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Characterization of Chitosan Extracted from Upper and Lower Squilla species Shells

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ABSTRACT

The characteristics of chitosan produced from upper and lower squilla species and compared to the analytical (commercial) chitosan were investigated. Two squilla species, *Squilla mantis* and *Oratosquilla massavensis*, were collected from Elanfoshy, Alexandria, Egypt, in Nov. 2022. Chitin and chitosan were extracted from the upper and lower shells of the species studied. The results showed that analytical (commercial) chitosan has low values of moisture, degree of deacetylation (DD), solubility, water binding capacity (WBC), and fat binding capacity (FBC), while the intrinsic viscosity and MW were higher in values than upper and lower squilla species chitosan samples. Four squilla chitosan samples exhibited regular absorption with little differences. Thermogravimetric analysis**(**TGA) curves showed that chitosan samples recorded a high thermal stability (200°C). Lower shells chitosan of *S. mantis* recorded the best radical scavenging activity followed by lower and upper shells chitosan samples of *O. massavensis*, and upper shells chitosan of *S. mantis*. Furthermore, the minimum inhibition concentration (MIC) for investigated chitosan samples has no general trend. In conclusion, this study recommended that squilla chitosan is a promising shellfish and is considered a good substitute for shrimp chitosan, with additional applicability in high-temperature food processing.

INTRODUCTION

Crustacean shells are good sources of chitin and chitosan production. It is well known that chitosan varies from chitin in the number of D-glucosamine and N-acetyl-D—glucosamine groups and type of solubility solution. The characteristics of extracted chitosan are more influenced by the original source and the deacetylation process. **(Sanchez *et al.*, 2005; Kumari *et al.*, 2016; Amiri *et al.*, 2022)**. Both chitin and chitosan are characterized by their safety, biological value, physiological compatibility, digestibility, adsorption, and chelating ability. To characterize the chitosan quality and efficiency parameters, some characteristics; chitosan yield, degree of deacetylation (DD),

solubility, water and fat binding capacities, antioxidant and antimicrobial activities have been reported by several workers. The crystalline index, solubility, tensile strengths, coagulant-flocculant properties, and bacteriological activity depend on molecular weight of chitosan. The solubility of chitosan in acidic solutions is due to the presence of both amine and NH_2 groups. Furthermore, the crystallinity and availability of amine groups in chitosan affect its adsorption capacity **(Aranaz *et al.*, 2009; Miretzky & Cirelli, 2009; Lee *et al.*, 2011; Peng *et al.*, 2013; Shukla *et al.*, 2013; Parthiban *et al.*, 2017)**. With regard to economic evaluation, **Parthibanet *et al.* (2017)** reported that utilization of shrimp shells to produce the chitosan was more economical and biological values than that produced from the crab and squilla shells. They explained that the yield (15.40%), viscosity (5300 cPs), solubility (97.65%) and DD (81.24%) of shrimp shells chitosan have better values than other ones. Therefore, the present work aimed to investigate the characteristics (moisture, ash, DD, MW, viscosity, solubility, WBC, FBC, FTIR, TGA, antioxidant and antibacterial activities) of chitosan produced from upper and lower *Squilla mantis* and *Oratosquilla massavensis* shells.

MATERIALS AND METHODS

Squilla samples

About 5kg of each two fresh squilla species, *Squilla mantis* (61± 8g) and *Oratosquilla massavensis* (36± 6g), samples were collected from the commercial catch, Elanfoshy, Alexandria, Egypt, during Nov. 2022 (Fig. 1). Iceboxes were used to transfer the samples to the laboratory of fish processing and technology Lab., Fisheries Division, NIOF.

Squilla mantis *Oratosquilla massavensis*

Fig. 1. Squilla species samples

Analytical (commercial) chitosan

It was obtained from Sigma –Aldrich Chemical Co. (St. Louis, USA).

Bacterial strains

Pathogenic strains; *Staphylococcus aureus* EMCC1351 and *Clostridium botulinum* ATCC3584 as Gram-positive, and *Salmonella* spp*., Vibrio fluvialis* and *Escherichia coli* BA12296 as Gram-negative strains (MERCIN, Fac. of Agri, Ain Shams Univ, Egypt) were used to display the antimicrobial potentials for chitosan samples. The test was held and the strains were maintained in 20% glycerol/ LB culture at -80^{o}C.

Preparation of samples

Two squilla samples were placed in an air blast freezer and frozen at a temperature of -30°C for 10 minutes; the shells were cutoff from the abdomen up to the thorax, packed in polyethylene bags and frozen at -20°C till using. Frozen two squilla

shells were thawed under room temperature, divided into upper (hard) and lower (soft) shells. All batches were steamed for 10 minutes and mixed with tap water at a ratio of 1:2 (W:V) using an electrical mixer. Samples were ground, dried at 60°C, cooled at room temperature, and filled in glass jars with tight lids.

Extraction of chitin and chitosan

Chitin samples were extracted **(Abouzeed *et al.*, 2015)** as follows: squilla shells were twice demineralized by 3% HCl for 3h and deproteinised by 4% NaOH for 24h between the two HCl treatments and washed with tab water, and then dried at 50°C. Additionally, chitosan was extracted by deacetylation (50% NaOH at 120°C for 1h, and repeated three times) from chitin obtained from the first stage. Fig. (2) shows the flowchart of chitosan production steps.

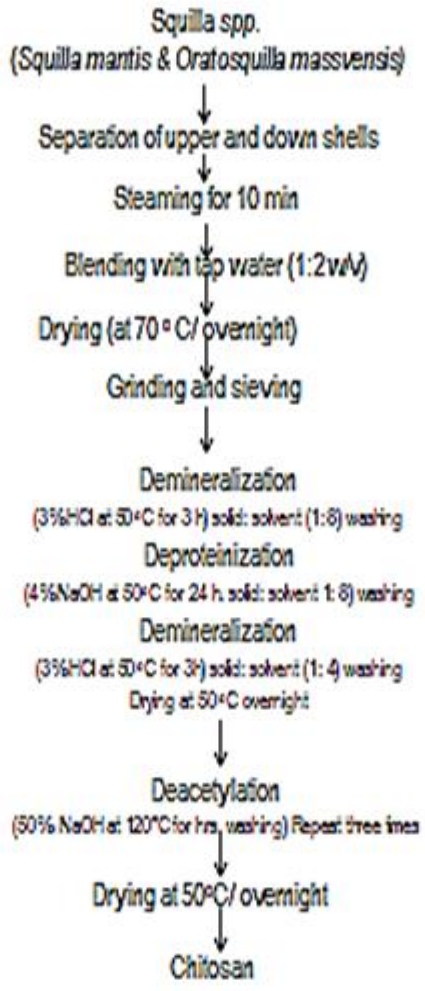

Fig. 2. Flowchart of chitin and chitosan extractions from two squilla shells

Analytical methods

Moisture and ash content of the extracted chitin and chitosan were determined according to the methods outlined by **AOAC (2005)**.

Degree of deacetylation (DD)

DD of chitosan samples was determined **(Sabnis & Block, 1997)** by using FTIR spectroscopy at a range of 500- 4000cm-1(Brucker Tensor 37) as follows: 40mg sample was well mixed with 120mg KBr and prepared as salt disc (1cm). The absorbance (A) of IR spectrum were measured and calculated as follows:

$DD = 97.67— \{26.486 \times (A_{1655}/A_{3450})\}$

Viscosity and molecular weight (MW)

MW average of the viscosity was determined **(Wang *et al.*, 2004)**; the chitosan samples were dissolved in 0.5M acetic acid with 0.2M sodium acetate solutions, and the intrinsic viscosity was measured using the Oswald viscometer. The MW of chitosan samples were calculated **(Wang *et al.*, 1991; Terbojevidh & Cosani, 1997)** using the Mark-Houwink equation related to intrinsic viscosity with empirical viscometric constants, as follows:

$[\eta] = KMa$

[η]: Intrinsic viscosity

$K= 3.5 \times 10–4cm3/ g$

$a = 0.76$

Solubility

Solubility of chitosan samples were measured **(Fernandez-Kim, 2004)** by dissolving 1% chitosan in 1% acetic acid solution (w/v) through stirring for 24h, followed by centrifugation. % insoluble chitosan was determined as follows: 0.1g squilla chitosan powder samples were centrifuged and then dissolved with 10ml of 1% acetic acid for 30min using incubation at 25°C and 240rpm. Subsequently, the solution was centrifuged at 10,000rpm for 10min, and the supernatant was decanted. Undissolved particles were washed in distilled water (25ml), and centrifuged again at 10,000rpm. The supernatant was removed; undissolved pellets were dried at 60°C for 24 hr, weighed, and calculated, as follows:

$$\% \text{ Solubility} = \frac{(\text{Initial wt. of tube + Chitosan}) - (\text{Final wt. of tube + Chitosan})}{(\text{Initial wt. of tube + Chitosan}) - (\text{Initial wt. of tube})} \times 100$$

Water and fat binding capacities (WBC and FBC)

Water and fat binding capacities (WBC and FBC) of chitosan were determined **(Wang & Kinsella, 1976)**, with little modifications as follows: 0.5g chitosan sample with 10ml water and 10ml soybean oil added in centrifuge tubes, respectively, and mixed by a vortex mixer for 1min. Subsequently, they were left at room temperature for 30min with intermittently shakes for 5sec every 10min, and centrifuged at 3500rpm for 25min. The supernatant was decanted, and the tubes were weighed again. WBC and FBC were calculated, as follows:

WBC (%) = {Water bound (g)/ Initial sample wt. (g)} × 100

FBC (%) = {Fat bound (g)/ Initial sample wt. (g)} × 100

Fourier transforms infrared (FTIR)

FTIR spectra of four chitosan samples were measured **(Cerqueira *et al.*, 2011)** by using FTIR spectroscopy (Shimadzu FTIR-8400 S, Japan, equipped with ATR 8000A) at range of 4000– 400cm^{-1}.

Thermogravimetric analysis (TGA)

Thermal stability characterization of chitosan samples for potential heating processes was determined **(Cerqueira *et al.*, 2011)** as follows: all samples were heated till 200°C (with a rate of 20^{o}C/ min underflow of N_2), and measured using TG analyzer (Shimadzu TGA–50, Japan).

Antioxidant activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of chitosan samples was determined according to **Zhang *et al.* (2003)**. Briefly, 0.5ml freshly DPPH (0.3mM) was mixed with 0.5ml of chitosan solution (1% chitosan in 1% acetic acid). The mixture was incubated in a dark place at room temperature for 60min, measured at 517nm and calculated as follows:

% Inhibition = (Abs. Blank –Abs. sample/ Abs. Blank) × 100

Minimum inhibitory concentration (MIC)

Agar well diffusion assay **(Kadaikunnan *et al.*, 2015)** was used to examine antimicrobial activity of chitosan samples against pathogenic bacteria. The bacterial strains were grown in a nutrient broth at 37°C/ 24h. 100µL of overnight activated culture of each pathogen strain (10^6cfuml^{-1}) was aseptically spread over nutrient agar plates. Chitosan samples were dissolved in 1% acetic acid (0.01g/ mL) followed by a set of 3 concentrations with double folds dilution of samples (100, 50, and 25ml sample/ ml acetic acid) thatwere compared to the control (1% acetic acid). All plates were incubated at 37°C for 18h, and the formed inhibition zones (IZ) were measured by the diameter of (IZ) around the well (mm) including the well diameter. The average values of all duplicates were calculated.

Statistical analysis

The data (n=3) obtained were statistically analyzed using analysis of variance (ANOVA). Duncan's test was performed (SPSS, Ver.16) for mean comparison at $P\leq$ 0.05. Data are expressed as mean ± SD.

RESULTS AND DISCUSSION

1. Physical characteristics of chitosan

Some physical characteristics of chitosan produced from upper and lower shells of two squilla species are presented in Table (1).

1.1. Moisture

Chitosan produced from upper and lower shells of *O. massavensis* contained moisture values of 6.88 and 7.06%, while the corresponding content recorded 7.94 and

7.52%, respectively, of *S. mantis.* Significant difference ($P \leq 0.05$) was found in moisture values among all samples of two species investigated.

Table 1. Physical characteristics of chitosan extracted from upper and lower shells of squilla species

Characteristic (%)	Chitosan			
	O. massavensis		*S. mantis*	
	Upper	Lower	Upper	Lower
Moisture	6.88±0.11[c]	7.06±0.07[c]	7.94±0.03[a]	7.52±0.08[b]
Ash	0.51±0.13[a]	0.14±0.01[b]	0.70±0.07[a]	0.13±0.01[b]
Chitin yield	22.41±0.57[b]	16.89±0.47[c]	28.30±0.16[a]	21.09±0.74[b]
Chitosan yield	74.50±0.22[b]	76.33±0.222[a]	74.90±0.10[b]	76.58±0.11[a]

Mean values in a row with different superscripts are significantly different at $P \leq 0.05$.

These results are in accordance with the results obtained by **Hossain and Iqbal (2014)**; the moisture of chitin produced from squilla, shrimp, and crab shells was recorded <10%. Additionally, **Parthiban *et al.* (2017)** found that the moisture values were 9.10, 8.30, and 9.32% of produced chitin products from squilla, shrimp, crab, respectively, while the corresponding values of chitosan were 7.56 %, 7.62 and 7.67 %, respectively. The data in this work are within the standard limit of commercial chitosan (< 10% moisture), as reported by **Sukumaran *el al.* (1987).** Furthermore, **Balde *et al.* (2022)** found that moisture of squilla (*Harpiosquilla annandalei*) chitosan was less than 10%**.**

1.2. Ash

The ash contents (Table 2) of chitosan for upper and lower shells were 0.51 and 0.14% for *O. massavensis*, 0.70 and 0.13% for *S. mantis*, respectively. Furthermore, the data indicate that ash content is higher in upper shells than lower shells, attributed to the reaction of carbonate salts with protein and chitin, resulting in increased solidity. Insignificant difference ($P \leq 0.05$) was found between upper samples and also lower samples of species studied. **Parthiban *et al.* (2017)** showed that ash content recorded 0.36% of shrimp, 0.58% of squilla, and 0.76% of crab chitosan. A decrease in ash content of chitosan is due to efficiency of used demineralization process and this also indicates that chitosan extracted either from upper or lower shells has a high quality grade, as reported by **No and Meyers (1989)** and **Hossain and Iqbal (2014)**; ash of chitosan is closely related to demineralization step affecting solubility and viscosity parameters.

2. Chitin and chitosan yield

There is no doubt that the structure of the upper shells of squilla varies from that of the lower shells. The results presented in Table (1) show that the values of yield chitin of upper and lower *S. mantis* were higher (28.30 and 21.09%) than those (22.41 and 16.89%) produced from *O. massavensis*, respectively. This indicates that the upper and lower shells of *S. mantis* are good sources of chitin compared to *O. massavensis*. On the other hand, chitosan extracted from lower shells recorded higher yield (76.33 and 76.58%) than that extracted from upper shells (74.50 and 74.58%) for *O. massavensis* and *S. mantis*, respectively. Significant differences ($P \leq 0.05$) were noted in the yield of chitin between different treatments, with the exception of upper samples for the first species and

lower samples for the second species. Additionally, significant differences ($P \leq 0.05$) in chitosan yield were found inside each species. Our results of chitosan yield for two squilla species are higher than 24% of *Squilla mantis* **(Rhazi *et al.*, 2000)**, 6.45% of *Squilla empusa* **(Rao *et al.*, 2007)**, 10.725% of *Oratosquilla nepa* and 10.625% of *O. quinquedentata* **(Thirunavukkarasu *et al.*, 2011)**, and 20.48% of *Oratosquilla nepa* **(Yarnpakdee *et al.*, 2022)**. Furthermore, **Parthiban *et al.* (2017)** found that the chitin yields were 17, 14.5, and 13% for shrimp, crab and squilla, while chitosan yield recorded 15.40, 13.45 and 12.56%, respectively. **Mohan *et al.* (2021)** found that values of chitin yield were 23.75, 20.00, 21.25, and 17.5% of squilla, shrimp, crab, and lobster, respectively. This variation in chitin yield depends on various factors, such as raw material source and processing conditions.

2.1. Characteristics of chitosan

Table (2) shows some characteristics of chitosan produced from upper and lower shells of squilla species.

2.1.1. Degree of deacetylation (DD)

Values of DD recorded 81.91 and 84.98% of lower and upper chitosan of *O. massavensis*, while they were 81.28 and 84.28% for *S. mantis*, respectively (Table 2). Hence, chitosan produced from upper shells has a higher DD% than that from lower shells for the two squilla species under study. No significant different ($P \leq 0.05$) was found in DD between the upper and also lower samples of two species studied. All chitosan samples in this work, are in high qualities based on the range of DD (56-99%), as reported by **No and Meyers (1989)**. In addition, an increase in DD% refers to procedures done throughout extraction processes. Moreover, these results are higher than those of **Parthiban *et al.* (2017)**; DD values were 65.54, 71.58, and 63.53% for squilla, shrimp, and crab chitosan, respectively. In this work, the results are lower than the results mentioned by **Ibrahim *et al.* (2019)**; DD values were 95.5, 93.0 for and 88.5 of shrimp chitosan obtained by autoclaved, microwaved and traditional techniques, respectively, and they are higher than 75% of squilla *(Harpiosquilla annandalei)* chitosan **(Blade *et al.*, 2022)**, and also the average (73.56- 75.56) of squilla chitosan (*Oratosquilla nepa*) **(Yarnpakdee *et al*., 2022)**.

Table 2. Characteristics of chitosan produced from upper and lower shells of squilla species

Characteristic	Shells			
	O. massavensis		*S. mantis*	
	Upper	Lower	Upper	Lower
DD (%)	84.98 ± 0.91^{a}	81.91 ± 0.45^{b}	84.06 ± 0.38^{a}	81.28 ± 0.39^{b}
Intrinsic viscosity (dl/g)	1.7442 or 1744	1.0478 or 1047	1.0346 or 1034	0.7879or 787
Mw (Dalton)	3830.83	2275.22	2246.56	1710.87
Solubility (%)	100	100	100	100
WBC (%)	747.51 ± 7.59^{c}	802.67 ± 11.00^{b}	692.99 ± 1.45^{d}	873.53 ± 13.33^{a}
FBC (%)	594.47 ± 16.39^{bc}	614.56 ± 18.56^{b}	552.65 ± 14.57^{c}	674.79 ± 12.91^{a}

DD: Degree of deacetylation; Mw: Molecular weight; WBC: Water binding capacity; FBC: Fat binding capacity.

Mean values in a row with different superscripts are significantly different at $P \leq 0.05$.

2.1.2. Viscosity

The values of viscosity were 1.7442 and 1.0478g/ dl of chitosan produced from upper and lower *O. massveniss* shells, which decreased markedly to 1.0346 and 0.7879g/ dl in *S. mantis* samples, respectively (Table 2). The results in this work are lower than 1.31dl/ g of squilla chitosan *(Harpiosquilla annandalei)* **(Blade *et al.*, 2022)**, and also the average (2.97- 3.58dl/g) of squilla (*O. nepa*) chitosan **(Yarnpakdee *et al.*, 2022)**. The decrement in viscosity values refers to the increasing time and high temperature used in deacetylation process of hydrolysis stages **(Jia *et al.*, 2001; Kumari *et al.*, 2016)**. The viscosity depends mainly on MW, DD, the bleaching steps, concentration, ash content, pH, ionic strength, and residual presented in solution **(Moorjani *et al.*, 1975; Bough *et al.*, 1978; No *et al.*, 2000; Toan, 2009)**.

2.1.3. Molecular weight (Mw)

The values of MW were 3830.83 and 2275.22 Dalton of chitosan extracted from lower and upper shells of *O. massavensis*, while they were 2246.56 and 1710.87 Dalton for *S. mantis*, respectively, as shown in Table (2). These data are lower than the MW range of commercial chitosan (100,000– 1,200,000 Dalton). as reported by **Li *et al.* (1992)**, **Roberts (1997)** and **Rout (2001**), and also lower than 1050 KDa of shrimp chitosan **(Hossain & Iqbal, 2014)**; 21.1, 18.8, and 11.4 KDa of traditional, microwaved and autoclaved chitosan samples, respectively **(Ibrahim *et al.*, 2019)**; 50.18 KDa of squilla (*Harpiosquilla annandalei*) chitosan **(Blade *et al.*, 2022)**; and the average 1.44-1.12×10^6 Da of squilla (*Oratosquilla nepa*) chitosan **(Yarnpakdee *et al.*, 2022)**. MW of chitosan is closely related to the original source and method used in extraction. Moreover, temperature, concentration of alkali, reaction time, chitin concentration, particle size, dissolved oxygen and shear stress affect the degradation of chitosan. The variation of MW values in this study and other studies is due to main factors, such as reagents, the constant K, chitosan source, time, and temperature employed throughout the extraction stages which affected the DD and Mw of chitosan, subsequently influencing some physical properties too.

2.1.4. Solubility

All chitosan samples extracted from upper and lower samples recorded high solubility (100%), as shown in Table (2). Chitosan is insoluble in most solvents and it is soluble in few acids with stirring **(Chung *et al.*, 2005; Qin *et al.*, 2006)**. Although the solubility% in this work agree with that of shrimp chitosan (99.5%), as mentioned by **Islam *et al.* (2016)**, however it was higher than the values recorded in the study of **Kumari *et al.* (2016)**; the solubility values reached 75, 70 and 90% of fish, shrimp and commercial chitosan, respectively. Additionally, **Parthiban *et al.* (2017)** found that the solubility of squilla, shrimp, and crab chitosan recorded 89, 97, and 85%, respectively. This increase in solubility is due to low ash content in chitosan samples investigated, and this explanation is supported by **Hossain and Iqbal (2014)**.

2.1.5. Water binding capacity (WBC)

The results (Table 2) show that WBC% of chitosan produced from upper and lower shells of *O. massavensis* were 747.51 and 802.67%, while they were 692.99 and

873.53% in case of *S. mantis* chitosan, respectively. Furtherrmore, WBC% of chitosan produced from lower sqiulla shells has more than that extracted from upper shells. A significant differece ($P \leq 0.05$) was found in WBC among different samples. The range of WBC (692.99- 873.53%) in this study is in higher value than the results obtained by **Kumari *et al.* (2016)**; 358, 492 and 520% of shrimp, fish scale and commercial chitosan, respectively **(Kumari *et al.*, 2016)**, and also 548% **(Balde *et al.*, 2022)**. This variation is asigned to the increase of DD, which provides with more amino groups to bind water, while the decreased crystallinity increased with water binding **(Rout, 2001)**. Moreover, **Ibrahim *et al.* (2019)** found that values of WHC were 412.45, 454.45, and 631.24% of shrimp chitosan obtained by autoclaved, microwaved and traditional techniques, respectively.

2.1.6. Fat binding capacity (FBC)

FBC% of chitosan produced from the upper and lower shells of *O. massavensis* were 594.47 and 614.56%, while they were 552.65 and 674.79% of *S. mantis* chitosan, respectively (Table 2). These values of FBC are more than the results obtained by **Kumari *et al.* (2016)**; the FBC values of shrimp, fish scale and commercial chitosan were 226, 246, and 446%, respectively. A high significant difference ($P \leq 0.05$) was found between chitosan samples from the second species. This variation in value of FBC is due to chitosan source and sequences of its hydrolysis steps **(Moorjani *et al.*, 1975)**. The range of FBC (552.65- 674.79%) in this study is higher than the range of 170- 315% **(Knorr, 1982)**, 314- 535% **(Young *et al.*, 1998)**, 537.29% **(Hossain & Iqbal, 2014)**, 393.75- 587.76% **(Ibrahim *et al.*, 2019)**, and 369% **(Balde *et al.*, 2022)**. Moreover, it was lower than 706% **(Rout, 2001)**. Generally, WBC values of chitosan ranged from 581 to 1.150%, and this depends on both demineralization and deproteinization steps which affect WBC and FBC. It could be found that demineralization of crawfish shells gave higher WBC than deproteinized shells. On the contrary, decoloration step decreased both WBC and FBC compared to unbleached crawfish chitosan, and also decoloration affected the viscosity **(Rout, 2001)**.

2.1.7. Squilla and analytical (commercial) chitosan

Comparison between studied squilla and analytical chitosan is presented in Table (3). Squilla chitosan composed the range of 6.88- 7.94% of moisture, 0.13- 0.70% of ash content, while analytical chitosan composed 3.50 and 1.80 %, respectively. Furthermore, it has 81.28- 84.98% of DD, 0.7879- 1.7442dl/ g of intrinsic viscosity, 1710.87- 3830.83 Dalton of MW, 100% solubility, 692.99- 873.53% of WBC, and 552.65- 674.79% of FBC. The corresponding values of analytical chitosan were 71, 384.5, 7194, 87.8, 548.7, and 370.2, respectively. Analytical chitosan had low values of moisture, DD, solubility, WBC, and FBC, while it recorded higher values of intrinsic viscosity and MW compared to squilla chitosan. This variation is due to the original source of shell, reagents

concentration, extraction method, sequence and repetition of production and decoloration steps (**No *et al.*, 2000; Rout, 2001; Toan, 2009; Abozeed *et al.*, 2015; Parthiban *et al.*, 2017; Ibrahim *et al.*, 2019)**.

Table 3. Comparison between studied squilla and analytical chitosan

Item	Squilla chitosan	*Analytical chitosan
Moisture (%)	6.88 - 7.94	3.5
Ash (%)	0.13 - 0.70	1.8
DD (%)	81.28 - 84.98	71
Intrinsic viscosity (dl/g)	0.7879 - 1.7442	384.5
Mw (Dalton)	1710.87 - 3830.83	7194
Solubility (%)	100	87.8
WBC (%)	692.99 - 873.53	548.7
FBC (%)	552.65 - 674.79	370.2

DD: Degree of deacetylation; Mw: Molecular weight; WBC: Water binding capacity; FBC: Fat binding capacity.

Fourier transforms infrared (FTIR)

The FTIR spectra of four extracted chitosan samples are shown in Fig. (3) (A to D) indicating great similarity. As an amino polysaccharide, the four chitosan samples exhibit absorption typical of polysaccharides, characterized peaks for OH groups (3852-3855 cm^{-1}) **(Shi *et al.*, 2016; Shehata *et al.*, 2020**). Peaks (2871- 2869cm^{-1}) indicated (C– H stretch) group. The characteristic peak's assignment of chitosan (at 1640-1579cm^{-1}) is due to Schiff base (C=N) formed by a cross linking reaction between the amino group and the aldehydic group of carbaldehyde **(Kumar & Koh, 2012**). The (C=O) of secondary hydroxyl groups showed intense peak at 1022- 1023cm^{-1}. Overall, these results revealed that the four samples showed regular absorption peaks, with no significant differences between their spectra. This trend agrees with the spectra decided by **Kumar and Koh (2012**).

2.1.8. Heat stability via thermogravimetric analysis (TGA)

TGA curves (Fig. 4A-D) show that weight loss in the sample indicates the thermal degradation behavior of extracted chitosan samples. The weight losses in upper and lower *O. massavensis* chitosan (Fig. 4A, B) occurred in three main stages. The first ~22.37-99.84, ~29.23-85.41°C that caused 10.2, 8.6 % weight loss in upper and lower *O. massavensis* chitosan samples, respectively, can be attributed to the evaporation of the free water of chitosan **(Darwish *et al.*, 2018)**. While, the 2nd stage was at ~99-116 and 85-112°C, and the 3rd stage was at ~116-199 and 112-199 in upper and lower *O. massavensis* chitosan, respectively, showing that the total weight losses did not exceed 3% for both samples due to slow decomposition of the residue from the previous process.

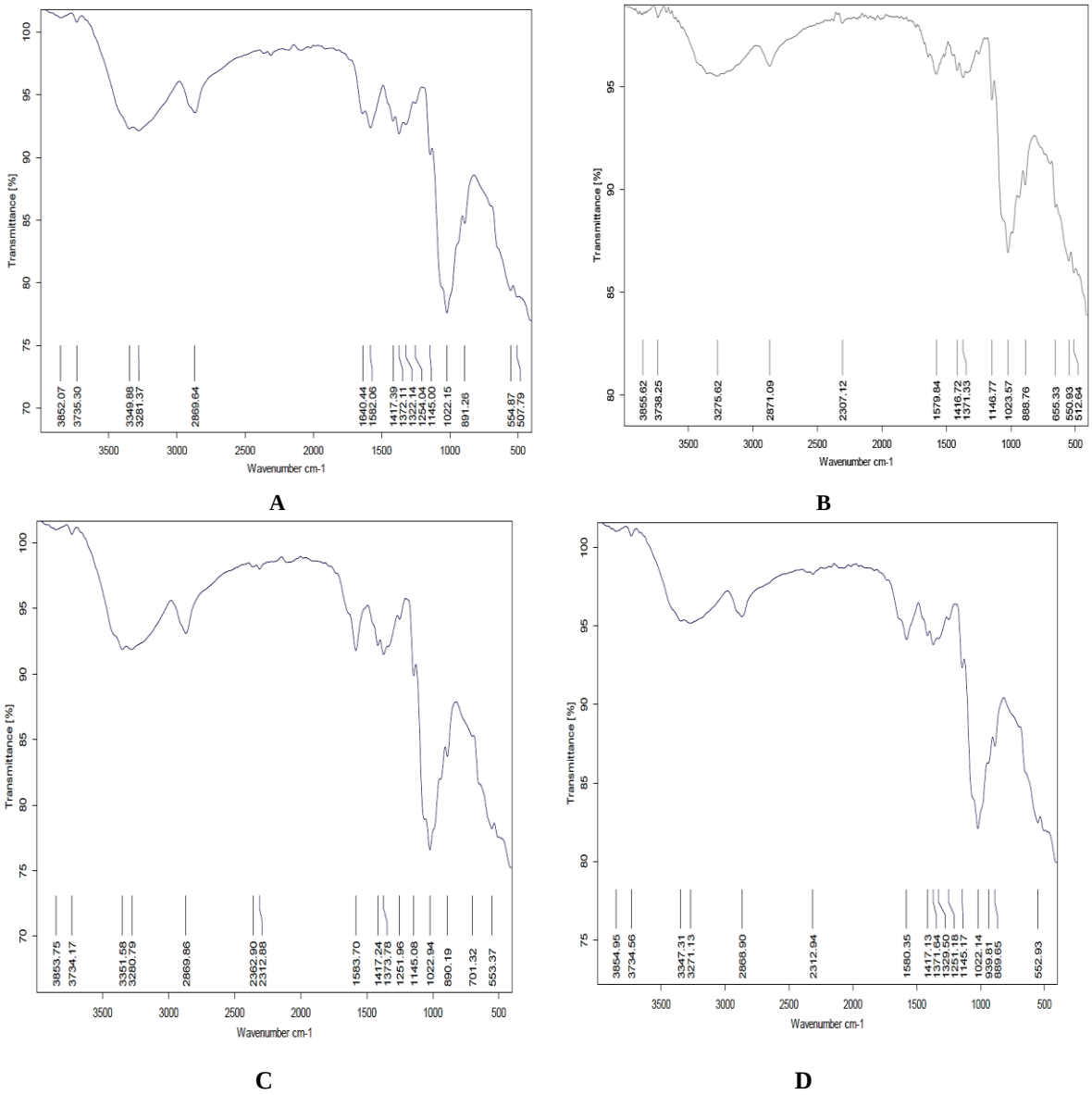

Fig. 3. FTIR analysis of chitosan extracted from upper and lower shells of *Squilla* species showing: **A.** Upper *O. massavensis* chitosan, **B.** Lower *O. massavensis* chitosan, **C.** Upper *Squilla mantis* chitosan, and **D.** Lower *Squilla mantis* chitosan

Weight losses in upper and lower *S. mantis* chitosan (Fig.4C, D) occurred in two stages. The first stage at ~23-102, ~26-107 °C caused 9.3, 9.0 % weight loss in upper and lower *S. mantis* chitosan samples, respectively, while the 2nd stage at ~102-199 and 107-199°C caused weight loss (1.8, 1.6%) in upper and lower *S. mantis* chitosan samples, respectively. All extracted chitosan samples showed to be heat stable as up to 200°C; the samples' losses did not exceed a total of 12% of their initial weights (Fig. 4C). Generally, the TGA curves showed that chitosan samples had excellent thermal stability (200°C), and they can be applied in high-temperature food processing.

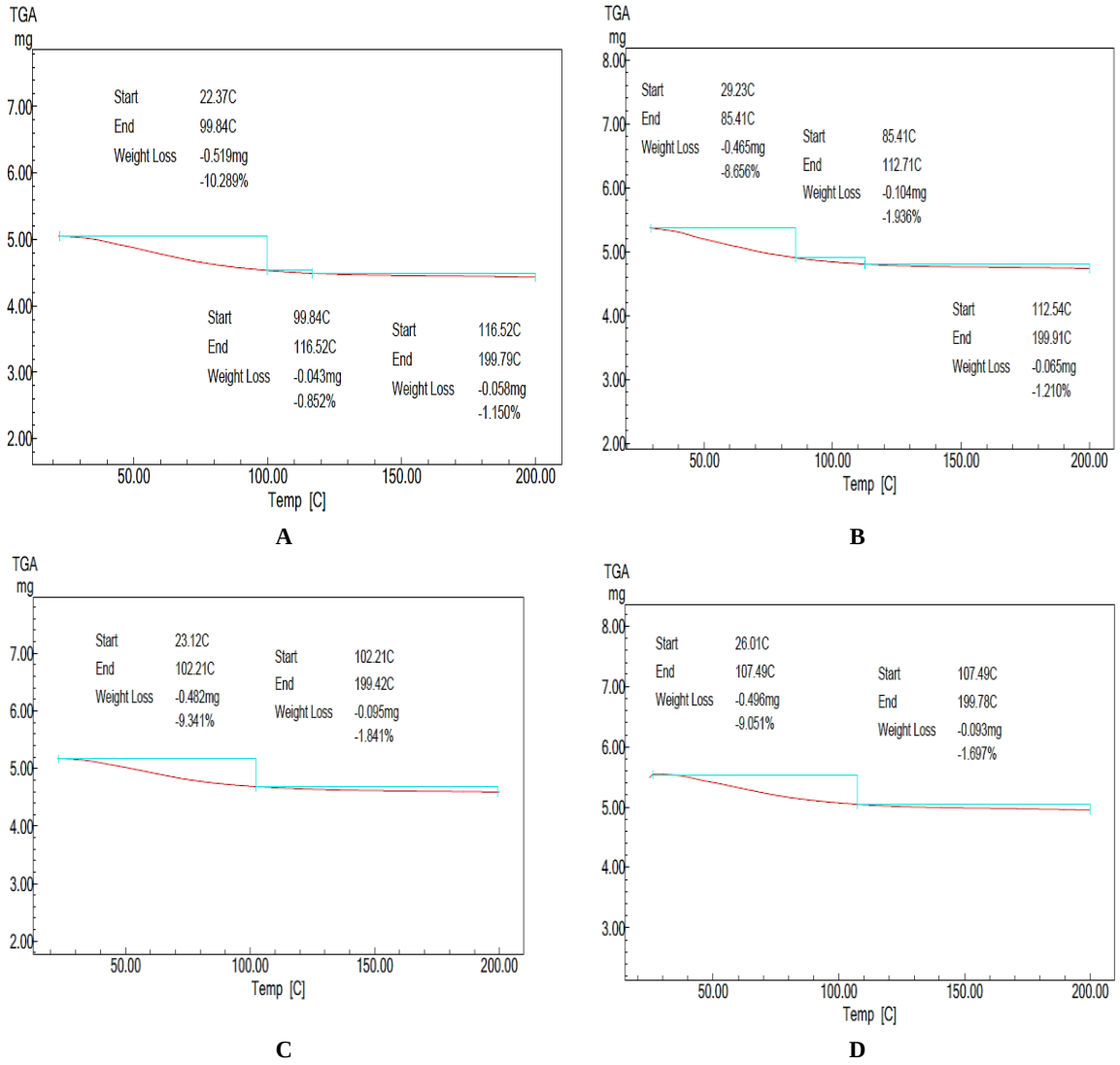

Fig. 4. Heat stability via thermogravimetric analysis (TGA) of chitosan extracted from upper and lower shells of *Squilla* species showing: **A.** Upper *O. massavensis* chitosan, **B.** Lower *O. massavensis* chitosan, **C.** Upper *S. mantis* chitosan, and **D.** Lower *S. mantis* chitosan

2.1.9. Antioxidant activity

The results in Table (4) reflect the antioxidant scavenging potentials represented as IC_{50} (mg/ mL), the inhibitory concentration at which 50% of DPPH radicals are scavenged. Lower shells chitosan has a higher radical-scavenging activity ($IC_{50,}$ 7.52-10.89) than upper shells chitosan ($IC_{50,}$ 14.65- 16.29) of two squilla species investigated. Additionally, lower shells chitosan of *S. mantis* was higher in radical scavenging activity than that of the same part for *O. massavensis*. Therefore, lower shells chitosan (7.52) of *S. mantis* was the best radical scavenging activity, followed by lower (10.89) and upper

(14.65) shells chitosan samples of *O. massavensis*, and upper shells chitosan (16.29) of *S. mantis*.

This variation in antioxidant activity compared with other studies is closely attributed to the MW of chitosan. Similar trend was decided by **Kim and Thomas (2007)**; antioxidative effect of chitosan concentrations (0.2, 0.5, and 1.0%, w/ w) in salmon was affected by the MW (30, 90 and 120KDa). Moreover, the MW (30kDa) was the highest radical-scavenging activity. Although scavenging activities of chitosan increased with increasing its concentration, no significant effects were detected when 120KDa was found.

Table 4. Antioxidant potentials of chitosan samples

Characteristic	Chitosan			
	O. massavensis		*S. mantis*	
	Upper	Lower	Upper	Lower
*IC_{50} (mg/ mL)	14.65±0.61	10.89±0.50	16.29±0.77	7.52±0.52

* IC_{50}: Half maximal inhibitory concentration.

In a higher MW chitosan, effect of intra-molecular hydrogen bonds is strong, and leads to weaken the activity of OH and NH_2 groups. Therefore, the lower the radicals scavenging activity suggests a potential restriction in the exposure of these active moieties. In contrary, high hydroxyl radical scavenging activity of lower MW chitosan is partially attributed to its metal chelating ability. The origin of the scavenging ability of chitosan activities is due to OH and NH_2 groups presented in the polymer chains **(Jeon *et al.*, 2000; No *et al.*, 2007; Feng *et al.*, 2008)**.

2.1.10. Antibacterial activity and minimum inhibitory concentration (MIC)

The antibacterial activity and MIC of chitosan extracted from upper and lower shells of *S. mantis* on pathogenic strains is shown in Table (5). All chitosan extracts showed antimicrobial effect against Gram positive/ negative tested pathogens with MIC values ranged from 25 to 50µL. The least antimicrobial effect recorded was of lower *O. massavensis* chitosan against *Salmonella* spp. with MIC of 100µL. The chitosan antimicrobial potentials were previously reported **by Junior *et al.* (2016)**. The effect of upper *O. massavensis* chitosan exhibited higher inhibition zones (IZ) for *Staph. aureus*, *Salmonella* spp., Vibrio *fluvialis*, and *E. coli* than its lower chitosan, while IZ values of *Cl. botulinum* were similar. In case of *O. mantis*, lower shells chitosan showed higher IZ for *Staph. aureus*, *Cl. Botulinum*, *Vibrio fluvialis*, and *E. coli* than upper chitosan, whereas IZ for *Salmonella* spp. was similar. The minimum inhibition concentration (MIC) for investigated chitosan samples recorded no general trend. Our results showed that the inhibition zones increased with increasing concentrations; 25, 50, 100ml sample/ ml acetic acid (1%).

Eminently, chitosan is considered as an alternative agent to synthetic chemical used in seafood preservation. The effect of chitosan as antimicrobial is due to numerous

factors as reported by several researches, such as **Raafat *et al.* (2008)**, **Raafat and Sahl (2009)** and **Kong *et al.* (2010)**. These factors include the microbial intrinsic factors, molecular intrinsic of chitosan, physical state, and environmental factors. On the practical side, most foods are composed of various compounds that can interact with each other, potentially leading to a loss or gain in its antibacterial activity **(Devlieghere *et al.*, 2004)**. Furthermore, both chitosan alone and its derivatives have been found to be more effective against $Gram^-$ than $Gram^+$ bacteria **(Kong *et al.*, 2010)**. On the other side, **Raafat and Sahl (2009)** showed that $Gram^+$ bacteria were higher susceptible than $Gram^-$ bacteria to chitosan. To address this variation, **Kong *et al.* (2010)** stated that various factors need to be taken into account when assessing the antimicrobial activity of chitosan.

CONCLUSION

Recently, squilla species has gained recognition as a promising shellfish, both aas a food or original source of bioactive compounds, such as chitin, chitosan, and also pigments compared with the past decades where they didn't have acceptance by most consumers. Therefore, squilla is a good substitute to obtain good characteristics of chitosan, especially when deacetylation process was repeated three times based on the results of this work compared with shrimp chitosan. In general, this study recommends the applicability of chitosan in high-temperature food processing.

Table 5. Effect of chitosan produced from upper and lower shells of *O. massavensis* and *O. mantis* on antibacterial activity and minimum inhibitory concentration (MIC) of pathogenic strains

Pathogenic strain	IZ (mm)** of upper and lower *O. massavensis* chitosan										IZ (mm)** of upper and lower of *O. mantis* chitosan									
	Upper 100	lower	Upper 50	lower	Upper 25	lower	Upper Control	lower	Upper MIC	lower	Upper 100	lower	Upper 50	lower	Upper 25	lower	Upper Control	lower	Upper MIC	lower
Gram-positive bacteria																				
Staph. aureus EMCC1351	32	28	30	25	27	19	12	11	25	25	22	25	21	22	19	13	11	11	25	25
Cl. botulinum ATCC3584	15	16	12	13	12	13	11	12	25	25	18	21	17	17	13	11	11	11	25	50
Gram-negative bacteria																				
Salmonella spp.	29	13	27	12	15	12	12	12	25	100	18	18	13	13	11	12	11	11	50	25
Vibrio fluvialis	24	17	20	15	18	12	12	11	25	25	20	24	18	18	15	15	11	11	25	25
E. coli BA12296	29	21	24	15	17	13	12	13	25	50	18	29	13	20	11	11	11	11	50	50

IZ: Inhibition zone diameter (mm)
MIC; minimum inhibition concentration, diameter included 4 mm well diameter.

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