

# Characterization and cytotoxicity of low-molecular-weight chitosan and chito-oligosaccharides derived from tilapia fish scales

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## ABSTRACT

The present study evaluated the physicochemical characterization and cytotoxicity activity of chitosan and chito-oligosaccharides (COSs). The extraction of chitosan and COSs was executed by chemical hydrolysis. The physicochemical characterization and deacetylation (DA) value were determined using an FTIR. The molecular weight was determined by using the Mark-Houwink equation. The physical parameters such as solubility, water-binding capacity (WBC), and fat-binding capacity (FBC) were determined as per equation (i), (ii), and (iii) respectively. The cytotoxic activities of chitosan and COS against MCF-7, HepG2, HeLa-6, and 3T3 were performed by MTS assay. The COS induced enhance cytotoxicity with  $IC_{50}$  0.87 and 2.21 mg/ml against MCF-7 and HepG2 respectively. However, COSs seem to be more sensitive toward the cell lines with the relative potential of MCF-7 > HepG2 > HeLa. Hence, the results showed promising future perspectives of chitosan and COS to develop biodegradable, antibacterial, cytotoxic naturally derived polysaccharides for cancer drug delivery and smart wound dressings.

**Key words:** Biodegradable polymer, cancer, chito-oligosaccharides, chitosan, cytotoxicity, MTS

## INTRODUCTION

Naturally, derived biopolymers have gained much attraction in various fields. Due to biocompatibility, biodegradability,

and various physicochemical features, these bio-proteins and bio-polysaccharides mimic inside the extracellular matrix of a biological system. The biopolymers are composed of (i) glycosaminoglycan, (ii)  $\beta$ -linked D-glucosamine, and N-acetyl-D-glucosamine, which possess physicochemical as well as biomedical properties. These various derivatives of biopolymers, having desire molecular weight (MW) and chemical modifications, make them undefine composition to unique and targeted applications. Chitin is a polymer of N-acetylglucosamine, present profoundly from unicellular to multicellular organisms.<sup>[1]</sup> Chitosan is derived from chitin and is composed of N-acetyl-D-glucosamine and  $\beta$ -(1,4)-linked-D-glucosamine, widely used in various

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biomedical applications, targeted drug delivery systems, and nanomedicines. Due to its nontoxic, biocompatible, and biodegradable nature chitosan used in drug delivery.<sup>[2,3]</sup> The utilization is considered to be safe and approved by the Food and Drug Administration in wound healing applications.<sup>[4,5]</sup> Furthermore, the chitosan microspheres and microcapsules have previously been studied in controlled release therapy for drugs and proteins.<sup>[6,7]</sup> Chitosan-based nanomedicine also gained massive attention due to its nontoxic and biodegradable nature. The chitosan-based nanoformulation improves the presence of drug in blood circulation as well as sustained drug release.<sup>[8-10]</sup> Moreover, the non-immunogenic nature unable to trigger the immune response in body.<sup>[11,12]</sup> Chito-oligosaccharide (COS) is short oligomers of chitosan, with degree of polymerisation (DP) <55 and molecular weight (MW) is 10 kDa.<sup>[13]</sup> COSs have been used as a drug delivery system for disease treatment also as food.<sup>[14,15]</sup> Furthermore, chitosan, along with other chemicals/polymers/cross-linkers and receptor ligands, has been shown to improve the efficacy of potential drug therapeutic. In this study, physicochemical characterization of chitosan and COSs was conducted along with cytotoxicity on selected cancer cell lines, including MCF-7, HepG2, HeLa, and fibroblast 3T3.

## MATERIALS AND METHODS

### Pretreatment of raw materials and isolation of chitosan

The tilapia fish scales were obtained from a commercial fish processing plant situated in West Malaysia. Chitosan extraction from fish scales was done by using the modified method given by Toan.<sup>[16]</sup> The fish scales were soaked in 2% HCl at ambient room temperature with a solid-to-solvent ratio of 1:5 (w/v) for 24 h. Then, the dried residues were treated with 4% NaOH at ambient room temperature with a solid ratio of 1:5 (w/v) for 24 h. Then, chitin was treated with 80% NaOH at 120°C for 6 h.

### Extraction of chito-oligosaccharides

COS was extracted by chemical hydrolysis following the methods described by Trombotto *et al.*<sup>[17]</sup>

### Degree of deacetylation

The degree of deacetylation (DA) was determined by the method described by Muzzarelli and Rochetti (1985).<sup>[18]</sup> The chitosan and COS samples were first pelleted with KBr at a sample/KBr ratio of 1:60.

### Physicochemical and functional properties

Crude protein, moisture, and ash were determined using official methods described by the Association of Official Analytical Chemists (1984).

### Molecular weight

Firstly, a series of mixture ranging from 0.02% to 0.1% of chitosan and COS in 0.1M acetic acid and 0.2M sodium chloride was prepared. Then, the MWs of the respective

samples were determined based on the Mark-Houwink equation.<sup>[19]</sup>

### Solubility

For solubility, 0.5 g of chitosan and COS was placed in a 50 mL tube, dissolved with 50 mL of 1% acetic acid, and mixed in a vortex mixer for 10 min. The residues were weighed, and the percentage of solubility was calculated using the formula as below:<sup>[20]</sup>

$$\text{Solubility \%} = \frac{\text{Precipitated pellet (g)}}{\text{Initial sample weight (g)}} \times 100 \quad \text{eq. (i)}$$

### Water- and fat-binding capacity

The water binding capacity (WBC) and fat binding capacity (FBC) of chitosan and COS were determined by previously reported method.<sup>[21]</sup> The WBC value was determined using the formula below:

$$\text{WBC (\%)} = \frac{\text{Precipitated pellet [g]}}{\text{Initial sample weight [g]}} \times 100 \quad \text{eq. (ii)}$$

The FBC value was determined using the formula below:

$$\text{FBC (\%)} = \frac{\text{Precipitated pellet [g]}}{\text{Initial sample weight [g]}} \times 100 \quad \text{eq. (iii)}$$

### Cytotoxicity analysis

The cytotoxicity of chitosan and COSs against MCF-7, HepG2, HeLa-6, and 3T3 was determined. The cell proliferation assay was conducted using the CellTiter 96™ Aqueous One Solution Cell Proliferation Assay.<sup>[22,23]</sup> The absorbance was then read at a wavelength of 490 nm using an ELISA reader (Multiskan, Thermo Fisher, USA).

### Statistical analysis

The experimental data were subjected to two-way ANOVA analysis (ANOVA), using Origin 8 SR4.

## RESULTS

### Physicochemical and functional properties

The physicochemical properties of chitosan and COSs are given in Table 1. The degree of DA for chitosan and COS was reported as 92.235% and 94.65%, respectively. Furthermore, the MW of chitosan and COS was 11.58 and 4.6 kDa, respectively. The degree of degree of acetylation (DA) of chitosan and its derivatives influenced by various factors such as sample location, extraction method and analytical instrumentation used.<sup>[24,25]</sup> Previous studies supported the value of DA; the degree of DA of chitosan ranged from 56% to 99%.<sup>[26]</sup>

Interestingly, the solubility of COS reporting is 98.65%, which is higher than the solubility of solubility chitosan (78.65%). However, protein contaminants have

a significant impact since the solubility is associated with the reaction of the amino groups.<sup>[27]</sup> Furthermore, the WBC for both chitosan and COS was considerably high, reporting 860% and 930%, respectively, whereas for the FBC, chitosan and COS reported 572% and 640%, respectively.

### Cytotoxicity analysis

The cytotoxicity of chitosan and COSs against MCF-7, HepG2, HeLa, and 3T3 cell lines [Figure 1] was evaluated. There is a dose-dependent increase in cytotoxicity noticed after chitosan and COS treatment in all cell lines. The COS inhibited the growth of MCF-7 cells at a concentration of 0.25 mg/ml in all three cancer cell lines. Interestingly, HepG2 is more sensitive toward chitosan with cytotoxicity of 26.40% versus COS inhibition of 14.40%. Similarly, HeLa cells are more sensitive toward COS (23.40%) inhibition versus chitosan (13.80%). Moreover, the highest cytotoxicity, 92.80%, was observed in MCF-7 after treatment with 1 mg/ml of chitosan. However, HepG2 seems to be less sensitive toward chitosan. The  $IC_{50}$  values of chitosan against HepG2

were above 4 mg/ml [Table 2]. Furthermore, 3T3 cells exhibited the least growth inhibition against chitosan and COS. Hence, chitosan and COS could be used in wound healing as they are safe against fibroblast even at high concentrations (4 mg/ml). The MCF-7 shown to be more sensitive against COS, followed by HepG2 and HeLa cells. However, the  $IC_{50}$  values for 3T3 unable to achieved in given concentration of 4mg/ml.

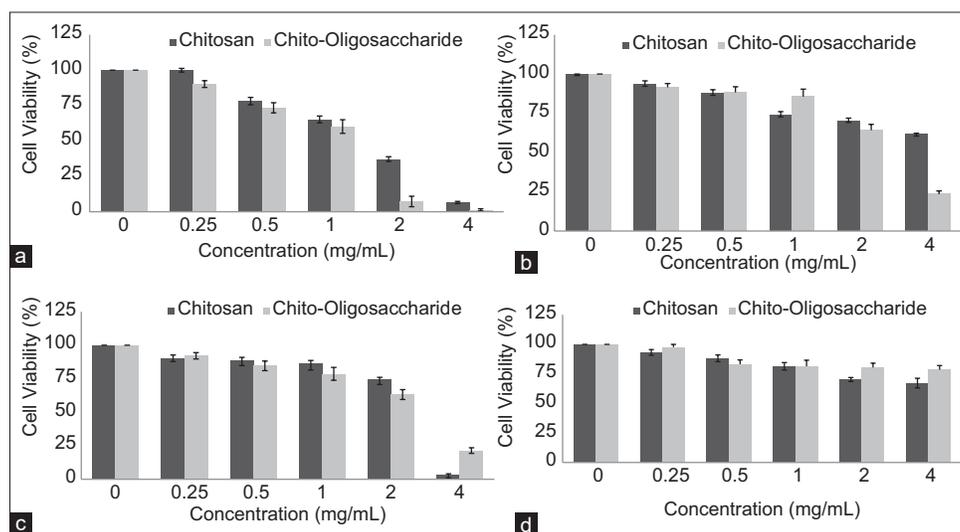
### DISCUSSION

The biopolymers could play a remarkable role in therapeutic drug development. In our study, chitin from fish scales converted into low MW, chitosan, and COSs with a high degree of DA. The chitosan samples were tested for their cytotoxicity properties against four cell lines, including MCF-7, HepG2, HeLa, and 3T3 cell lines. The role of chitosan nanoparticles and the effects of MW in cellular uptake and cytotoxicity activities have been proven in many studies. Huang *et al.*<sup>[28]</sup> demonstrated that the increasing DA degree had a much significant impact on the cellular uptake capability of the chitosan samples. The increase in DD% from 61% to 88% significantly increased the cytotoxic activities. Cytotoxic activities were highly dependent upon the three-dimensional arrangement of cationic residues along with the charge density of chitosan. Chitosan with a higher degree of DA binds more readily with cell membranes due to its extended conformation caused by the charge repulsion. A previous cytotoxicity study was done on three different marine sources, including shrimp and crab shells and cuttlefish bones against RT112 bladder cancer cells. The cytotoxicity depended on the chitosan MW.<sup>[29]</sup> The chitosan and COS used in this study were found not to induce cytotoxic activities on 3T3 fibroblast cells. Even at high concentrations of 4 mg/ml, the cell viability percentage of 3T3 cells treated with chitosan exhibited 67.2%, and COS

**Table 1: The physicochemical properties of chitosan and chito-oligosaccharides extracted from fish scales**

Physicochemical properties	Chitosan	COS
Degree of DA (%)	92.23	94.65
Molecular weight (kDa)	11.58	4.6
Moisture content (%)	9.87	4.13
Ash content (%)	1.66	1.12
Protein content (%)	2.42	1.65
Solubility (%)	78.65	98.65
WBC (%)	860.45	930.12
FBC (%)	572.13	640.67

COS: Chito-oligosaccharide, DA: Deacetylation, FBC: Fat-binding capacity, WBC: Water-binding capacity



**Figure 1:** The cell viability activity of chitosan and chito-oligosaccharides against MCF-7 (a); HepG2 (b); HeLa (c); 3T3 (d) lines at 72 h. Results are mean  $\pm$  standard deviation ( $n = 8$ )

**Table 2: IC<sub>50</sub> values of chitosan and chito-oligosaccharides against cell lines (mg/mL)**

Samples	IC50 value			
	MCF-7	HepG2	HeLa	3T3
Chitosan	1.46±0.2	>4.00	2.66±0.4	>4.00
COS	0.87±0.3	2.21±0.2	3.51±0.3	>4.00

COS: Chito-oligosaccharide

revealed 78.5% with an undetectable IC<sub>50</sub> value. The results from this study were comparable to the ones conducted by Nor *et al.*, Lim *et al.*, and Abdull Rasad *et al.*<sup>[30-32]</sup> The IC<sub>50</sub> value of the chitosan nanoparticles used in their study reported was 5.3 µg/mL after 48 h treatment. The fibroblast cells treated with chitosan possessing a higher degree of DA exhibited significantly higher proliferation rates than lower degree DA chitosan.<sup>[33]</sup> Moreover, chitosan was used as a polymeric vehicle for delivering drugs, i.e. pioglitazone, heparin, and bemiparin, for diabetic wounds.<sup>[34,35]</sup> Also, chitosan oligosaccharide nanofiber promotes wound healing by activating TGFβ1 Smad signaling pathway.<sup>[36,37]</sup> However, the potential toxicity of chitin limits its utilization due to potentially harmful effects on the human body.<sup>[38]</sup> Interestingly, chitosan and oligosaccharide have water-soluble nature and have reported minimal toxicity via oral routes.<sup>[39,40]</sup> Hence, chitosan and oligosaccharide could be a huge potential in developing targeted drug therapeutics with enhanced efficacy.

## CONCLUSION

Scientific breakthroughs are seen in the last decade in chitosan-based nanomedicines in treating cancer. Unfortunately, both chitosan molecules exhibited comparable cytotoxicity. However, fibroblast cells are not sensitive toward these biopolymers, which show the potential to be used in disease treatment and therapeutics. The *in vivo* study on the pipeline strengthens the future perspectives of chitosan and COS for biodegradable, antibacterial, cytotoxic naturally derived polysaccharides for cancer drug delivery and innovative wound dressings.

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## Conflicts of interest

There are no conflicts of interest.

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