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RESEARCH ARTICLE

Effects of chitin and its derivatives on human cancer cells lines

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Abstract The present study is focused on the effect of chitin derivatives against human cancer cell lines RD and Hep2. As an outcome from this research, chitin was cytotoxic at IC50= 400 μg/ml and 200 μg/ml against Hep2 cells and RD cells lines, respectively. Irradiated chitin had an IC50 value of 450 μg/ml for Hep2 and an IC50 of 200 μg/ml for RD. The lowest IC50 is attributed to chitosan, 300 μg/ml in Hep2 and 190 μg/ml in RD.

Keywords Chitin · Chitosan · Cytotoxicity · Biopolymers · Enzymatic hydrolysis

Introduction

Chitin and chitosan are biopolymers of growing importance and have a significant impact on research and development in diverse fields such as in chemistry, biology, health, and the environment protection. Their outstanding properties explain the craze for these natural macromolecules extracted from crustacean shells, considered so far as waste. Chitin is a linear polymer which has a similar structure to cellulose, but it is an amino polysaccharide with acetamide groups, C-2 instead of the hydroxyl (Benhabiles et al. 2012; Laribi-Habchi et al. 2015). Its chemical structure is a sequence of monomeric units linked by a glycosidicbond (1-4) *N-acetyl*-(beta)-D-glucosamine. Recently, chitin extraction from marine debris is expanding. Chitin has the major advantage of being non-toxic, biodegradable, biocompatible, and bioactive and allows many applications in a variety of fields: food (Benhabiles et al. 2012), agriculture, water treatment (Zemmouri et al. 2011), cosmetics, and the biomedical and pharmaceutical field. Lately, this type of molecule has been used for the fight against cancer (Salah et al. 2013). Antitumor effects of chitin and its derivatives have been the subject of several studies (Lee et al. 2005). This work treats the improvement of the extraction of chitin, and its derivatives with antitumor properties, from shrimp shells. Therefore, the cytotoxicity (IC50) of an irradiated chitin and chitosan was evaluated for two human cancer cell lines: Hep2 (Human larynx carcinoma cell line) and RD (Human embryo rhabdomyosarcoma cell line) at various concentrations of chitin and its derivatives.

Experimental

All chemicals used in this study were analytical grade and purchased from Sigma Chemical Co. (St. Louis Mo). Shrimp shells were obtained from a seafood restaurant. In addition, it was confirmed that all shells came from a single group of shrimp "Parapeneuslongirostris." Test materials shrimp shells were obtained from a seafood restaurant. It was confirmed that all shells were from a single species of shrimp Parapenaeus longirostris.

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Test materials

Shrimp shells were obtained from a seafood restaurant. It was confirmed that all shells were from a single species of shrimp Parapenaeus longirostris.

Chitin extraction

For extraction of chitin, shrimp shells were washed under running warm tap water to remove soluble organics, adherent proteins, and other impurities. The shells where then collected and boiled in water for 1 h to remove the tissue, followed by drying in an oven (Prolabo, model Volca MC18, French) at 160 °C for 2 h to make them more brittle and breakdown the crystalline structure. At the end, the dried shells were ground micro into a fine powder using a standard grinder (Model KU-2, PredomMesko, Skarzyskoka, Poland).

After that, the powder was demineralized to remove the calcium carbonate. For that, only dilute hydrochloric acid (1.5 M) was used to prevent hydrolysis of chitin within 30 min of incubation.

Preparation of chitin and chitosan

1. The shrimp shells were first stripped of their legs and antennae, washed, and boiled in water for an hour. After that, they were dried at 163 °C in an oven (Prolabo, model Volca MC18, France). The dry tissues could be removed easily.
2. Next stage is "quenching." The shell was heated in an oven at 80 °C for 48 h and cooled down immediately in iced acetone (Mukherjee 2001). Finally, the dried shells were grounded into a fine powder using a standard grinder (Model KU-2, PredomMesko, SkarzyskoKam., Poland). Demineralization calcium carbonate, calcium phosphate, and other mineral salts found in shell waste were extracted with dilute acids. Calcium carbonate constitutes the main inorganic component of the shells. To remove the calcium carbonate, only diluted hydrochloric acid at a concentration ranged from 0.25 to 2.5 M was used to prevent hydrolysis of chitin (No and Meyers 1995) with a reaction time varying from 5 to 150 min. The ratio of dried shells to acid solution used during the extraction of chitin ranged from 1/5 to 1/30 (*w*/*v*).

The experiments were carried out at room temperature under constant stirring speed of 150 rpm. The decalcified shells were collected on a 250-μm sieve, washed with tap water and neutralized with deionized water, and then oven dried at 80 °C overnight. The rate of demineralization was evaluated by determining the ash contents in the solids.

Deproteinization

Similar experimental conditions were applied for the demineralization of dried shells. The sodium hydroxide concentration varied from 0.5 to 3 M, the reaction time ranged from 20 to 150 min, and the temperature varied from 20 to 70 °C. The ratio of dried shells to aqueous sodium hydroxide solution used during the extraction of chitin ranged from 1/5 to 1/30 (*w*/*v*).

After this process, the material was filtrated, washed, and dried, as previously described in the demineralization process. In order to evaluate the extent of deproteinization, the protein concentration in the supernatant was determined according to Biuret's method (Fine 1935) which remains as the most widely used method for protein determination.

Bleaching

The chitin residue was mixed with acetone at a solid/solvent ratio of 1:10 (*w*/*v*) for 10 min, filtered, dried for 2 h at room temperature, and followed by bleaching with 0.315 % NaOCl during 5 min at the same solid/solvent ratio (No and Meyers 1995). The discolored chitin was washed and filtered as described beforehand.

Deacetylation

The conversion of chitin to chitosan involved deacetylation, a suggested process by other researches (Mirada, et al. 2002 and Kurita et al. 2003). For the purpose of N-deacetylation, 1 g of purified shrimp chitin was treated with 10 ml of 50 % sodium hydroxide solution at 140 °C for 60 min. After filtration, it was washed until neutral pH with deionized water and oven dried at 80 °C overnight.

Characterization of chitin and chitosan

Ashes were determined by incineration of 1 g sample in a muffle furnace at 900 °C for the duration of 2.5 h (Mirada et al. 2002). The calculation of ashes allowed the determination of the final yield of shell demineralization (Rao et al. 2000).

Total proteins in the solid fraction were determined according to the Kjeldahl procedure (A.O.A.C 1990). The final yield of shell deproteinization was then deduced (Rao et al. 2000). The degree of deacetylation (DD) was assessed by using the method FTIR spectroscopy (Sabnis and Block 1997). The infrared spectra of the samples were recorded with an infrared Fourier spectrometer of 380 Nicolets, type in transmission mode in the spectral range of 4000 to 400 cm^{-1}. For each spectrum, 64 scans were performed with a resolution of 4 cm^{-1}. The spectra were processed by the software OMNIC®. The DD was calculated using Baxter's equation (Baxter et al. 1992).

The different degrees of acetylation (DA) of polysaccharides were calculated using the following equation:

$$DA = (A1655/A3450) \times 100/1.33 \quad (1)$$

With A1655 and A3450absorbances at 1655 cm^{-1} of the amide I-band, this is a measure of N-acetyl groups and at 3450 cm^{-1} of hydroxyl band, indicating the internal standard for correction.

The factor 1.33 is the value of A1655/A3450 when all groups of the polymer are N-acetylated. The degree of deacetylation (DD) is deduced from the following equation (Khan et al. 2002).

$$DD = 100\,DA \quad (2)$$

The viscosity-average molecular weight, Mv, was determined with an Ubbelohde viscosimeter (Technico, ASTM D. 445) at room temperature with constant k= 0.01 cS/s. To measure the viscosity, the chitosan was dissolved in a mixture of acetic acid and 0.2 M sodium acetate 0.3 M, while chitin was dissolved in a mixture of LiCl and dimethylacetamide 5 % (Mirada, et al. 2002). The average molecular weight of the polymer prepared in this work was determined using the Mark-Houwink equation:

$$[\mu] = K(Mv)^a \quad (3)$$

Cytotoxicity assay

Cytotoxicity assay of Hep2 and Rd cells line

Hep2 (Human larynx carcinoma cell line) cell line and Rd (Human embryo rhabdomyosarcoma cell line) cell lines were obtained from Pasteur Institute of Algeria.

These cells were maintained in MEM supplemented with 10 % of FBS, 2 mM of glutamine, 100 $UIcm^{-3}$ of penicillin, and 100 g cm^{-3} of streptomycin. Cultures

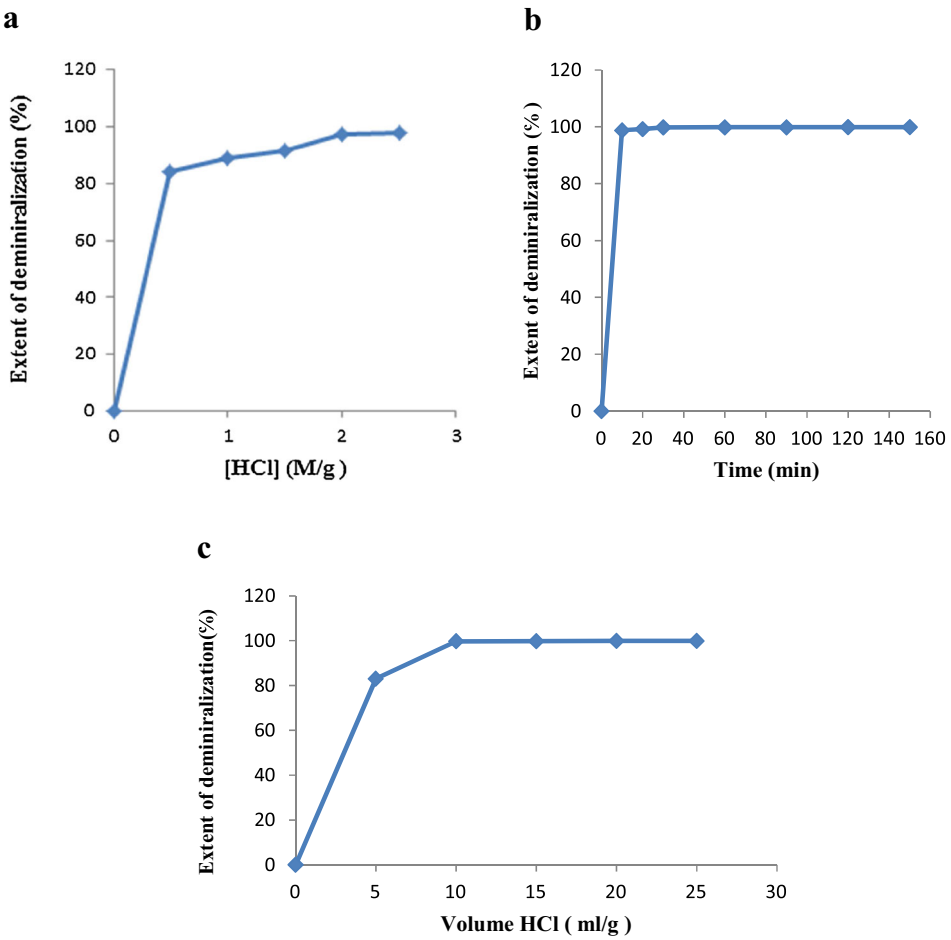

Fig. 1 **a** Effect of the concentration of HCl on extent of demineralization (ambient temperature solid/solvent 1/20 (g/ml), t=24 h). **b** Effect of reaction times on extent of demineralization (ambient temperature; [HCl] 1.5 M; solid/solvent 1/15 (g/ml)). **c** Effect of solids to solvent ratio on extent of demineralization (ambient temperature; [HCl] 2 M; 1 g of solid; t=30 min)

Table 1 The optimized conditions for demineralization of 1 g shells

Temperature (°C)	Time (min)	Volume of HCl (ml)	Concentration of HCl (M)
Ambient	30	15	2

were maintained in a humidified atmosphere with 5.5 % CO_2 at T=37 °C.

To study the inhibitory effect of chitin, chitosan, and the irradiated chitin, cells were seeded in 24-well plates. After a 24-h growth, cells were treated with varying concentrations of the test molecules (1, 50, 250, 500, 1000, 1500, 2000, 2500, and 3000 μg/ml). The cells were incubated at 37 °C for a period of 24 h and then stained with trypan blue to test the viability after 48 h culture. All the experiments and measurements were done in triplicate.

Trypan blue exclusion assay

About 300 μl of cell suspension was sampled and mixed with an equal volume of trypan blue. The total number of live cells was then counted using a hemocytometer under light microscopy. The cytotoxic activity was calculated by the following equation, established by Cuesta et al. (2003).

Results and discussions

Extraction, demineralization, and deproteinization of chitin

The shells contained relatively high protein content (49 %) and ash (35 %) on a dry weight. These values were in agreement with those obtained in other studies (No and Meyers 1995). However, it should be noted that these results are not constant because quantitative and qualitative changes in chitin may be observed in crustaceans due to the physiological stage of the organism or seasonal variations and according to the species (Fernandez-Kim 2004). The extent of demineralization ranged from 84 to 100 % (*wt*/*wt*) as a result of varying HCl concentrations, reaction time, and solid solvent ratios (Fig. 1). Demineralization of the dried shrimp shells was achieved with 1.5 N HCl and 30 min reaction time at ambient temperature (Table 1). The deproteinization reaction occurred at low concentrations of NaOH and its peak at 2 M. The deproteinization was proportional to the reaction time up to 90 min (Fig. 2). In addition, a plateau was reached. The deproteinization can be performed at room temperature, but it is optimal at 55 °C. The optimal volume of NaOH to deproteinize the shrimp shells was about 20 ml. The deproteinization shell *Parapenaeus longirostrisis* was achieved by using the optimum experimental conditions

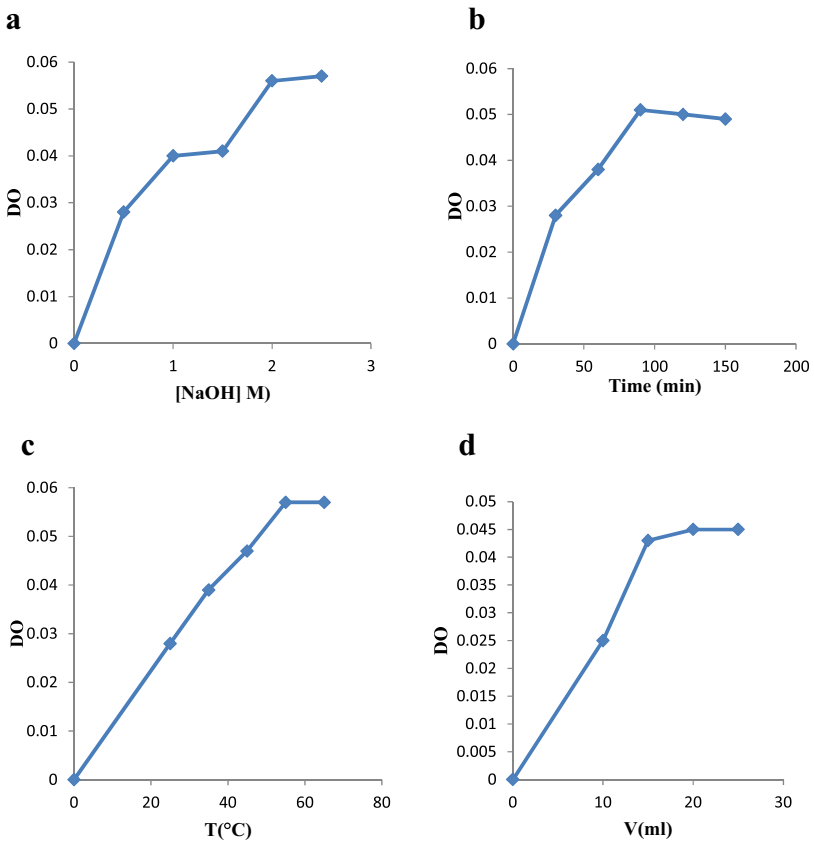

Fig. 2 **a** Effect of concentration of NaOH on extent of deproteinization, ambient temperature solid/solvent 1/20 (g/ml); t=3 h. **b** Effect of reaction time on extent of deproteinization at room temperature; [NaOH] 2 M; solid/solvent 1/20 (g/ml). **c** Effect of temperature on extent deproteinization [NaOH] 2 M; solid/solvent 1/15 (g/ml); t= 90 min. **d** Effect of solid/solvent ratio on deproteinization [NaOH] 2 M; T, 55 °C; 1 g of solid; t= 90 min

Table 2 The optimized conditions for deproteinization of 1 g of shells

Temperature (°C)	Time (min)	Volume of NaOH (ml)	NaOH concentration (M)
55	90	20	2

20 ml of 2 M NaOH at 55 °C, stirring during 90 min and a ratio solid/solvent of 1/20 g/ml (Table 2).

Molecular weight and deacetylation degree of chitin and chitosan

The viscosity-average molecular weight, Mv, of chitin obtained in this study was determined to be 350, 000 Da and equal to 20,050 Da for chitosan. The irradiated chitin molecular weight at a dose of 210 kGy is 11 300 Da (Dziril et al. 2015).

The deacetylation degree of chitosan and chitin was calculated by infrared spectrophotometry. DD are approximately 60, 39, and 17 % for chitosan, chitin, and for the irradiated chitin, respectively (Figs. 3, 4, and 5).

The infrared spectrophotometry results of chitin are similar to those found by Brugnerotto et al. (2001). However, the results of infrared spectrophotometry of chitosan differ from those already published (Hurt et al. 2014). This difference is probably due to the partial reactions of chitin deacetylation.

Study of the inhibitory effect of chitin and its derivatives against cancer cell lines

Figures 6 and 7 indicate that chitin and its derivatives showed a marked inhibition of the growth of Hep2 and RD cells after 24 h of contact. It is also observed that chitin and its derivatives exerted a dose-dependent inhibition. Indeed, as the later figures show, cell viability decreased and hence the cytotoxicity of chitin, irradiated chitin, and chitosan have increased with an augmentation of the tested molecules concentrations. The results clearly showed that chitin was cytotoxic with an IC50=400 μg/ml (Table 3), and the total cytotoxicity (100 %) is reached at a concentration of 2000 μg/ml for Hep2 cells. For RD line (Table 4), the IC50 is 200 μg/ml and total cytotoxicity (100 %) is not reached even at a concentration of 3000 μg/ml. Compared to other derivatives, the lowest IC50 is attributed to chitosan. Indeed, it was 300 μg/ml in Hep2 and 190 μg/ml in RD. The death of all cells was observed at a concentration of 3000 μg/ml, whereas it was not reached for Hep2. The results of the antiproliferative effect of chitin and its derivatives on the Hep2 line outlined that the chitosan has an IC50= 300 μg/ml; all tested molecules have an IC50 range of 400 to 500 μg/ml. The effect of tested molecules on cells of the RD line was slightly different from the results obtained for Hep2. Indeed, one can notice that the IC50 of molecules tested on RD are smaller than those observed in Hep2 and vary from 190 to 300 μg/ml. In both cases, there is an antiproliferative

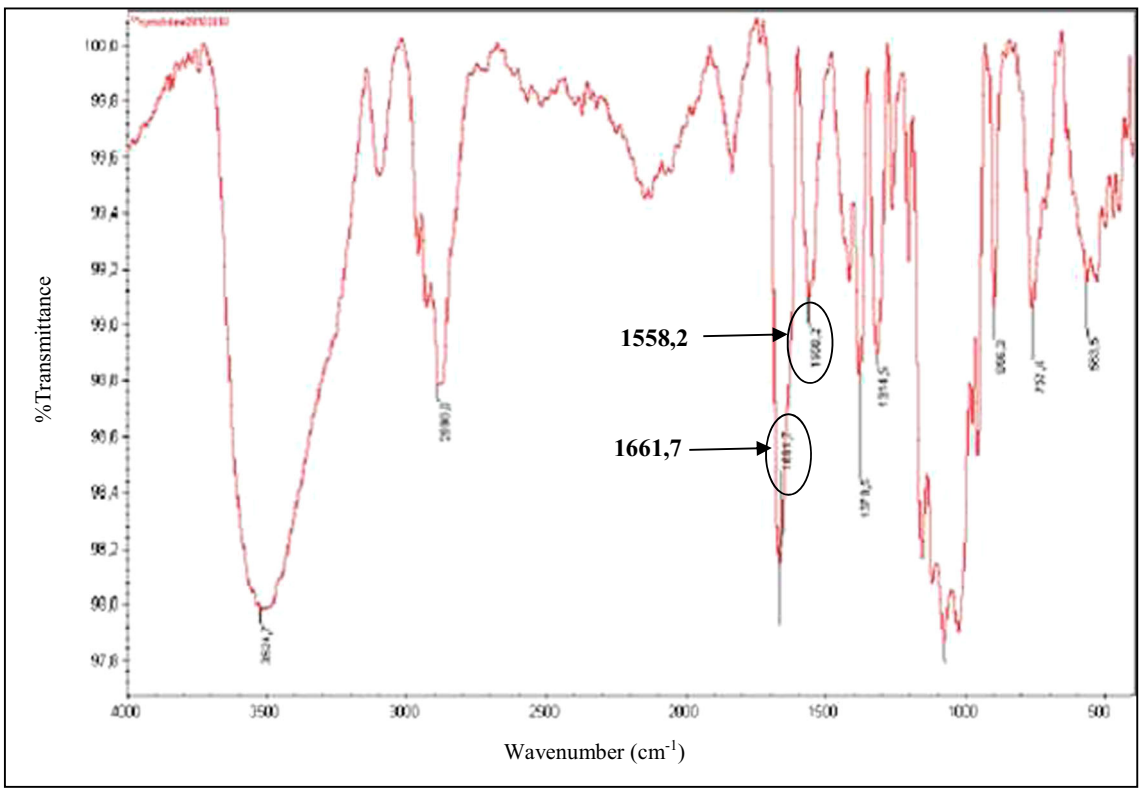

Fig. 3 Infrared spectrum of chitin

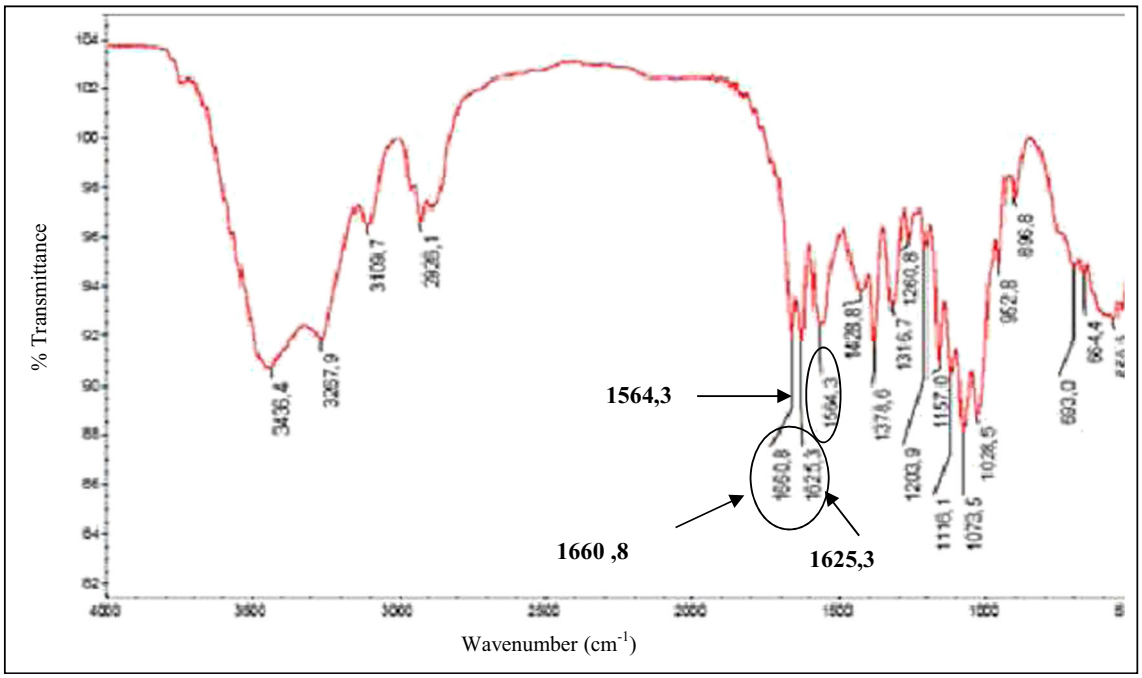

Fig. 4 Infrared spectrum of the irradiated chitin

effect on cancer cells. Chitosan has the lowest IC50 and therefore the greater efficiency. The difference in cytotoxicity on the two lines with respect to the different molecules tested may be related to their morphological and structural characteristics but also the effectiveness of their damage compensation system. Moreover, effectiveness of chitosan may be attributed to its cationic polyelectrolyte character and thus its positive charges. In fact, the presence of amine groups in the units of D-glucosamine added a feature to chitosan compared to other polysaccharides. Chitosan acquires positive charge at pH 6.2, allowing it to bind the negatively charged molecules (lipids, collagen glycosamunoglycans, lignosulfonates,

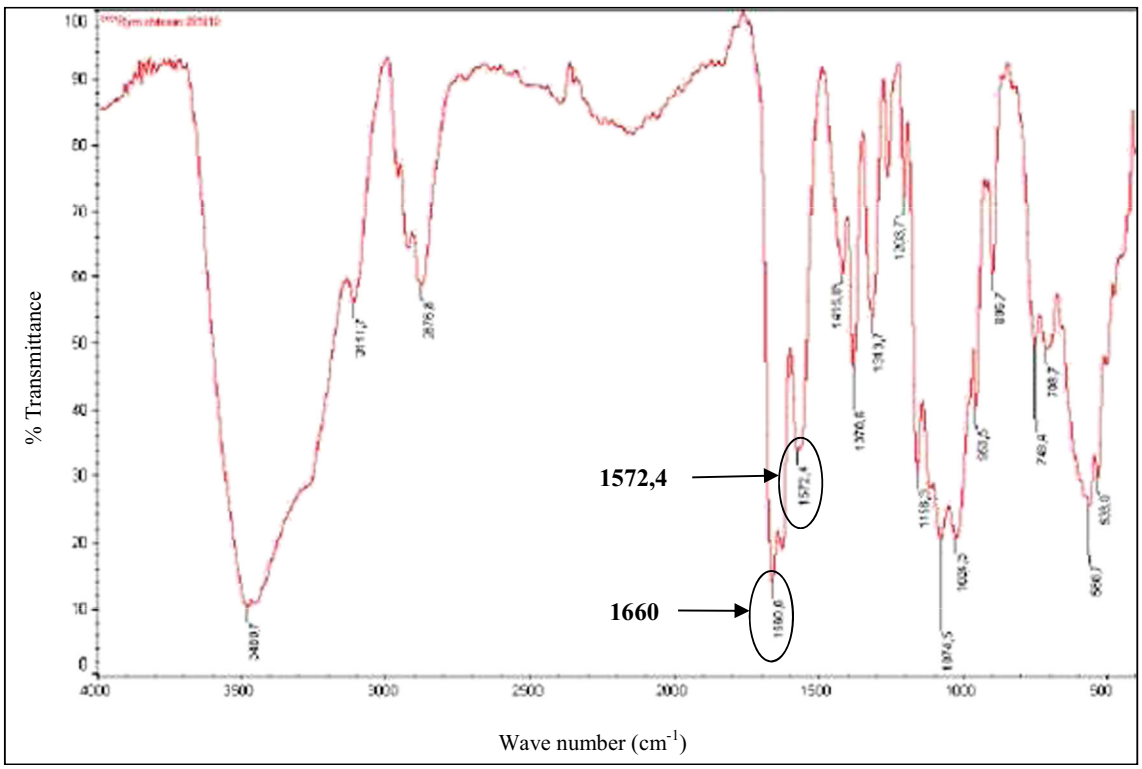

Fig. 5 Infrared spectrum of chitosan

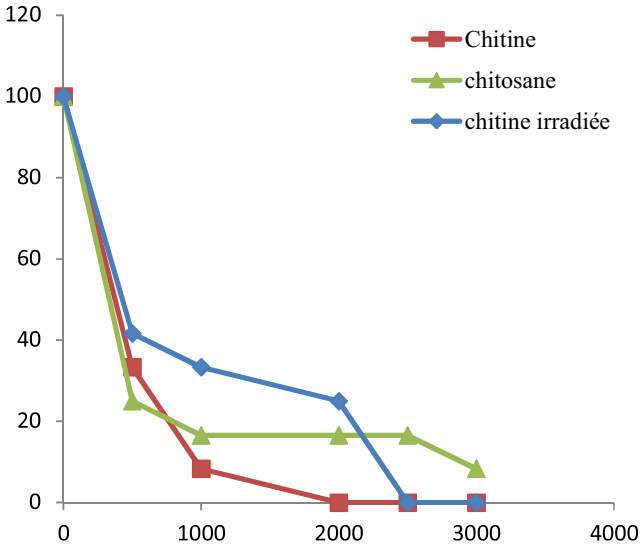

Fig. 6 Cytotoxic effect of chitin, the irradiated chitin, and chitosan on Hep2 cells

alginates, and DNA) by electrostatic interactions; and thus a complex poly-electrolytes (CPE) may be formed. The formation and properties of these CPE strongly depend on pH, the DD of chitosan, and the two polymers load; (Gamzazade and Nasibov 2002). The formation of DNA/chitosan complex was demonstrated by Lee et al. (2005). Study focused on the cytotoxicity of cationic chitosan derivatives against human cancer cell lines. It concluded that the cytotoxicity is probably due to interactions between the negative charges groups of tumor cells and the positively charged groups of the derivatives. The same conclusion, as the previous authors, was given by Salah et al. (2013) who studied the cytotoxicity of chitosan derivatives by varying degrees of deacetylation towards human cancer cell lines. Many other authors (Aspden et al. 1997) also attributed the effectiveness of chitosan in its DD, its electric charge, morphology, and size. Salah et al. 2013 determined the passage of the molecule through the plasma membrane

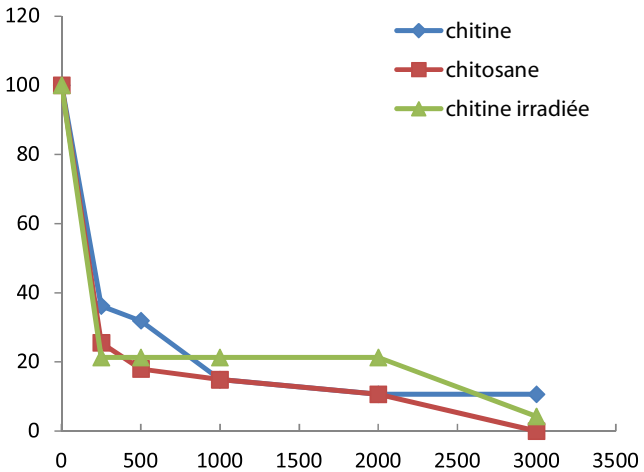

Fig. 7 Cytotoxic effect of chitin, the irradiated chitin, and chitosan on RD cells

Table 3 IC50 of chitin and its derivatives (Hep2)

Molecules tested	IC_{50} (μg/ml)
Chitosan	300
Chitin	400
Irradiated chitin	450

and cell wall. The passage through the pores of polar molecules is possible for MM <40 kDa. The results obtained with chitin have not reached the same conclusions as the latter, which is an uncharged molecule. It is interesting to note that all molecules have an inhibitory and cytotoxic effect against Hep2 and RD lines. It can be deduced that there are several types of interactions between the charged groups of molecules and tumor cells such as electrostatic interactions between the negative charges of groups of tumor cells and the positive charges of groups of molecules tested and also interactions between hydrophobic groups and tumor-derived cells.

Characterization of chitin and its derivatives by infrared spectroscopy

The absorption spectra of chitin and chitosan prepared from *Parapenaeuslongirostris* shells exhibit the same peaks mentioned in the literature (Brugnerotto et al., 2001 and Kasaai 2008) (Figs. 3, 4, and 5). The FTIR spectra of chitin and chitosan have confirmed the extraction of chitin, and its deacetylation was realized satisfactory. Indeed, chitin and chitosan characteristic peaks are demonstrated. These are as follows:

- Strip of OH stretching vibration at 3450 cm^{-1};
- Band of CH stretching vibration between 2870 and 2880 cm^{-1};
- Bands of COC stretching vibration at 1030 or 1070 cm^{-1};
- The tape of the deformation vibration-CH2 bond at 1420 cm^{-1};
- Strip antisymmetrical stretching vibration COC at 1160 cm^{-1};
- The band of the amide III between 1315 and 1320 cm^{-1}
- The band bending vibration of the NH-NH 2 between 1620 and 1630 cm^{-1}
- The band COC 890–900 cm^{-1}glycosidic bond.

Effective deacetylation of chitin is translated by the displacement of the amide II band present at 1558.2 cm^{-1} in the chitin to 1572.4 cm^{-1} in chitosan.

Table 4 IC50 of chitin and its derivatives (RD)

Molecules tested	IC_{50} (μg/ml)
Chitosan	190
Chitin	300
Irradiated chitin	200

Conclusions

This study revealed that the composition of the shell *Parapenaeus longirotris* is 39 % protein and 35 % minerals. The demineralization optimization of the *Parapenaeus longirotris* shell is achieved using 15 ml of 2 M HCl per gram of shells at room temperature with a stirring during 30 min and that the optimum deproteinization is achieved by using 20 ml of 2 M NaOH per gram of shells at 55 °C with stirring for a period of 90 min. The degree of chitosan and chitin deacetylation was determined by infrared spectrophotometry. DD are approximately 39 and 60 % for chitosan and chitin, respectively. The results obtained also demonstrated that chitin and its derivatives have a dose-dependent cytotoxic effect against Hep2 and RD cell lines, indicating that there are several types of interactions between the charged groups of molecules and tumor cells such as electrostatic interactions between the negative charges of tumor cells groups and the positive charges of groups tested and interactions between hydrophobic groups and tumor cells derived molecules. However, it would be of such interest to improve the properties of the molecules tested in the present study and to focus our future work on the optimization of the production processes. It would also be necessary to expand the range of cell lines studied and further investigate the chitin, chitosan, and their derivatives exact mechanisms of action on cancer cells. Consideration should be given subsequently to the possible resistance mechanism of cancer cells to the inhibitory effect of these substances.

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