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β-Chitin and chitosan from squid gladius: Biological activities of chitosan and its application as clarifying agent for apple juice

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ABSTRACT

Chitin is the second most abundant polysaccharide in biomass after cellulose and the term chitosan usually refers to a family of polymers obtained after chitin deacetylation. The aim of this work was the preparation and the characterization of chitin and chitosan from the gladius (pen) of the European squid (*Loligo vulgaris*). A high level of deproteinization (more than 80%) was recorded using Alcalase® with an enzyme/protein ratio of 10 U/mg. The demineralization of the gladius was completely achieved within 8 h at room temperature in HCl. ^{13}C NMR, FTIR, and XRD diffractograms of prepared chitin and chitosan were taken and then degree of deacetylation of chitosan was calculated using ^{13}C CP/MAS-NMR Spectroscopic. Further, *in vitro* antioxidant capacity of chitosan was evaluated on 1,1-diphenyl-2-picrylhydrazyl method (IC_{50} = 3.2 mg mL^{-1}) and the β-carotene bleaching assay (IC_{50} = 3.3 mg mL^{-1}). Antimicrobial activity was also investigated and assays indicated that prepared chitosan exhibited marked inhibitory activity against all microbial strains tested. Additionally, chitosan was tested such as clarifying agent for apple juice and showed powerful clarification capability, without affecting nutritional value. Furthermore, the results suggested that prepared chitosan could be used as alternative additive in pharmaceutical preparations and food industry.

1. Introduction

A significant increase in the amount of waste resulting from the industrial processing of seafood has become a problem, both for the environment and for the processing plants [1]. The new fisheries management constraint is how to adopt new development strategies by-products. Currently, they are mostly converted into the meal and oil but it is a low value added processing. Improved economic performance for a better application of the by-products is therefore necessary. Hence, the management of fishery resources and new product development using underutilized species and processing discards is very important in the world's fishing industry. Although there are many approaches to further utilization of these resources, interest has been expressed in isolating value-added components from such raw material [2,3]. In recent years, recovery and characterization of chitin and chitosan from fishing by-products has taken place, and this has led to the emergence of some interesting new applications of these molecules.

Chitin is a copolymer of *N*-acetyl glucosamine and *N*-glucosamine residues linked via β-1,4 glycosidic bonds, with a high fraction of acetylated residues. It is the second most important natural biomaterial in the world, next to cellulose. Chitin can be obtained from the cell wall of fungi, the exoskeleton of arthropods, the radula of mollusks, and the internal shells and the beaks of cephalopods including cuttlefish, octopuses and squids [4]. Chitin, on the other hand, is relatively difficult to process and due to its insolubility in aqueous solution, has become less "accessible" to biological laboratories and has been a stumbling block in its appropriate utilization.

Chitosan is a deacetylated form of chitin having D-glucosamine repeating units linked by β (1-4) glycosidic bond. It is soluble in aqueous acidic medium due to the presence of amino groups. Chitosan is characterized by its degree of *N*-acetylation and this degree influences not only its physicochemical characteristics but also its biological properties. Chitosan is considered as a versatile biopolymer that can be developed into different forms, such as gels, films and nanoparticles [5]. Due to its solubility, biodegradable,

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biocompatible, non-toxic and non-antigenic properties, chitosan has a wide range of applications in diverse fields, ranging from waste management to in agriculture, textiles, food processing, cosmetics, medicine and biotechnology. Chitosan has so far attracted notable research interest and numerous works can be found in the literature [6,7].

The squid *Loligo vulgaris* is a large squid belonging to the family Loliginidae. It is found throughout the Mediterranean and in the eastern Atlantic Ocean. Its skeleton is represented by a chitin structure called gladius or pen that generally is a by-product produced in the industrial processing of squids. The species is extensively exploited by commercial fisheries [1]. Considering the potential for use, the present study was undertaken to extract chitin from the gladius of squid (*L. vulgaris*) using an eco-friendly process based upon demineralisation by acid treatment and enzymatic deproteinization by Alcalase. Also, we describe for the first time the antioxidant and antimicrobial activities of prepared chitosan and its potential application in the clarification of apple juice was also tested.

2. Material and methods

2.1. Reagents

The chemicals and solvents used in the present study were purchased at the analytical grade or highest level of purity available. Alcalase® 2.4 L was obtained from Novozymes® (Bagsvaerd, Denmark). All chemicals and reagents were used at the analytical grade, and all solutions were freshly prepared in distilled water.

2.2. Raw material

Squid gladius (averaged length ≈ 12 cm; averaged width ≈ 1 cm) were obtained from a marine processing industry "CALEMBO", Tunisia. The gladius were packed in polyethylene bags, placed in ice and transported to the research laboratory. The gladius were washed twice with water, dried, pulverized using pestle and mortar and stored at −20 °C until further use.

2.3. Extraction of chitin from gladius

2.3.1. Enzymatic deproteinization

Squid gladius were mixed with water at a ratio of 1:3 (*w/v*), minced then cooked for 20 min at 90 °C. The cooked sample was then homogenized in a homogenizer (MAMMONLEX, R.O.C, model no JW. 1001, Taiwan). The pH and temperature of the mixture were adjusted to 8.0 and 50 °C, respectively. Then, the sample proteins were digested with Alcalase® using different enzyme/protein (E/S) ratio (Units of enzyme/mg of protein). After incubation for 3 h at 50 °C, the reaction was stopped by heating the solution at 90 °C during 20 min. The solid phase was washed and then dried at 50 °C. Deproteinization (DP) was expressed as percentage and computed by the following equation as described by Rao et al. [8].

$$DP(\%) = \frac{(P_o \times O) - (P_R \times R)}{P_o \times O} \times 100$$

where P_O and P_R are protein concentrations (%) before and after hydrolysis; while, O and R represent the mass (g) of original sample and hydrolyzed residue in dry weight basis, respectively.

2.3.2. Demineralization

Demineralization was carried out in HCl medium according to a method developed by Madhavan and Nair [9]. Solid fractions obtained after hydrolysis were treated with HCl (1.5 M) at a ratio of 1:10 (w/v) for 2, 4, 6 and 8 h at 50 °C under constant stirring. The chitin residues were filtered through four layers of gauze with the aid of a vacuum pump, washed to neutrality with de-ionized water and then drying in a dry heat incubator at 50 °C. Degree of demineralization (DDM) was expressed as a percentage and computed by the following equation [8]:

$$\%DDM = \frac{[(A_O \times O) - (A_R \times R)]}{A_O \times O} \times 100$$

where, A_O and A_R are ash contents (%) before and after demineralization, respectively. While O and R represent the mass (g) of initial and demineralized residue respectively on dry weight basis.

2.4. Deacetylation of chitin: preparation of chitosan

The chitin was treated with 50% (w/v) NaOH at 120 °C for 4 h until it was deacetylated to chitosan perfectly soluble in might acidic conditions. After filtration, the residue was washed with distilled water and the crude chitosan was obtained by drying in a dry heat incubator at 50 °C overnight.

2.5. Physicochemical characterization

2.5.1. Chemical composition

Moisture and ash contents were determined according to the AOAC standard methods 930.15 and 942.05, respectively [10]. Protein contents in the gladius of *L. vulgaris* and crude chitin were determined (in three replicates) by extracting (0.5 g/50 mL) samples with 10% (w/v) NaOH for 20 min at 120 °C. The supernatant was retained by filtration and diluted to 100 mL with distilled water. This extract was used for protein determination (total nitrogen contents × 6.25) according to the Kjeldahl procedure. Crude fat was determined gravimetrically after the Soxhlet extraction of dried samples with hexane.

2.5.2. Water activity

Water activity (aw) was measured at 25 °C by a NOVASINA aw Sprint TH-500 apparatus (Novasina, Pfäffikon, Switzerland).

2.5.3. Determination of color

The samples were placed between two steel dishes with a hole of 5.7 cm diameter. The color of films was determined with a tristimulus colorimeter (CHROMA METER CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale (C/2°), where L^*, a^* and b^* refer to the parameters measuring lightness, redness, and yellowness, respectively. A standard white plate was used as a reference.

2.5.4. Infra-red spectroscopic analysis

The absorption spectra of the samples were obtained using FTIR spectroscopy (Analect Instruments fx-6 160). The FTIR spectra of the prepared materials were recorded between 400 and 4000 cm^{-1} in a NICOLET spectrometer. The transmission spectra of the samples were recorded using the KBr pallet containing 0.1% of sample.

2.5.5. ^{13}C CP/MAS-NMR spectroscopic analysis

Samples structural analysis was carried out by 13C NMR with CP/MAS technique (cross-polarization, magic-angle-spinning) using a BRUKER-ASX300 instrument. NMR spectra were recorded at a ^{13}C frequency of 75.5 MHz (field of 7.04 T). CP/MAS sequence was used with the following parameters: the ^{13}C spin lattice relaxation time was 5 s, powdered samples were placed in an alumina rotor used for the double air-bearing-type MAS system and spun as fast as 8 kHz. Contact time was 8 ms.

The degree of acetylation (DA) of the sample was determined by dividing the intensity of the resonance of the methyl group carbon by the average intensity of the resonances of the glycosyl ring car-

bon atoms. The DA was calculated using the following relationship Ottøy et al. [11]:

$$DA(\%) = \frac{100 \times I[CH3]}{(I[C1] + I[C2] + I[C3] + I[C4] + I[C5] + I[C6])/6}$$

where, ***I*** is the intensity of the particular resonance peak.

2.5.6. X-ray diffraction (XRD)

The X-ray diffraction patterns of samples were recorded at room temperature on an X-ray diffractometer (D8 advance, Bruker, Germany). The data was collected in the 2θ range 10–60° with a step size of 0.02° and a counting time of 5 s/step. The crystallinity index (CI) of samples was calculated using the following expression [12]:

$$CI(\%) = ((I_{110} - I_{am})/I_{110}) \times 100$$

where I_{110} is the maximum intensity of the diffraction peak at 2θ = 19.4° and I_{am} correspond to the amorphous diffraction intensity at 2θ = 12.6°.

2.6. Determination of antioxidant activity

2.6.1. Free radical-scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity of prepared chitosans was determined as described by Bersuder et al. [13]. A volume of 500 μL of each sample, at different concentrations (1–5 mg mL^{-1}) in 0.1% acetic acid, was mixed with 375 μL of 99.5% ethanol and 125 μL of 0.02% DPPH in 99.5% ethanol. The mixtures were then incubated for 60 min in the dark at room temperature and the reduction of DPPH radical was measured at 517 nm. A control was conducted in the same manner, except that distilled water was used instead of chitosan sample. In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by antiradical compounds. DPPH radical-scavenging activity was calculated as follows:

$$\text{Radical-scavenging activity}(\%) = \frac{\text{Absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.6.2. The ß-carotene bleaching method

The ability of chitosan samples to prevent the bleaching of β-carotene was determined as described by Koleva et al. [14]. A stock solution of β-carotene/linoleic acid was prepared by dissolving 0.5 mg of β-carotene, 25 μL of linoleic acid, and 200 mg of Tween-40 in 1 mL chloroform. The chloroform was completely evaporated under vacuum in a rotatory evaporator at 45 °C, then 100 mL distilled water was added, and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. Aliquots (2.5 mL) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 mL of each chitosan sample at different concentrations in 0.1% acetic acid. The tubes were immediately placed in a water bath and incubated at 50 °C for 2 h. Thereafter, the absorbance of each sample was measured at 470 nm. 0.5 mL of distilled water instead of the chitosan solution is used as negative control

2.7. Antimicrobial activity

The antibacterial activities of the chitosan were tested against 8 strains of bacteria, namely four Gram-negative (*Salmonella enterica* ATCC 43972, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 13883) and four Gram-positive (*Micrococcus luteus* ATCC 4698, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 and *Bacillus* cereus ATCC 11778) bacteria. The antifungal activities were tested using *Fusarium solani*, *Botrytis cinerea*, and *Alternaria solani*. Antimicrobial activity assays were performed according to the method described by Berghe and Vlietinck [15]. Chitosan sample (40 mg mL^{-1}) was prepared under stirring in 0.1% acetic acid. Culture suspensions (200 μL) of the tested microorganisms (10^6 colonyforming units mL^{-1} of bacteria cells and 10^8 spores mL^{-1} of fungal strains) were spread on Muller–Hinton agar and Potato Dextrose Agar medium, respectively. Then, bores (3 mm depth, 5 mm diameter) were made using a sterile borer and loaded with 50 μL of chitosan sample. A well with 50 μL of 0.1% acetic acid was used as a negative control. The Petri dishes were kept, first for 3 h at 4 °C, and then incubated for 24 h at 37 °C for bacteria and 72 h at 30 °C for fungal strains. Antimicrobial activity was evaluated by measuring the diameter of growth inhibition zones in millimeters.

Table 1
Proximate composition of the gladius of *L. vulgaris*. Values represent averages ± standard deviations for triplicate experiments.

Protein (%)	Fat (%)	Ash (%)	Chitin (%)	Color
36.52 ± 0.61	0.32 ± 0.04	2.57 ± 0.01	31.2 ± 1.53	Transparent

2.8. Clarification of apple juice

Apples were purchased from a local market in Sfax city and washed with tap water. Juice was extracted from the fruits by homogenizing in a homogenizer (MAMMONLEX, R.O.C, model no JW. 1001, Taiwan) for 5 min followed by filtration through cheese cloth. To each 45 mL of fruit juice, 5 mL of different concentrations of chitosan solutions were added [16]. Control tube received only 5 mL of water instead of chitosan solution. Tubes were then incubated at room temperature for one hour. Titratable acidity was measured by titrating 10 mL of juice with 100 mM NaOH using phenolphthalein as indicator. The turbidity of the supernatant fractions was determined as percent transmittance (% T) at 660 nm. Reducing sugar was estimated using DNS method. Ascorbic acid was determined titrimetrically using 2,6-dichlorophenolindophenol [16].

2.9. Statistical analysis

Analytical values were determined by using three independent determinations. The values of the different parameters were expressed as the mean (n = 3). The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 11.0. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Composition of European squid gladius

The proximate composition of the gladius of European squid (*L. vulgaris*) is shown in Table 1. Gladius showed high chitin content (31.2%). Similar result was reported by Cortizo et al. [17] for the gladius of *Illex argentines* (31%). The chitin contents of squids gladius were around of 25.5% and 39.7%, for *Todarodes pacifica* [18] and *Sepioteuthis lessoniana* [19], respectively. The ash content of *L. vulgaris* gladius was 2.57%. Lavall et al. [20], Tolaimate et al. [21] and Cuong et al. [22] were reported that the ash contents of squids gladius were 1.9%, 1.7% and 1.37%. Results of the proximate analysis indicate also that squid (*L. vulgaris*) gladius could serve as an excellent source of protein (36.52%). In compared to previous works, there is a different amount of protein in squid gladius, which is attributed to different squid species [18,19,22]. Cortizo et al. [17] reported that

Table 2
Proximate composition of chitin extracted from the gladius of *L. vulgaris*. Values represent averages ± standard deviations for triplicate experiments.

	Chitin
Protein (%)	3.05 ± 0.36
Fat (%)	ND
Ash (%)	0.09
aw	0.308 ± 0.02
Color	Whitish–light
L^*	90.01 ± 0.2
a^*	−1.26 ± 0.01
b^*	8.42 ± 0.28
Yield[a] (%)	21.65 ± 1.33

Physicochemical composition was calculated based on the dry mater.
ND: none detected.
[a] Calculated based on wet weight of gladius.

the squid (*I. argentinus*) gladius contain were 1% of ash, 2.3% of fat, 64% of protein and 31% of chitin.

3.2. Extraction of chitin from gladius

Two successive steps (enzymatic deproteinization and demineralization) were used for chitin extraction and then chitosan was prepared by *N*-deacetylation. The extraction of chitin and preparation of chitosan scheme is presented in Fig. 1.

3.2.1. Deproteinization

For efficient chitin extraction, associated proteins should be removed. Chemical treatment can cause deacetylation and depolymerization of chitin. Many reports have demonstrated the application of proteases for the deproteinization of marine sources to produce chitin [7,23,24]. Deproteinization treatments were conducted for 3 h at pH 8.0 and 50 °C using Alcalase® which contains both endoprotease and exoprotease activities, which offer the ability to achieve higher deproteinization degree. Previous studies reported that enzymatic deproteinization was found to be dependent on the enzyme/substrate (E/S) ratio. Thus, E/S ratios between 0.5 and 15 were used to compare the deproteinization efficiency. As shown in Fig. 2A, the percentage of protein removal increased with increasing E/S ratio (32.2 ± 25% using 0.5 U/mg; 74.85 ± 1% using 5 U/mg). Beyond a ratio of 10, no significant increase in the deproteinization rate was observed (80.2 ± 1.2% and 81.06 ± 1.7% for 10 and 15 U/mg, respectively). The deproteinization activity of Alcalase® was better than many other proteases reported in previous studies [24,25]. The fact that deproteinization cannot reach 100% may be explained by the non-accessibility of enzymes to some proteins protected by chitin.

3.2.2. Demineralization

One of the factors determining the good quality of chitin is the low mineral content. Thus, to extract chitin from the gladius, associated minerals should be removed. As a consequence, the by-products were treated with HCl for 2, 4, 6 and 8 h at 50 °C under constant stirring in order to remove minerals. The results presented in Fig. 2B shows that the demineralization of gladius, deproteinized by enzymatic treatment, increased with the demineralization time (40.3, 72.38 and 91.51% after 2, 4 and 6 h, respectively). The most efficient demineralization time was 8 h (96.05 ± 1.7%).

3.3. Chitin characterization

Table 2 shows the proximate composition of the chitin extracted from squid (*L. vulgaris*) gladius. Low protein content was obtained after enzymatic deproteinization, using Alcalase®. The protein content of chitin extracted from the gladius of *L. chenisis* was 0.63% [22]. The demineralization reduces the mineral content in gladius to

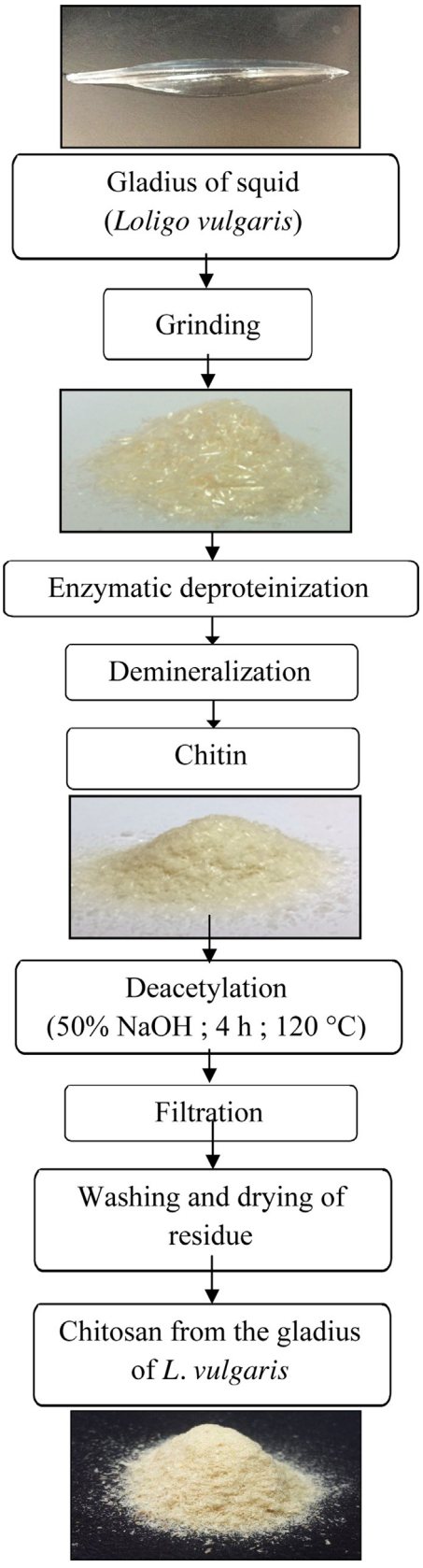

Fig. 1. Flow diagram for the preparation process of chitin and chitosan from the gladius of squid (*L. vulgaris*).

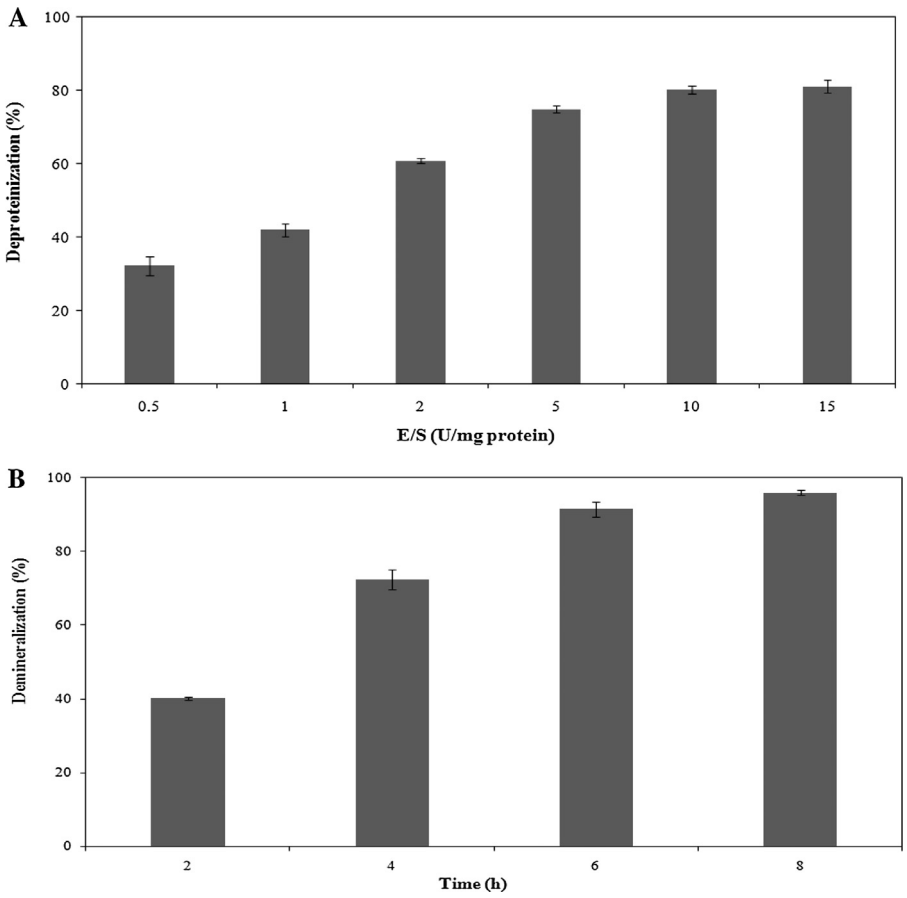

Fig. 2. Chitin extraction: Effect of the E/S ratio on the deproteinization of gladius using Alcalase® (A) and chemical demineralization of deproteinized gladius (B).

permissible limits in the chitin. Indeed, the ash content was reduced to 0.09%. Subhapradha et al. [19] reported that β-chitin extracted from *S. lessoniana* has negligible minerals content. Interestingly, extracted chitin shows a negligible amount of fat. Similar result was reported by Cuong et al. [22]. The chitin extraction yield was 31.27% on the basis of wet weight. This yield was similar to those obtained from shrimp waste by Hajji et al. [23]. In comparison, it is lower than that from the gladius of *L. lessoniana* (36.06%) [26]. The color values of extracted chitin expressed in terms of L^* (lightness), a^* (redness) and b^* (yellowness) are also shown in Table 2. Result indicated that extracted chitin had a white and bright color. In fact, color influences the overall acceptability of products. Chitin extracted from the gladius of L. vulgaris showed low water activity (0.327), which is known to prevent bacteria, mould and yeast development.

FTIR spectrum was performed in the 4000–400 cm^{-1} region to further characterize the extracted chitin. As shown in Fig. 3A, The β-chitin polymorphic form was confirmed by a unique band at 1660 cm^{-1} assigned to the stretching vibration of C=O group (amide I) hydrogen bonded to N—H of the neighboring intra-sheet chain. Furthermore, the existence of following bands allows to characterize the polysaccharide [27]: 3420 cm^{-1} (O—H) a broad band which is attributed to the intra molecular hydrogen bond —O—H—O— from the ring, 1560 cm^{-1} (N—H, amide II), 1367 cm^{-1} (C—H, CH_3), 1312 cm^{-1} (C—N and N—H, amide III), 1160 cm^{-1}, 1106 cm^{-1}, 1058 cm^{-1} and 1025 cm^{-1}, are assigned to the C—O—C and C—O stretching vibrations modes [17]. Besides, other bands were identified at 2916 cm^{-1} (C—H) and 652 cm^{-1} (O—H bending). All detected bands were in agreement with those reported by Cortizo et al. [17] for β-chitin from *I. argentinus* squid gladius. The squid gladius is mainly composed by β-chitin in association with proteins. β-Chitin is less abundant than α-chitin but could be obtained in an important amount from squid gladius [17]. β-Chitin was found to show higher solubility, higher reactivity and higher affinity toward solvents and swelling than α-chitin [26]. These characteristics are due to weaker intermolecular hydrogen bonding ascribable to the parallel arrangement of the main chains.

^{13}C CP/MAS-NMR spectrum of the chitin extracted from the gladius of *L. vulgaris* is shown in Fig. 3B. Seven signals are assigned to the eight carbon atoms of the *N*-acethylglucosamine repetitive unit which appear at the following chemical shifts: δ = 173.1 (C=O), 104.2 (C-1), 85.0 (C-4), 75.6 (C-3 and C-5), 59.7 (C-6), 55.6 (C-2) and 23.0 (CH_3). The C=O signal appears as a sharp and symmetric profile, indicating a unique conformational state, typical of a β-chitin structure [17]. The signals of C-3 and C-5 merge into single resonance centered at 75.6 ppm, while in the α-chitin spectra that signals were reported to appear as a doublet [27]. The differences between the two polymorphous (α- and β-chitin) were attributed to different C-3 and C-5 configurations resulting from the hydrogen bonds established. The degree of deacetylation of the chitin extracted from the gladius of *L. vulgaris* was determined as described by Ottøy et al. [11]. This degree was calculated as 3.8%. Different degree of deacetylation values from chitin extracted of other sources have been reported: 4% for gladius of *I. argentinus* squid [17], 19.7% for gladius of *L. chenisis* squid [22], and 19.2–4.6% for gladius of *Loligo sp*. squid [20]. These differences in the degree of acetylation could be attributed to the different isolation conditions of chitin, due to the demineralization step or due to the deproteinization step [17].

The crystalline structure of the chitin isolated from the gladius of *L. vulgaris* was analyzed by X-Ray diffraction. Fig. 3C, shows the corresponding diffractogram which exhibits two broad peaks at

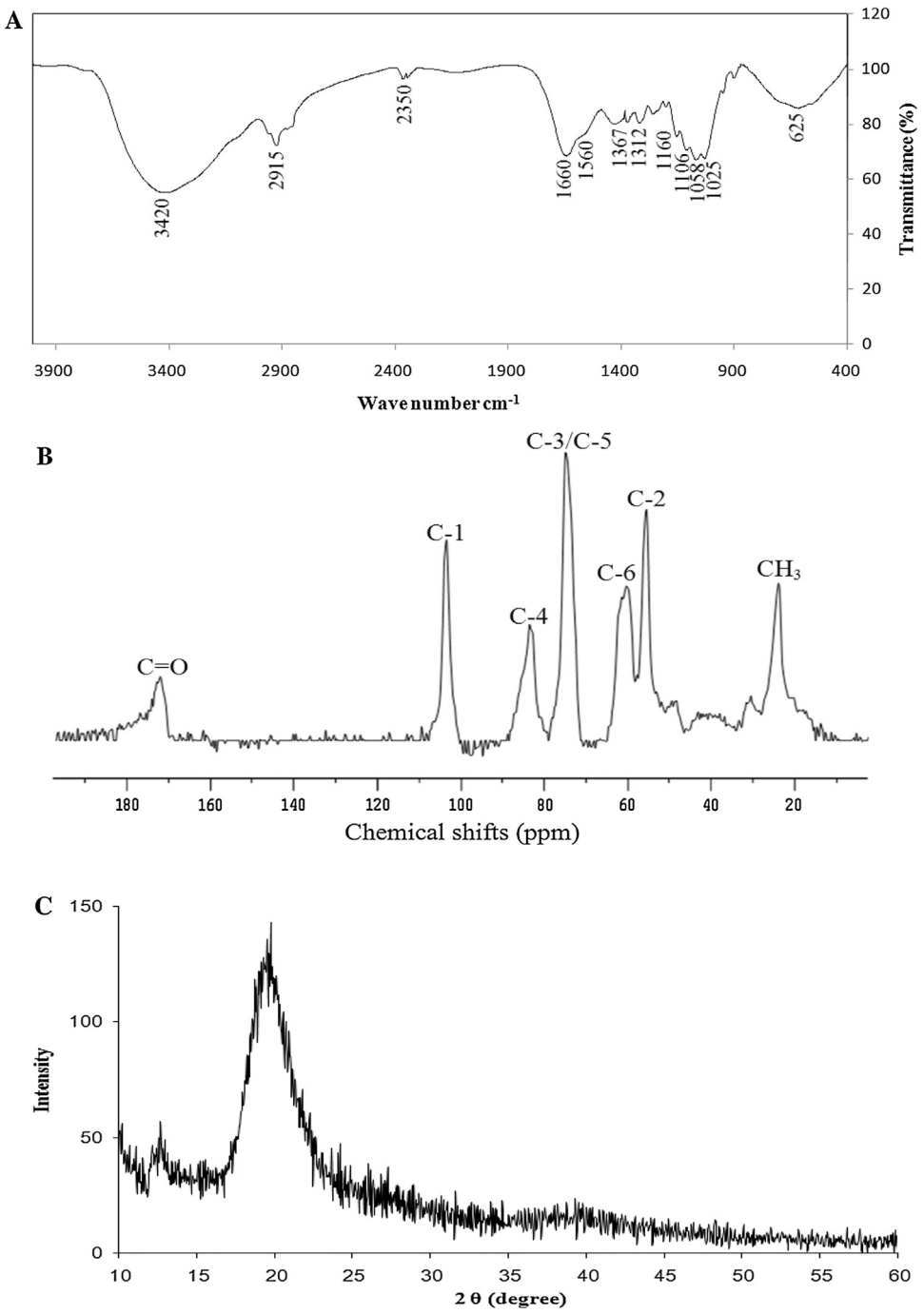

Fig. 3. Characterization of β-chitin extracted from the gladius of *L. vulgaris*: FT-IR spectrum (A), solid state ^{13}C NMR spectra (B) and X-ray diffraction pattern(C).

2θ = 12.6° and 2θ = 19.4°.The crystallinity index (CI) of the extracted chitin was determined as described by Focher et al. [12]. In the present work, the crystallinity index of the β-chitin from the gladius of squid (*L. vulgaris*) was 57.8%. This index is lower than that reported by Cortizo et al. [17] for β-chitin from *I. argentinus* squid gladius (CI = 74.9%).

3.4. Chitosan preparation and characterization

Chitosan is obtained by partial deacetylation of chitin and composed of glucosamine and *N*-acetylglucosamine. The chitosan extraction yield was 19.52% on the basis of wet weight of squid gladius. Chandumpai et al. [26] reported an extraction yield 28% (The value was calculated on the basis of the weight of dried squid gladius powder). Hajji et al. [28] reported that the chitosan extraction yields from shrimp, crab and cuttlefish were 14.9%, 5.3% and 1.2%, respectively.

The process of deacetylation involves the removal of acetyl groups to NH_2 on C-2 glucosamine position. Conventionally, a deacetylated chitin with a rate of 70–90% and low protein content is considered as a good final product for application. ^{13}C CP/MAS-NMR spectrum of the chitosan prepared from the gladius of *L. vulgaris* is shown in Fig. 4A. The deacetylation degree of the prepared chitosan was determined as 71%. This deacetylation degree was similar to that obtained by Hajji et al. [23] for chitosan from cuttlefish bones (70.1%) but lower than that obtained by Younes et al. [29] for chi-

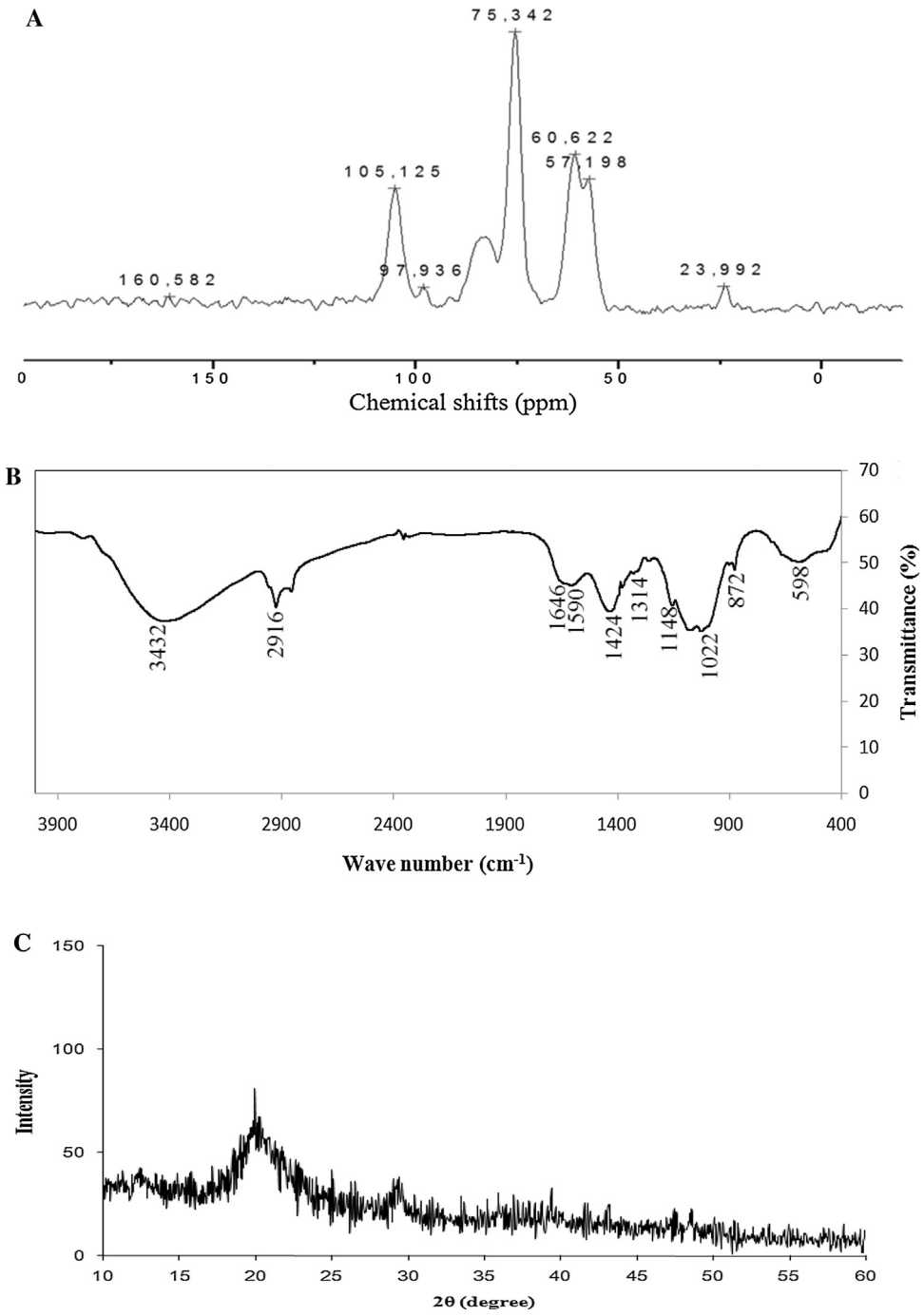

Fig. 4. Chitosan prepared from squid gladius: Solid state ^{13}C NMR spectra (A), Infrared spectra (B) and X-ray diffraction pattern (C).

tosan from shrimp shell. Subhapradha et al. [19] reported that the deacetylation degree values of chitosan prepared from the gladius of *S. lessoniana* and commercial chitosan were 88.2% and 88.3%, respectively. Deacetylation degree is highly dependent on the morphology and the source of chitin (larger reactivity of the β-form). As shown in Fig. 4A, there is no peak at 173 ppm that correspond to the carbonyl group (C=O). The residual methyl group (CH_3) appears at around 23 ppm. The lower the signal of methyl group is, the more efficient the deacetylation reaction is. The other peaks correspond to C-1, C-2, C-3, C-4, C-5 and C-6. Note in the spectrum that the removal of proteins were efficient during the extraction, once there are no other peaks, suggesting a great purity of the product.

Fourier transform infra-red (FT-IR) spectroscopy was performed in the 400–4000 cm^{-1} region to further characterize the prepared chitosan (Fig. 4B). According to this spectrum, the bands at the range of 3432 cm^{-1} were attributed to the stretching vibration of O—H and N—H. The bands at 1646 cm^{-1} and 1314 cm^{-1} were referenced as amides I and III, respectively [30]. The amide II band was at 1590 cm^{-1} [30]. The bands at 1148 cm^{-1} and 1022 cm^{-1} were groups of C—O—C and C—O. The band at 872 cm^{-1} was assigned to the absorption peaks of β-(1,4) glycosidic in chitosan. The out plane bending —OH vibrations are observed at 670 cm^{-1}.

The X-ray diffraction pattern for the chitosan of the *L. vulgaris* gladius was given between 10 and 60 of 2 θ in Fig. 4C. The prepared chitosan has only one peak at $2\theta = 19.9^{\circ}$ (crystalline peak).

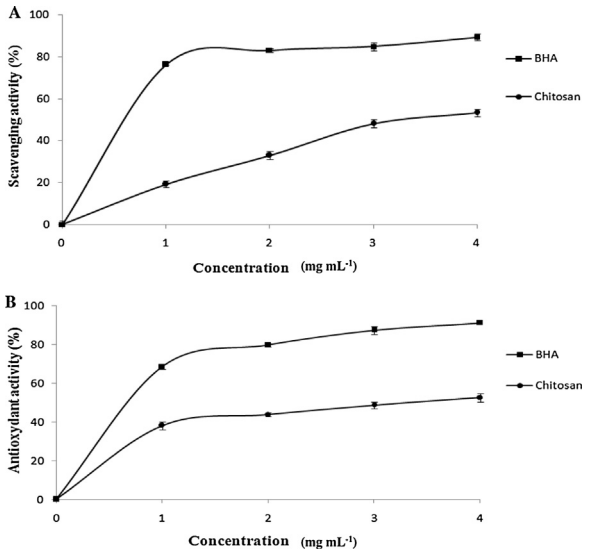

Fig. 5. Antioxidant activities of prepared chitosan at different concentrations: DPPH free radical-scavenging activities (A) and inhibition of β-carotene bleaching (B). Values are means of three replications ± SD.

Similar result was described by Yen et al. [31] for the extracted crab chitosan (One crystalline peak at 2θ = 19.4°).

3.5. Antioxidant activity of chitosan

Antioxidant is defined as any substance that significantly delays or inhibits oxidation of a substance when present at low concentrations compared to that of an oxidizable substrate. Chitosan has been known to have different antioxidant mechanisms, such as free radical scavenging ability, chelating ability, and reducing ability [32]. To evaluate antioxidant actions, we conducted tow *in vitro* antioxidant assays: the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging and the β-carotene bleaching inhibition.

3.5.1. DPPH radical-scavenging capacity

In this study, DPPH was used to determine the proton scavenging activity of the prepared chitosan. DPPH radical scavenging capacities of chitosan prepared from the gladius of *L. Vulgaris* and a positive control (BHA) are shown in Fig. 5A. The prepared chitosan showed a DPPH free radical scavenging activity in the range of concentrations tested. This scavenging ability was increased with their concentration increased. Similar result was reported by Youn et al. [18] for the chitosan from squid (*T. pacifica*) gladius. In the present investigation, the chitosan was a radical scavenger with an IC_{50} of 3.2 mg mL^{-1}. This activity was significantly ($p < 0.05$) lower to that of BHA. At 4 mg mL^{-1}, the scavenging capacity of prepared chitosan was 48.24%. At the same concentration, Mahdy et al. [33] reported that the scavenging capacity of chitosan extracted from shrimp wastes was in the range of 20.54–36.43% and that of commercial chitosan was 77.84%. The chitosan from cuttlebone *Sepia kobiensis* showed the scavenging ability of 46.17% at 10 mg mL^{-1} on DPPH radicals [7]. Chitosan may eliminate various free radicals by the action of nitrogen on the C-2 position. The scavenging mechanism of chitosan is related to the fact that the free radicals can react with the hydrogen ion from the ammonium ions (NH_3^+) to form a stable molecule [34].

3.5.2. β-carotene bleaching inhibition assay

As displayed in Fig. 5B, Antioxidant activity of prepared chitosan was analyzed using the β-carotene–linoleate bleaching inhibition assay in comparison with BHA at different concentrations (1; 2; 3 and 4 mg mL^{-1}). Antioxidant activity of chitosan increased with increasing chitosan concentration. At 1 and 4 mg mL^{-1}, the antioxidant activity of chitosan was 38.17% and 52.56%, respectively, while those of BHA were 68.6% and 91.16%, respectively. Younes et al. [29] reported that thye antioxidant capacity of chitosan extracted from shrimp wastes (chitosan-C) was 33% at 1 mg mL^{-1}. The prepared chitosan was an antioxidant activity with an IC_{50} of 3.3 mg mL^{-1}. Hajji et al. [28] reported that, at 5 mg mL^{-1}, the ability of cuttlefish, crab and shrimp chitosans to prevent bleaching of β-carotene were 82.3, 70 and 48.6%, respectively. These results demonstrated that chitosan prepared from the gladius of *L. Vulgaris* was strong effect against the discoloration of β-carotene and that this chitosan has a potential use as scavengers of free radicals in emulsion-type systems and can be explored as novel potent antioxidant in food industry.

Table 3
Diameters of inhibition zones against different bacteria and fungi in the presence of prepared chitosan. Inhibition zones: +++: >1.5 cm; ++: 0.5–1.5 cm; +: <0.5 cm; –: no activity.

Microorganisms		Chitosan (40 mg mL^{-1})
Gram +	*Bacillus cereus*	+
	Staphylococcus aureus	++
	Enterococcus faecalis	–
	Micrococcus luteus	++
Gram -	*Escherichia coli*	++
	Salmonella enterica	+
	Pseudomonas aeruginosa	–
	Klebsiella pneumoniae	+++
Fungi	*Fusarium solani*	++
	Botrytis cinerea	+++
	Alternaria solani	++

Acetic acid 0.1% as negative control.

3.6. Antimicrobial activities of chitosan

Chitosan was shown to have several advantages over other disinfectants, as it possesses a higher antimicrobial activity, a broader spectrum of activity, a higher kill rate, and lower toxicity towards mammalian cells [35,36]. The antimicrobial activity of chitosan is related to physical status, type of microorganism, environmental factors, and intrinsic factors such as molecular weight and deacetylation degree [37,38].

3.6.1. Antibacterial activity

To examine the antibacterial properties of the prepared chitosan, *S. enterica*, *E coli*, *P. aeruginosa K. pneumonia*, *M. luteus*, *E. faecalis*, *S. aureus* and *B. cereus*, which are very significant pathogens in the food industry and agriculture, were tested. Antibacterial activity was assessed by evaluating the diameter of the clear zone of growth inhibition.

As shown in Table 3, Chitosan prepared from the gladius of *L. vulgaris* markedly inhibited the growth of all bacteria tested except *E. faecalis* and *P. aeruginosa*. At a concentration of 40 mg mL^{-1}, the inhibition zones diameter was in the range of 0.5–2.3 cm. The prepared chitosan showed antibacterial activity against both Gram-positive and

Gram-negative bacteria. For instance, in the present work the levels of inhibition of *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) were similar. Arancibia et al. [39] reported that chitosan prepared from *Litopenaeus vannamei* showed antimicrobial activity against both Gram-positive and Gram-negative bacteria. However, Jeon et al. [40] reported that chitosan was more effective against Gram-positive than Gram-negative bacteria. The most feasible hypothesis is a change in cell permeability due to interactions between the positively charged polysaccharide (chitosan at pH lower than 6.5) and the negatively charged membrane. The

Table 4
Evaluation of the nutritional quality of apple juice clarified by chitosan prepared from the gladius of *L. Vulgaris*. Values represent averages ± standard deviations for triplicate experiments.

	pH	Transmittance (%)	Reducing sugars ($g\,L^{-1}$)	Ascorbic acid ($mg\,L^{-1}$)	Titrable acidity ($g\,L^{-1}$)
Before clarification	4.17^{A}	7.9 ± 0.4^{A}	83.7 ± 2^{A}	27.6 ± 0.9^{A}	1.83 ± 0.21^{A}
After clarification	4.3^{A}	91.1 ± 0.2^{B}	81.4 ± 1.8^{A}	24.2 ± 1.1^{A}	1.51 ± 0.12^{A}

Different superscripts in the same column indicate the significant differences ($p < 0.05$).

mechanism underlying the inhibition of bacterial growth should be that the positively charged polymer combines with anionic components such as *N*-acetylmuramic acid, sialic acid and neuraminic acid, on the cell surface [36]. On the other hand, Tokura et al. [41] suggested that antibacterial activity was related to the suppression of the metabolic activity of bacteria by blocking nutriment penetration through the cell wall rather than the inhibition of the transcription from DNA. It has been also suggested that antimicrobial activity can be due to the metal binding capacity of chitosan inhibiting various enzymes activities in the cell leading to the death of microorganisms [42].

3.6.2. Antifungal activity

As shown in Table 3, the antifungal activity of prepared chitosan from gladius squid (*L. vulgaris*) was tested using fungal strains: *F. solani*, *B. cinerea* and *A. solani*. The results revealed that prepared chitosan exhibited marked inhibitory activity against the three strains. Besides, the antifungal activity is more potent against *B. Cinerea* (inhibition zones = 1.9 cm). At $40\,mg\,mL^{-1}$, the inhibition zones value of *F. solani* was 1.5 cm. Chitosan activity on *Fusarium* fungus was previously studied by many authors [29,43,44]. Also, it has been reported that chitosan can reduce the infection of *F. oxysporum* in celery and inhibits the spread of *Sphaerotheca pannosa* var. rosae, *Peronospora sparsa* and *Botrytis cinerea* on roses [45]. Concerning the mechanism, it is suggested that chitosan forms a permeable film at interface and has two functions: direct interference of fungal growth and activation of several defense processes [46].

3.7. Effects of chitosan on the clarification of apple juice

Chitosan possesses several intrinsic characteristics that make it an effective coagulant and/or flocculant for the removal of contaminants. It has characteristics of both coagulants and flocculants, i.e., high cationic charge density, long polymer chains, bridging of aggregates and precipitation [46]. The object of fining a beverage is to produce a product that is near perfect in terms of aroma, taste, color and clarity. Chitosan is used in the production of fruit juices for clarification and precipitation of substances causing turbidity, and also for reducing the concentration of polyphenols such as tannins and anthocyanogens [47].

Prepared chitosan was assayed at different concentrations ($0.2–1\,g\,L^{-1}$) to clarify apple juice. An apparent fall in juice turbidity was observed following the addition of the different concentrations of chitosan solution. Ghorbel-Bellaaj et al. [16] were used chitosan from shrimp by-product for the clarification of apple, lemon and orange juices and similar results were noted. The maximum rates of apple juice clarification were attained with a chitosan concentration of $0.6\,g\,L^{-1}$. Further increases in concentration (0.8 and $1\,g\,L^{-1}$) did not improve significantly the level of clarification. In fact, chitosan is a cationic flocculant; it can be combined with the negatively charged pectin, soluble starch, protein and microparticles through positive and negative charge attraction to form floc precipitation [48]. In order to determine the change in composition due to treatment with clarifying aids, apple juice clarified with $0.6\,g\,L^{-1}$ of chitosan was subjected to analysis of pH, transmittance, reducing sugars, ascorbic acid content and titrable acidity. As displayed in Table 4, change in pH and titrable acidity of apple juices before and after clarification, was not significant. A slight reduction in reducing sugar and ascorbic acid contents of the juice has been observed which practically have no impact on the nutritional value of the juice. This indicates that concentrations of organic acids and sugar present in the apple juice remain practically unaltered. The clarifying agent was noted to significantly improve the transmittance of the apple juice, which increased to reach 91%. Clarification experiments show that chitosan the fruit juices with natural, harmless and nontoxic characteristics.

4. Conclusion

In this study, enzymatic deproteinization process and treatment with dilute HCl solution for demineralization were applied for chitin recuperation from the gladius of squid (*Loligo vulgaris*). The prepared chitin has been characterized with several techniques. The polysaccharide exhibited a β-chitin structure according to FT-IR and ^{13}C NMR spectra. Chitin was then converted to chitosan by deacytilation. The deacetylation degree of the prepared chitosan was determined as 71%. The biological activities of chitosan were studied. Prepared chitosan was found to be an effective antioxidant in different *in vitro* assays. The results of this study indicate that the prepared chitosan exhibited a good antioxidant activity and they can be used in food systems as natural antioxidants. The findings revealed also that chitosan exhibited high antimicrobial activity. The work demonstrated also that chitosan can be used as clarifying agent for apple juice. Nevertheless, these important properties make chitosan an exciting and promising excipient for agriculture, food and pharmaceutical industry.

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