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Characterization and In Vitro Evaluation of Cytotoxicity, Antimicrobial and Antioxidant Activities of Chitosans Extracted from Three Different Marine Sources

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Abstract Chitins in the α and β isomorphs were extracted from three Tunisian marine sources shrimp (*Penaeus kerathurus*) waste, crab (*Carcinus mediterraneus*) shells and cuttlefish (*Sepia officinalis*) bones. The obtained chitins were transformed into chitosans, the acid-soluble form of chitin. Chitosans were characterized and their biological activities were compared. Chitosan samples were then characterized by Fourier transform infrared spectroscopy (FTIR). The results showed that all chitosans presented identical spectra. Antimicrobial, antioxidant, and antitumor activities of the extracted chitosans were investigated. In fact, cuttlefish chitosan showed the highest DPPH radical-scavenging activity (83 %, 5 mg/ml), whereas it was 79 % and 76 % for shrimp and crab chitosans, respectively. However, in linoleate−β−carotene system, cuttlefish and crab chitosans exerted higher antioxidant activity (82 % and 70 %, respectively), than shrimp chitosan (49 %). Chitosans were tested for their antimicrobial activities against three Gram-negative and four Gram-positive bacteria and five fungi. Chitosans markedly inhibited growth of most bacteria and fungi tested, although the antimicrobial activity depends on the type of microorganism and on the source of chitin. In addition, chitosans showed high antitumor activity which seemed to be dependent on the chitosan characteristics such as acetylation degree and especially the molecular weight.

Keywords Chitin · Chitosan · Antitumor · Antimicrobial · Antioxidant activity

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Introduction

Chitosan, a linear β-1,4-D-glucosamine, is a biocompatible, non-toxic polymer mainly obtained by deacetylation of chitin, the major component of crustaceans shells such as shrimp, crab, and crawfish [1]. Chitosan possesses many beneficial biological properties such as enzyme inhibitory effect, immunostimulant, antimicrobial, anticoagulant, anticancer, anticholesterolemic, and wound-healing property [2–7]. For this reason, chitosan has so far attracted notable research interest and numerous works can be found in the literature [8]. Due to its intrinsic properties, chitosan is considered as a versatile biopolymer that can be developed into different forms, such as gels, films, nanoparticles, etc., and find numerous applications in various fields, including food, pharmaceutical, and biotechnological products, cosmetics, textiles, in waste water treatment, and in agriculture [9–10].

Lipid peroxidation is of great interest in the food industry because it leads to the development of undesirable flavors and odors and impair the nutritional value of foods. Therefore, prevention of lipid oxidation is necessary. To retard or prevent such changes, synthetic antioxidants are normally used. The possible toxicity of synthetic antioxidants makes the use of natural antioxidants positive for consumers. In this sense, the use of natural compounds with efficient antioxidant activity, become important [11]. Currently, natural antioxidants are widely used as ingredients of functional foods, with the aim of preventing chronic diseases, such as cancer, atherosclerosis, and heart disease [12, 13].

Recently, natural polysaccharides such as chitosan and their derivatives have been demonstrated to possess antioxidant activity [14, 15]. Peng et al. [16] showed that chitosans retard lipid oxidation by chelating ferrous ions present in the system, thus eliminating their prooxidant activity or their conversion to ferric ion. Besides, chitosan exhibits recognized antimicrobial activity and was suggested as food preservatives of natural origin. Chitosan has broad spectrum of activity and high killing rate, with a low toxicity toward mammalian cells. Several mechanisms have been proposed, such as the polycationic characteristics of chitosan in acidic medium and their interaction with the negative charge of the microbial cell membranes [17]. Chitosan are reported to be stacked over the microbial cell surface, blocking the nutrients or bind to DNA such as inhibiting transcription or permeability of the microbial cell wall [18]. Chitosan bacterial efficacy depends on various factors such as the deacetylation degree, molar mass, and the environmental conditions, especially pH, ionic strength of the medium, and bacterial species [19].

Cancer is the second largest non-communicable disease, and it has a sizable contribution in the total number of deaths. Urinary bladder cancer ranks the fourth most common cancer in men and ninth in the women with an alarming increase in cancer patients of this type every year [20]. Furthermore, chemical and dyestuff manufacturers are at particularly high risk of bladder cancer. Chitosans have various biological activities such as immune-enhancing activity and antitumor activity [21]. These activities were suggested mainly due to its cationic property exerted by amino groups, and the molecular weight also plays a major role for the antitumor activity. Recently, it was proved that strong electronic charge is an important factor for anticancer activity of chitosan [22].

Usually, chitosans, described in literature for their biological activities, are prepared mainly from shrimp waste and from other sources such as crabs and fungi. Although, to the best of our knowledge, there are no reports for biological activity of chitosan extracted from cuttlefish bones. The present work is the first systematic trial to investigate the effect of chitosan sources on their biological activities especially antimicrobial, antioxidant, and antitumor.

Materials and Methods

Raw Materials

Shrimp (*Penaeus kerathurus*) waste and cuttlefish (*Sepia officinalis*) bones were provided from a Tunisian processing factory (Calambo Congelation). Crab (*Carcinus mediterraneus*) shells were obtained from a local commercial fishery in Sfax (Tunisia).

Chemical Analysis

The moisture and ash content were determined according to the AOAC standard methods 930.15 and 942.05 [23], respectively. Total nitrogen content of shell was determined by using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25 [24]. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane. Analyses of calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}), and potassium (K^{+}) mineral contents in the raw materials were carried out using the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Model 4300 DV, Perkin Elmer, Shelton, CT, USA).

Chitin Extraction

Demineralization of Raw Materials

Chitins were prepared from shrimp, crab shells waste, and cuttlefish bones. Raw materials were washed thoroughly with tap water, dried at room temperature, and were milled (sieved from 2 to 5 mm). Dried wastes were demineralized in 0.55 M HCl (1:10; *w*/*v*) ratio for 15 min for each bath (2, 3, and 4 baths for shrimp, crab, and cuttlefish bones, respectively). The decalcified materials were collected, washed to neutrality with distilled water, rinsed with deionized water, and filtered to remove excess moisture. Demineralization ratio (DM) was calculated using the following equation [25] :

$$\%\mathrm{DM} = \frac{[(A_o \times O) - (A_R \times R)]}{(A_o \times O)} \times 100$$

where A_O and A_R are ash contents (%) before and after demineralization, respectively; while, O and R represent the masses (g) of initial and demineralized residues, respectively.

Enzymatic Deproteinization

The demineralized materials were deproteinized using *Bacillus mojavensis* A21 crude protease cited in our previous study [26]. Deproteinization was carried out in a thermostated stirred Pyrex reactor (300 ml). The demineralized materials were mixed with distilled water (1:3, *w*/*v*). pH and temperature of the mixtures were adjusted to 9.0 and 50 °C, respectively. These parameters were optimized by response surface methodology in the previous study [27]. Proteins were digested using this crude enzyme at different enzyme/substrate ratios (unit of enzyme/mg of protein: E/S) during 3 h. Deproteinization was stopped by heating at 90 °C for

20 min to inactivate enzymes. The residue was then washed and pressed manually through four layers of gauze. Deproteinization rate (DP) was evaluated using the following formula [25]:

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

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

Deacetylation of Chitins

Deacetylation was achieved by incubating chitin in 12.5 M NaOH at 140 °C for 4 h and with a solids/solvent ratio of 1:10 (*w*/*v*). The resulting chitosans were collected, washed, and dried at 50 °C for 12 h.

Physicochemical Characterization of Chitins and Chitosans

Intrinsic Viscosity and Molecular Weight Determination

Chitosan samples were dissolved in sodium acetate buffer (0.1 M sodium acetate and 0.3 M acetic acid). Intrinsic viscosity [η] was determined using an Ubbelohde viscometer immersed in a thermostated bath at 25 ± 0.1 °C. The efflux time of the solution was between 200 and 300 s. Intrinsic viscosity was calculated using the following equation:

$$[\eta] = \lim\ \eta_{\mathrm{sp}}/c, \ \text{ with } \ c \rightarrow 0$$

where [η] is the intrinsic viscosity, η_{sp} is the specific viscosity and c is the concentration of chitosan solution.

The viscometric-average molecular weight (MW) of chitosan samples was estimated using the Mark–Houwink relationship [28]:

$$[\eta] = K(\mathrm{MW})^a$$

where K = 7.95 10^{-2} and a = 0.79 [29]. The means of four replicates was taken for the viscosity measurements.

Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectra were measured by KBr-supported sample of chitosans over the frequency range 400–4000 cm^{-1} at resolution of 4 cm^{-1} using Bruker FTIR spectrometer. Chitosan samples were thoroughly mixed with KBr in 1:1 (*w*/*w*) ratio, and then blended using an agate mortar for 5 min. The dried mixtures were then pressed to result in a homogeneous sample/KBr disc.

Acetylation degree (AD) of the samples was determined by the method of Moore and Roberts [30]. Considering the –OH band at 3450 cm^{-1} as a reference,

the acetyl content (%) was determined from the ratio of absorbance using the following equation:

$$\mathrm{AD}\% = (\mathrm{A}_{1655}/\ \mathrm{A}_{3450}) \times 1.33$$

where A_{1650}: the area of the *N*-acetyl group, A_{3450}: the area of the hydroxyl band. The ratio of A_{1655}/A_{3450} was equal to 1.33 and zero for fully *N*-acetylated chitin and fully deacetylated chitosan, respectively.

Antioxidant Activities of Chitosans

DPPH Radical-Scavenging Activity

The ability of chitosan to scavenge DPPH radical was determined according to the method of Bersuder et al. [31]. A volume of 500 μl of each sample (chitosans) at different concentrations (0–5 mg/ml) was added to 500 μl of 99.5 % ethanol and 125 μl of 0.02 % DPPH in 99.5 % ethanol. The mixture was homogenized in a vortex (Phoenix, AP-56, Brazil) and kept for 60 min at room temperature (25 °C) in the dark. The absorbance of the solution was measured at 517 nm in a UV/VIS spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd., China). In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compounds. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. The control was conducted in the same manner, except that distilled water was used instead of sample. Butylated hydroxyanisole (BHA) was used as a standard. DPPH radical-scavenging activity was evaluated using the following formula:

$$\text{Radical scavenging activity}(\%) = \frac{A_c - A_s}{A_c} 100$$

where A_c is the absorbance of the control reaction and A_s is the absorbance of chitosan solution. The test was carried out in triplicate.

Reducing Power Assay

The ability of chitosans to reduce iron (III) was measured spectrophotometrically by the method of Yildirim et al. [32]. One milliliter of sample containing different concentrations of chitosan (0–5 mg/ml) was mixed with 1.25 ml of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of 1 % potassium ferricyanide. The mixtures were incubated for 30 min at 50 °C, and the reactions were stopped by addition of 1.25 ml of 10 % (*w*/*v*) trichloroacetic acid. The reaction mixtures were then centrifuged for 10 min at 1650×*g*. Finally, 1.25 ml aliquot of the supernatant solution, from each sample mixture, was mixed with 1.25 ml of distilled water and 0.25 ml of 0.1 % (*w*/*v*) ferric chloride. After a 10-min reaction, the absorbance of the resulting solution was measured at 700 nm. The increase in absorbance of the reaction indicates an increased reducing power. Values presented are the mean of triplicate analyses.

β-carotene Bleaching Assay

The ability of the chitosans to prevent the bleaching of β-carotene was determined according to the method of Koleva et al. [33]. A stock solution of β-carotene/linoleic acid was prepared

by dissolving 1 mg of β-carotene, 25 μl of linoleic acid, and 200 μl of Tween-80 in 1 ml chloroform. The chloroform was completely evaporated under vacuum in a rotatory evaporator at 45 °C. One hundred milliliters of distilled water were 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 sample at different concentrations. The tubes were immediately placed in a water bath and incubated at 50 °C for 2 h. Thereafter, the absorbance of each chitosan sample was measured at 470 nm. A control consisted of 0.5 ml of distilled water instead of the sample solution. BHA was used as positive standard. Antioxidant activity in β-carotene bleaching model was calculated with the following equation:

$$\beta\text{-carotene bleaching inhibition}(\%) = 1 - \frac{A_o - A_t}{A_o' - A_t'} \times 100$$

where A_0 and A_0' are the absorbances, measured at time zero, of the sample and the control, respectively. A_t and A_t' are the absorbances of the sample and the control, measured after 2 h, respectively. Tests were carried out in triplicate.

Antimicrobial Activity of Chitosans

For antibacterial activity tests, three Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Salmonella typhi* (ATCC 19430), and *Klebsiella pneumonia* (ATCC 13883) and four Gram-positive bacteria *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), and *Micrococcus luteus* (ATCC 4698) were used. Antifungal activities were tested using *Fusarium solani*, *Fusarium oxysporum*, *Aspergillus niger*, *Alternaria solani*, and *Botrytis cinerea*, provided by the Center of Biotechnology, Sfax-Tunisia.

Antimicrobial activity assays were performed according to the method described by Vanden Berghe and Vlietinck [34]. Chitosan samples (50 mg/ml) were prepared under stirring in 0.1 % acetic acid. Culture suspension (200 μl) of the tested microorganisms (10^6 colony-forming units/ml of bacteria cells estimated by absorbance at 600 nm and 10^8 spores/ml of fungal strains measured by Malassez blade) 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 samples at 50 mg/ml. A well with 50 μl of 0.1 % acetic acid solution was used as a negative control. Gentamycine and Cycloheximide were used as positive references for bacteria and fungi activities, respectively. The Petri dishes were kept, first for 1 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 (including well diameter of 5 mm).

Antitumor Activity of Chitosans

Cell Culture

Bladder cancer cell lines were cultured in RPMI 1640 medium containing 2 mM L-glutamine (Invitrogen Life Technologies, Cergy Pontoise, France) and supplemented with 10 % (*v*/*v*) fetal calf serum, 2.5 U/ml penicillin, and 2.5 mg/ml streptomycin (Invitrogen, Life

Technologies). Cells were cultured at 37 °C in a 5 % CO_2-humidified atmosphere and tested to ensure absence of mycoplasma contamination.

Clonogenic Assay of Cells In Vitro

RT112 bladder cancer cell lines (2×10^3 cells/well) were plated in a six-well plate and incubated for 24 h at 37 °C, before treatment with chitosans. Each chitosan sample was prepared under stirring in aqueous solution containing the stochiometric amount of HCl (1 mg/ml or ~6 mM). These mother solutions were then diluted with RPMI medium at pH = 6.5 (using 1 M HCl and HEPES as buffer) to get 500 μM of chitosan concentration. RT112 cells were treated with chitosan solutions during 2 h allowing chitosan penetration into cells and preventing cell death at pH 6.5 as demonstrated separately. Then, the initial mixture was replaced by pure RPMI medium at pH 7.5 for a total of 48 h. After incubation at 37 °C in a humidified incubator, cultures in the presence and the absence of chitosan samples were observed using an inverted microscope at ×200 magnifications (Nikon, Tokyo, Japan). The harvesting of cells was performed using trypsinization. Then, cell suspensions were diluted at 1/1000 and seeded into six-well plates for clonogenic assay according to the method elaborated by Franken et al. [35]. Plates were then placed in the incubator until cells in control dish have formed sufficiently large clones (7 days). Colonies were then fixed and stained using a mixture of 6.0 % glutaraldehyde and 0.5 % crystal violet.

MTT Assay

Cell proliferation assay was estimated using MTT assay. RT112 bladder cancer cells (3×10^3 cells/well) were seeded in 96-well plates and incubated for 24 h at 37 °C, before treatment with chitosans. Mother solutions of chitosan were progressively diluted with RPMI medium at pH 6.5 to get the vehicle at 50, 100, 500, and 1000 μM chitosan concentrations. RT112 cells were treated with these chitosan solutions during 2 h. Then, the initial mixture was replaced by pure RPMI medium at pH 7.5 for a total of 24, 48, or 72 h. Ten microliters of tetrazolium dye (MTT) (at 5 mg/ml in phosphate buffer PBS) was then added to each well and incubated for 2 h, after the plate was centrifuged at 1800×*g* for 5 min at 4 °C. After careful removal of the medium, 100 μl of DMSO was added to each well and plates were shaken. Absorbance was recorded on a microplate reader (Sunrise; Tecan, Lyon, France) at the wavelength of 570 nm. The effect of each chitosan sample on growth inhibition was assessed as percent cell viability, where vehicle-treated cells were taken as 100 % viable.

Results and Discussion

Chemical Composition of Raw Materials

Shrimp shells exhibited the lowest value of ash (48.8 %: *w*/*w*), whereas it was 59.8 % and 90.6 % for crab shells and cuttlefish bones, respectively (Table 1). The chitin content was around 5.8, 27.4 and 37.2 % (*w*/*w*) for cuttlefish, crab and shrimp shells, respectively. These raw materials were characterized by lower protein (<14 %) and lipid (0.2–0.9 %) contents than those reported in literature [36]. In fact, the main mineral component in shells is calcium carbonate which is necessary for the calcification of their exoskeleton [37].

Table 1 Characterization of the dried raw materials from Tunisian marine sources, extracted chitins, and chitosans

		Shrimp	Crab	Cuttlefish
Raw material	Ash[a]	48.8	59.8	90.6
	Protein[a]	13.1	12.1	3.4
	Chitin[a]	37.2	27.4	5.8
	Fat[a]	0.9	0.7	0.2
Chitin	Yield of extraction[b]	20	10	5
	AD	88.5	78.5	70.1
	Residual proteins[a]	–	–	–
	Residual ash[a]	0.4	0.5	0.8
Chitosan	Yield of extraction[b]	14.9	5.3	1.2
	AD	12	17	5
	MW	17030	6120	1030
	Residual proteins[a]	–	–	–
	Residual ash[a]	–	–	–

[a] % on dried material basis

[b] % on dried raw material

– not detected

Chitin Characteristics

The extracted chitins present a mineral content below 1 %. The average acetylation degree (AD) of each chitin sample was determined from FTIR data edited in our previous study [37]. AD of chitins extracted from cuttlefish bones and crab shells were 70 and 78 %, respectively, whereas shrimp shells was characterized by the highest AD among the studied species (88 %) (Table 1). It should be noted that the lowest AD was obtained from cuttlefish chitin (β-isomorph). These results are in good agreement with the results cited in literature [36, 38]. Acetylated chitins, with a rate of 70–90 % and low protein content, are considered as good final products.

Chitosan Preparation and Characterization

Chitosans were obtained through heterogeneous alkaline deacetylation of chitins extracted from shrimp, crab shells, and cuttlefish bones. The structures of the extracted chitosans were confirmed by FTIR analysis and reported in Fig. 1. The broad band at 3490 cm^{-1} was attributed to O–H stretching vibration that overlaps the N–H stretching vibration in the same region. The broad band between 2800 and 3100 cm^{-1} was attributed to C–H stretching vibration. The peak at 1558 cm^{-1} was due to the amide bending vibration (N–H). It is clear that the absorption patterns of the spectra were similar to that cited in literature and suggesting the good quality of chitosans [39].

Cuttlefish chitosan has a lower AD (5 %) than crustacean chitosans (12 and 17 % for shrimp and crab, respectively). These results confirm that AD is highly dependent on the morphology and the source of chitin (especially due to the different H-bond networks present in the initial material which modify the accessibility). It is proved that the cuttlefish chitin has a

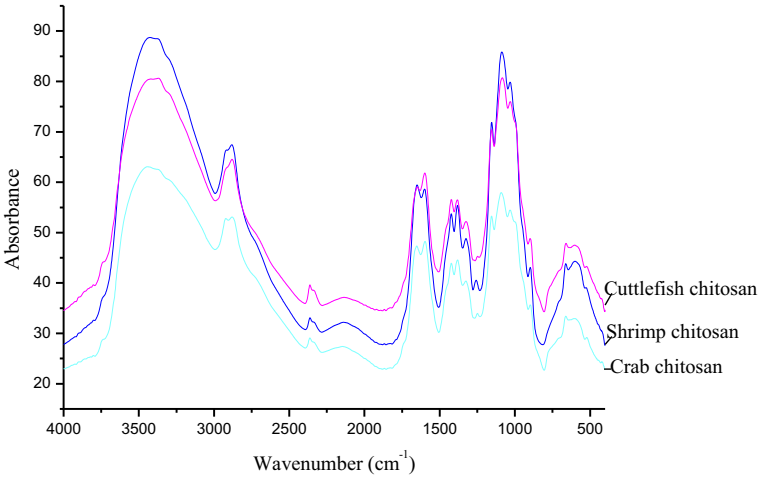

Fig. 1 FTIR spectra of chitosans extracted from shrimp waste, crab shells, and cuttlefish bones

higher reactivity against deacetylation than the crustacean chitin [40]. The low AD (<20 %) offers extraordinary potential in a broad spectrum of chitosan in industrial applications.

Viscosity-average molecular weights of prepared chitosans were also determined. The results showed that viscosity-average molecular weights varied with the source of chitins, the extraction method, and the residual aggregates in solution [41]. Among the three samples, shrimp chitosan showed the highest viscosity and molecular weight (175 ml/g; 17030 g/mol), while cuttlefish chitosan had the lowest (19 ml/g; 1030 g/mol). However, crab chitosan showed a viscosity and molecular weight at about 78 ml/g and 6120 g/mol, respectively. It is clearly demonstrated that the physicochemical characteristics of the extracted chitosans were very closely related to the used the extraction conditions (alkali solution, concentration of alkali, high temperature, reaction time, etc.) and the source of chitins.

Antioxidant Activity of Chitosans

DPPH Radical-Scavenging Assay

DPPH radical-scavenging assay is a widely used method for evaluating the ability of antioxidant to scavenge free radicals generated from DPPH reagent. This assay is based on the ability of DPPH, a stable free radical, to be quenched and thereby decolorize in the presence of antioxidants resulting in a reduction in absorbance at 517 nm [42]. The decrease in absorbance is taken as a measure for radical-scavenging activity.

As displayed in Fig. 2, both chitosans exhibited effective antioxidant activity against DPPH and their scavenging activities were concentration-dependent. For example, at a concentration of 5 mg/ml, cuttlefish chitosan was found to be the most active radical scavenger (83 %) followed by shrimp and then crab chitosans (79 and 76 %, respectively). However, chitosans showed lower radical-scavenging activities than butylated hydroxyanisolt (BHA) used as reference at the same concentration. The effective concentrations of chitosans for 50 % radical-scavenging activity (EC_{50}) determined using the regression equation were 1.2, 1.8, and 2.4 mg/ml for cuttlefish, shrimp, and

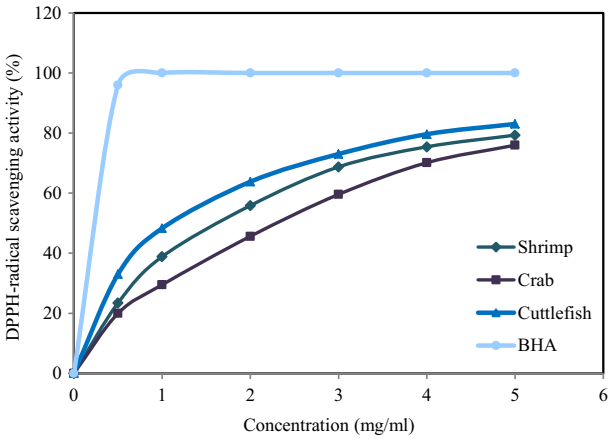

Fig. 2 Scavenging effect on DPPH free radical of chitosans at different concentrations. BHA (2.0 mM; 0.36 mg/ml) was used as positive controls. Values presented are the mean of triplicate analyses

crab chitosans, respectively. The lower EC_{50} value indicates the higher free radical-scavenging ability.

From these results, cuttlefish chitosan which have the lowest MW and lowest AD was found to be the most active radical-scavenging activity than those of chitosans with high MW (from crab and shrimp).

Reducing Power

The reducing power assay is often used to evaluate the ability of antioxidant to donate electron or hydrogen [43]. In this assay, the ability of chitosans to reduce Fe^{3+} to Fe^{2+} was determined; reducing power capacity of the three prepared chitosans was investigated at different concentrations (0- 5 mg/ml). Both chitosans showed low reducing power (OD_{700}: 0.34, 0.28 and 0.40 for shrimp, crab, and cuttlefish chitosans, respectively) than did BHA at the same concentrations, indicating that chitosans were not able to reduce Fe^{3+} to Fe^{2+} by donating electron (data not shown). These results are comparable with those reported by Yen et al. [44] mentioning that crab chitosan showed low reducing power (OD_{700} = 0.44; 10 mg/ml).

ß-carotene Bleaching Assay

The antioxidant assay using the discoloration of β-carotene is widely employed to measure the antioxidant activity of chitosans. In the absence of antioxidants, β-carotene undergoes rapid discoloration. The decrease in absorbance (A_{470nm}) indicates the oxidation of β-carotene caused by the oxidation process. When the oxidation of linoleic acid occurs, free radicals formed are able to attack the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidized, leading to the losses in chromophore and characteristic orange color of β-carotene [11].

Antioxidant activity of chitosans using the β-carotene-linoleate bleaching inhibition assay in comparison with BHA was tested at different concentrations (0–5 mg/ml). As shown in Fig. 3, all chitosans inhibited significantly the oxidation of β-carotene and their antioxidant activity increased with increasing chitosan concentrations. At 5 mg/ml, cuttlefish chitosan

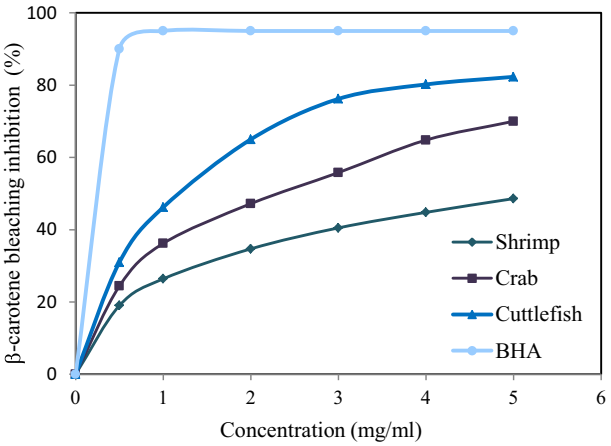

Fig. 3 Antioxidant activity using the β-carotene bleaching method of chitosans at different concentrations. BHA (2.0 mM; 0.36 mg/ml) was used as positive controls. Values presented are the mean of triplicate analyses

showed the highest ability to prevent bleaching of β-carotene (82.3 %), followed by crab (70 %) and then shrimp chitosans (48.6 %). However, all chitosans were characterized by lower antioxidant activities than BHA at the same concentration. Nevertheless, these results showed that chitosans extracted from cuttlefish bones and crab shells had strong effects against the discoloration of β-carotene. Results suggest that chitosan has a potential use as scavengers of free radicals in emulsion-type systems.

Antibacterial Activities of Chitosans

Antimicrobial activity of chitosans has been considered as one of the most important properties linked directly to their possible biological applications [45]. As seen in Table 2, chitosans

Table 2 Antibacterial activity of chitosans using diameters of inhibition zones against Gram-positive and Gram-negative bacteria

		Diameter of inhibition zones (mm)			
		Chitosans			
		Positive control	Cuttlefish	Crab	Shrimp
Gram −	*E. coli* (ATCC 25922)	20 ± 0.5	17 ± 0.5	14 ± 0.3	10 ± 0.8
	K. pneumonia (ATCC 13883)	12 ± 0.5	11 ± 1.0	10 ± 0.5	7 ± 0.5
	S. typhi (ATCC 19430)	18 ± 0.5	17 ± 0.8	12 ± 0.2	10 ± 0.2
Gram +	*S. aureus* (ATCC 25923)	25 ± 0.0	14 ± 0.5	12 ± 0.0	9 ± 0.5
	B. cereus (ATCC 11778)	20 ± 0.5	16 ± 0.5	12 ± 0.6	12 ± 0.1
	E. faecalis (ATCC 29212)	20 ± 1.0	16 ± 0.0	14 ± 0.1	12 ± 0.5
	M. luteus (ATCC 4698)	19 ± 0.5	14 ± 0.5	12 ± 0.5	10 ± 0.4

Diameter well, 5 mm. Chitosan samples pH 4.7. Gentamycine and acetic acid solution (0.1 %) were used as positive and negative controls, respectively

markedly inhibited the growth of all Gram-negative and Gram-positive bacteria tested, although the inhibitory effects differed with regard to the source of chitosan and the type of inhibited bacteria. Cuttlefish chitosan with the lowest MW and AD exhibited the highest antimicrobial activity against all bacteria tested as compared to crab and shrimp chitosans. Results suggest that chitosan characteristics such as AD and especially MW control the antimicrobial activity. These results are in accordance with other study [46] which showed that with Gram-negative bacteria, the antibacterial activity increased with decreasing MW and the highest MW (≥110 kDa) being less effective.

From literature, the antimicrobial activity of chitosans was claimed to depend on the protonated NH_2 groups in chitosan which interact with the negative residues at the cell surfaces [47]. Therefore, chitosan with a lower AD, which had a higher positive charge, would be expected to have a stronger antimicrobial activity. Tokura et al. [48] suggested that antimicrobial 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 [49].

Antifungal Activities of Chitosans

The antifungal activities were tested against five fungi *F. solani*, *F. oxysporum*, *A. niger*, *A. solani*, and *B. cinerea*. As seen in Table 3, chitosans inhibited the growth of all fungi tested; however, the inhibitory effects differed with the source of chitosan and the type of fungi. Otherwise, cuttlefish chitosan with the lowest MW and AD exhibited the highest antifungal activity (18–21 mm). Chitosan has been observed to act more rapidly on fungi and algae than on bacteria [50]. Younes et al. [46] reported that antifungal activity of shrimp chitosan was influenced by its molecular weight for *F. oxysporum*, by its acetylation degree for *A. solani* but no MW or DA dependences were observed with *A. niger*. Al-Hetar et al. [51] showed that mycelial growth, sporulation, and spore germination were affected by chitosan demonstrating its effects on various stages in the growth and development *Fusarium oxysporum* f. sp. *Cubense*. Guo et al. [52] showed that low MW chitosan shows stronger inhibitory effect (25 %) than high MW chitosan (19.6 %) against *Botrytis cinerea* Pers. In fact, chitosan characterized by a low MW chitosan can enter the fungal cell easily because of its small size.

Table 3 Diameters of inhibition zones (expressed in mm) against fungi in the presence of chitosans

	Diameter of inhibition zones (mm)			
	Positive control	Cuttlefish chitosan	Crab chitosan	Shrimp chitosan
F. oxysporum	14 ± 0.5	20 ± 0.2	17 ± 0.5	13 ± 0.5
F. solani	16 ± 0.1	21 ± 0.0	17 ± 0.5	13 ± 0.2
A. solani	20 ± 0.5	20 ± 1.0	15 ± 0.1	11 ± 0.3
B. Cinerea	18 ± 0.5	18 ± 0.5	15 ± 0.8	11 ± 0.0
A. niger	20 ± 0.5	21 ± 0.2	17 ± 0.0	13 ± 0.5

Diameter well, 5 mm. Chitosan samples at pH 4.7. Cycloheximide was used as positive control and acetic acid solution (0.1 %) as negative control

The mechanism for inhibition remains controversial. Two hypotheses are as follows firstly, the polycationic chitosan consumes the electronegative charges on cell surfaces and the cell permeability is changed; thus, this interaction results in the leakage of intracellular electrolytes and proteinaceous constituents. Secondly, chitosan enters fungal cells and then essential nutrients are adsorbed, which inhibit or slow down the synthesis of mRNA and protein [53]. Then the differences in the antifungal activity and chitosan characteristics dependence will also be influenced by the chitosan mechanism of inhibition which differs for each microorganism.

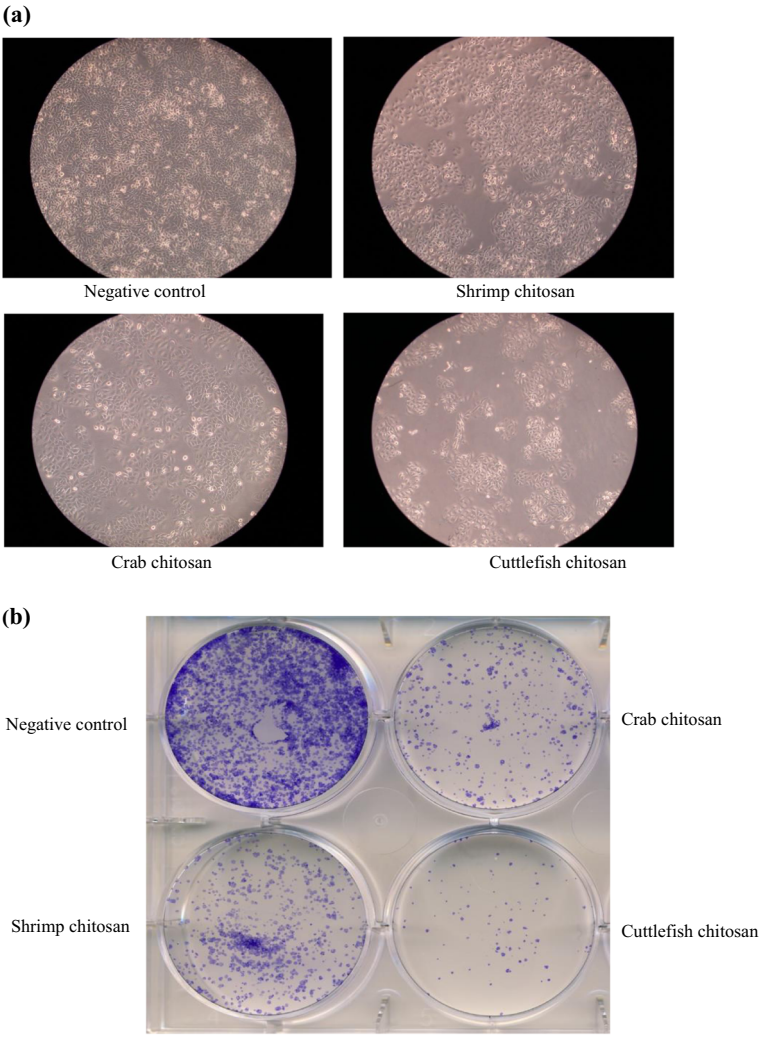

Fig. 4 Cytotoxic effects of chitosan samples on human bladder cancer (RT112) cells: **a** Morphological observation of chitosans tested after 48 h of incubation with RT112 cells. Cell morphology was observed using an inverted microscope at ×200 magnification (Nikon, Tokyo, Japan). **b** Clonogenic assay performed in six-well plates and comparative effects of chitosans on the growth of RT112 cells. The first well represent the negative control in which RT112 cells were incubated only with the medium

Antitumor Activity

Cytotoxic anticancer chemotherapeutic agents generally produce severe side effects, while reducing host resistance to cancer and infections. Currently, bladder cancer is estimated to be the seventh most common malignant neoplasm and the eighth leading cause of cancer-related death worldwide [20]. For this purpose, the effect of the prepared chitosans against human bladder cell carcinoma (RT112) was also investigated.

The microscopic observations (Fig. 4a) and cytogenic assays (Fig. 4b) of the bladder cancer RT112 cells after 48 h of treatment with chitosans showed an important reduction of cell number, as compared to the control. The results showed that there is a significant difference in growth inhibitory effects of the three chitosan samples. Cuttlefish chitosan with the lowest AD and lowest MW was more active on bladder human cancer cells than crab and shrimp chitosans (Fig. 4b).

The effect of the chitosans on the cells viability was also measured by the MTT assays, which reflects the cellular reducing activity. MTT assay as shown in Fig. 5 indicated that all chitosans inhibited the RT112 cells proliferation in a dose- and time-dependent manner. The

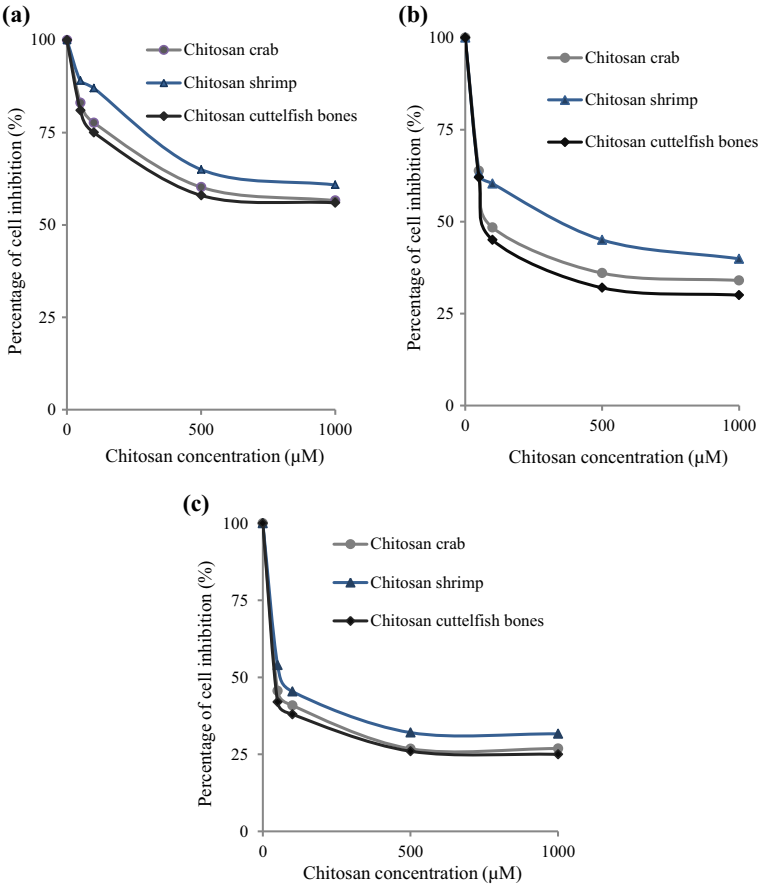

Fig. 5 MTT assays of each chitosan sample after 24 h (**a**), 48 h (**b**), and 72 h (**c**). The values are represented as the percentage of cell inhibition, where vehicle-treated cells were regarded as 100 %

antitumor activity of chitosan was dependent on their structural characteristics such as AD and MW. In fact, cuttlefish chitosan with lower AD and MW was most active, with an estimated EC_{50} (50 % inhibitory concentration) of 62 μM after 48 h (Fig. 5b) and 50 μM after 72 h of incubation with RT112 cells (Fig. 5c). On the other hand, the EC_{50} values of crab chitosan (AD: 12 %; MW: 6120 g/mol) were 100 and 50 μM after 48 and 72 h of incubation with RT112, respectively. Shrimp chitosan with higher AD (17 %) and MW (17030 g/mol) showed the lowest antitumor activity with an EC_{50} of 330 and 60 μM after 48 and 72 h, respectively. These values were comparable to values given in a previous study which showed that the EC_{50} values of shrimp chitosan with a higher MW of 321 and 52 μM after 48 and 72 h, respectively [54]. Our results showed that this effect seemed to be dependent on chitosan molecular weight. However, in-depth studies of this antitumor effect must be investigated further.

Previous reports have indicated that the inhibitory effect of chitosan on tumor growth was most likely related to its ability to induce lymphocyte cytokines by increasing T cell proliferation. Mainly, adaptive immunity is thought to have enhanced the antitumor mechanism of chitosan and derivatives by accelerating T cell differentiation, which in turn increases cytotoxicity and maintains T cell activity [55]. Studies have demonstrated that the antitumor effects of low molecular weight chitosans that form low viscosity solutions, in mice bearing sarcoma (180 tumors) can be attributed to an increase in natural killer cell activity [56]. Another separate report stated that a low molecular weight, water-soluble chitosan could prevent tumor growth by serving as immune modulator in enhancing the cytotoxic activity against tumors [57].

Conclusion

In this study, chitins were first extracted from shrimp, crab, and cuttlefish by-products from processing industries in Tunisia and then converted into chitosans. They were characterized by spectroscopic methods using FTIR. The current study demonstrated that chitosan extracted from the cuttlefish bones, characterized by lower molecular weight and lower acetylation degree, exhibited higher antioxidant, antimicrobial, and high antitumor activities on bladder carcinoma cells RT112 as compared to the other chitosans.

Owing to their excellent properties exhibited in antioxidant and antimicrobial activities, chitosans extracted from crustacean waste and cuttlefish bones could be proposed as a natural additive for foods and pharmaceutical industries.

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