitosan and commercial chitosan.

EDX analysis was used to evaluate the chemical composition and purity of the isolated chitosan from aquatic beetles' shells (Hydraenidae family) as shown in Fig. 4. The EDX spectra revealed the presence of carbon and oxygen elements for the chitosan sample (Shekhawat et al., 2017). Moreover, no impurity peaks were detected, which indicates high purity of the samples. The weight percentages of C and O in the chitosan sample were 61.9 and 38.1%, respectively.

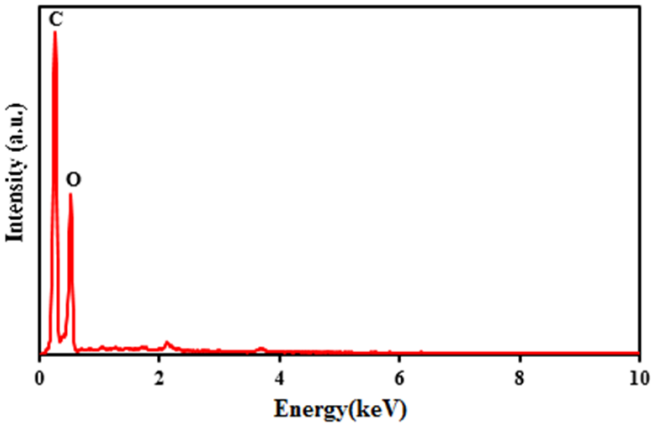

Fig. 4. EDX spectra for the isolated chitosan from aquatic beetles shells (Hydraenidae family).

SEM analysis was employed to predict the morphology of the chitosan sample (Fig. 5). It is observed that some regions are flat and some regions have an inhomogeneous surface (Kaya et al., 2013). Furthermore, the chitosan membrane obtained from the exoskeleton of the aquatic beetles is a compact and no damage. In some section, are observed particles, this case occurs because filtration and washing in the four-step process.

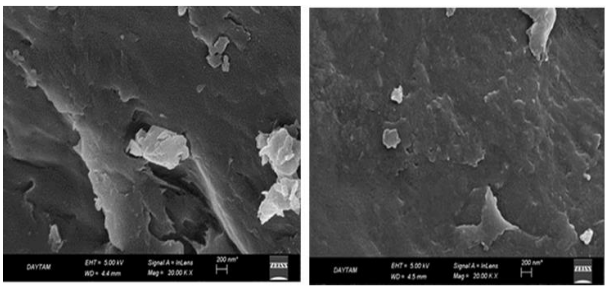

Fig. 5. SEM images from the isolated chitosan.

FT-IR spectra were also recorded to determine the functional groups in solid phase using the KBr pellet technique in the regions of 400 to 4000 cm^{-1}. The FT-IR spectra of chitosan obtained from the exoskeleton of the aquatic beetles and commercial chitosan are shown in Fig. 6. The stretching vibration around 3100 to 3600 cm^{-1} is usually attributed to intermolecular H bands from O–H to N–H. This absorbance represents O–H groups stretching in a certain frequency due to the intramolecular and inter-molecular hydrogen bonds of varying length and strength in the chitosan (Rumengan et al., 2014). The band observed at 2926 cm^{-1} is commonly assigned to carbon-hydrogen (C–H) stretching vibration. the deformation vibration (N–H, type II amid band) at 1574 cm^{-1} and for type I amide band of the carbonyl group (C=O) observed at 1646 cm^{-1} (Kasaai., 2008). Moreover, the C–H deformation vibration of the scissors of methylene groups was observed at 1436 cm^{-1} (Jiang et al., 2010). The derived chitosan was compared with commercial chitosan, and the results were observed indicating that samples have similar chemical compositions. The degree of deacetylation (DD) of chitosan samples was calculated by the following equation: DD (%) = 100 − [(A1658 / A3450) × 115]. The results suggest that based on the analysis FTIR, the deacetylation of the chitosan is 78%.

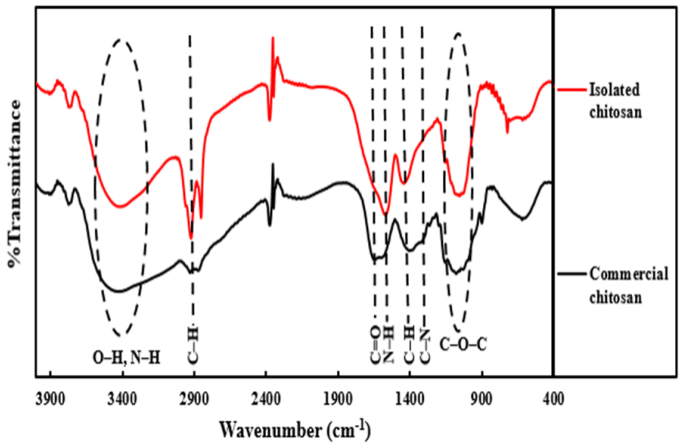

Fig. 6. FT-IR spectra of the isolated chitosan and commercial chitosan.

Figure 7 displays TGA curves for the commercial chitosan and chitosan extracted from the aquatic beetles' shells. As shown in Fig. 7, the samples weightlessness is divided into two steps. From 20 to 300◦C, weight loss of about 11.87% attributed to water molecules desorption in chitosan during the heating process (Kaya et al., 2014 Ma et al., 2015).

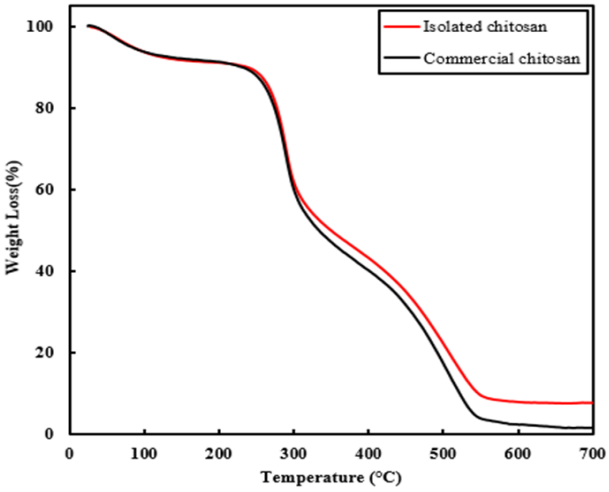

Fig. 7. TG analysis of the isolated chitosan and commercial chitosan.

UV–Vis DRS spectra provides information on the electronic absorption properties of the samples under light irradiation. DRS spectra of the resultant chitosan sample and commercial chitosan were obtained, and the results are shown in Fig. 8. In the range of 250 to 800 nm, the commercial chitosan sample shows absorption at 360 nm (Taheri et al., 2018). On the other hand, the absorption spectrum of the chitosan from Hydraenidae family was observed at 379 nm. The results obviously demonstrated the both samples are similar to each other. Hence, the results obtained from the DRS data were parallel with the EDX and FT-IR analysis.

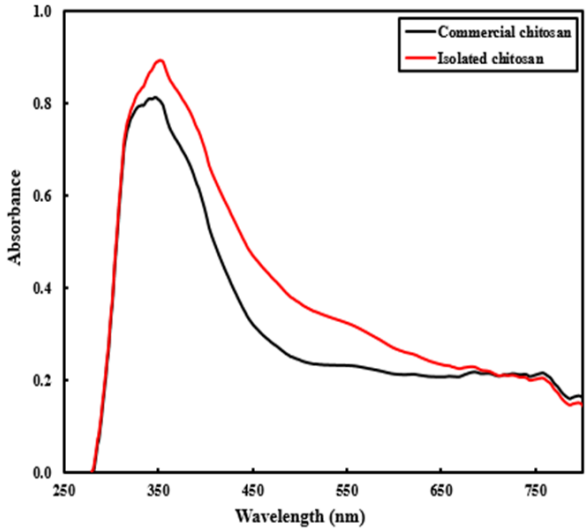

Fig. 8. UV–vis DRS spectra of the isolated chitosan and commercial chitosan.

The antibacterial activity of the isolated chitosan in the different concentrations was assessed to give an account on the potential applications of them. Figures 9 show the viability curves of *E. coli* and *S. aureus* bacteria under the studied condition. The differences in the viability of pathogenic bacteria among the different concentrations of the isolated chitosan were statistically significant by one-way ANOVA at $P < 0.05$, as indicated. The antibacterial activity at 0.1 mg/mL concentrations against both bacteria was less than that of other samples. Interestingly, the viability percentage of *E. coli* and *S. aureus* bacteria at 0.4 mg/mL concentrations was significantly reduced. Furthermore, isolated chitosan at 0.8 mg/mL concentrations not only prevented the bacterial replication, but also killed the pathogenic bacteria. It was concluded that the antibacterial activity of isolated chitosan against of *S. aureus* (10.9 ±0.78) was higher than that observed for *E. coli* (13.8 ±0.69) (Lima et al., 2017).

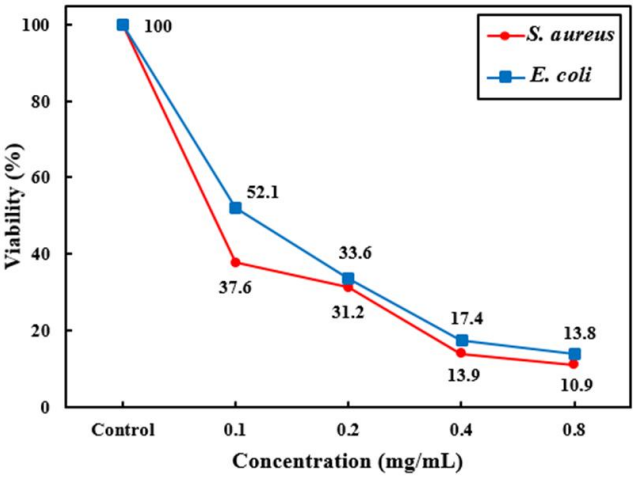

Fig. 9. Antibacterial activity of the isolated chitosan against *E. coli* and *S. aureus* bacteria.

For better insights, the *E. coli* and *S. aureus* bacteria were inactivated with the different concentrations of isolated chitosan and their optical images were shown in Fig. 10. In these optical images, the highest antibacterial activity belonged to the 0.8 mg/mL concentrations and the other concentrations; including 0.4, 0.2, and 0.1 mg/mL were in the next ranked, respectively. Moreover, in all these concentrations the number of cell colonies for *S. aureus* were less than those for *E. coli*. Thus, these optical images showed that isolated chitosan from the aquatic beetles induced dose-dependent toxicity in the pathogenic bacteria.

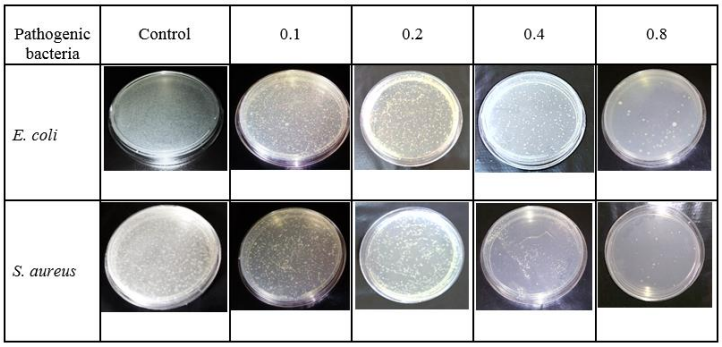

Fig. 10. Antibacterial activities of the isolated chitosan against *E. coli* and *S. aureus*.

2. Conclusion

Chitosan biopolymers were successfully produced from the aquatic beetles' shells (Hydraenidae family). The yield of chitin and chitosan from the aquatic beetles' shells were 38 and 23.5%, respectively. Furthermore, the average ash content for the isolated chitosan were calculated 0.34%. XRD analysis exhibited that the chitosan extracted from Hydraenidae family have high crystallinity. SEM results clearly indicated that the prepared chitosan is almost a smooth

surface. EDX analysis indicated the formation of the corresponding pure materials. No significant changes between the prepared chitosan from the aquatic beetles' shells (Hydraenidae family) and commercial chitosan were observed through FT-IR spectroscopy analysis. TG analysis revealed that the weight loss rates of chitosan from Hydraenidae family were lower than that of commercial chitosan. Based on the results, prepared chitosan by Hydraenidae family could be an alternative source with commercial chitosan and showed a great potential to be used applied in the biomedical and food industry applications. Finally, obtained results indicated that gram-positive bacterial strains were more sensitive in comparison to gram-negative strains against the tested isolated chitosan from Hydraenidae shells, and disclosed the potential application of the chitosan as appropriate candidates for successful minimization of bacterial infections that may occur.

Declaration of conflicting interests

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