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Evaluation Antioxidant and cytotoxic activities of novel chitooligosaccharides prepared from chitosan via enzymatic hydrolysis and ultrafiltration

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ABSTRACT

Our work was designed to evaluate antioxidant (free radical scavenging and reducing power) and cytotoxicity activities of chitooligosaccharides with different molecular weights. The antioxidant activities *in vitro* were assessed by three separated methods, including DPPH (1,1-diphenyl-2-picrylhydrazyl radical) and ABTS (2,2-azinobis (3-methyl-benzothiazoline-6-sulfonic) free radical-scavenging assay systems and ferric reducing power (FRAP). Results were compared with that of trolox which was used as a reference compound. There was correlation between antioxidant activity and molecular weight of chitooligosaccharides. In general, lower molecular weights showed better activity than higher one. Chitooligosaccharides with molecular weight lower than 1 KDa showed highest antioxidant activity in the all tested methods (86.97to 93.56 µgTE/mg). Chitooligosaccharides with molecular weight less than 1.0 KDa group was analyzed by high-performance liquid chromatograph (HPLC). It mainly contained N-acetylglucose amine (36.7 %), chitobiose (29.1 %) and chitotetrose (23.51 %). The chitooligosaccharides groups were also subjected to investigate cytotoxic activity on three different carcinoma cells lines (HEPG2, HCT and MCF7). Cytotoxicity was evaluated by sulfohodamine B (SRB) cell viability assay *in vitro*. Chitooligosaccharides with molecular weight 10 to 100 KDa showed IC_{50} 1.564, 1.84 and 2.208 µg/ml against HEPG2, HCT and MCF7 cells, respectively. While Chitooligosaccharides with molecular weight 1.0 to 10 KDa showed IC_{50} 12.948 and 11.952µg/ml against HEPG2 and MCF7 cells, respectively. They caused 75 to 80% inhibition of the tested cells. Thus, the chitooligosaccarides had valuable antioxidant and cytotoxicity, which related to their molecular weights. Chitooligosaccharides may be used as a functional food ingredient in pharmaceutical and food industries future.

INTRODUCTION

Chitooligosaccharides possess various biological activities such as antitumor activity, radical scavenging, antihypertensive, opioid, mineral binding, antimicrobial and wound healing **(**Li *et al.*, 2012, Aliakbarlu *et al.*, 2014, Jamshidi *et al.*, 2014 and El-Sayed *et al.*, 2017). The chemical spoilage and rancidity of foods was caused by excess oxidation (Colbert and Decker, 1991). Oxidative metabolism is urgent for the survival of cells. Excess of free radicals causes destructive and lethal cellular effects. They could be overcome by various antioxidant compounds. Therefore, many searches for separation and identification of new natural antioxidant compounds were required to overcome the deleterious effects in the biological systems. Low molecular weight chitooligosaccharides had high antioxidant activity and stability at different conditions besides high solubility in water and easy absorption. They also had high safety over the use of synthetic antioxidants, which may have potential carcinogenicity and genotoxicity (Bomhard *et al.*, 1992). The mechanism of chitooligosaccharides antioxidant effectmay be related to the presence of amino and hydroxyl groups attached topyranose ring at C-2, C-3 and C-6 positions (Je *et al.*, 2004; Charernsriwilaiwat *et al.*, 2012).

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They react with liable free radicals to form stable macromolecule radicals. Free radicals were involved in pathogenesis of cancers, diabetes and cardiovascular diseases, which may be prevented by antioxidant compounds (Aliakbarlu *et al.*, 2014).

The anti-tumor activity of chitooligosaccharides with several mechanisms including regulation of immunity had been proposed (Yu *et al*., 2004). The inhibitory effects of chitooligosaccharides, as antioxidant compounds on angiogenesis, had received some attention (Wang *et al*., 2007 and Wu *et al*., 2008). When tumor cells reached a certain size, new capillary blood vessels was formed from already existing blood vessels. Chitooligosaccharides inhibited the growth of tumor cells through influence immuno-enhancing effects and also by inhibiting angiogenesis (Wu *et al*., 2012). In intestinal intraepithelial lymphocytes, chitooligosaccharides improved the natural killer activity and decreased tumor growth in mice (Maeda and Kimura, 2004). They caused death of human hepatocellular carcinoma cells by regulation of the pro-apoptotic protein (Xu *et al*., 2008). Molecular weight was suggested to play a major role in activity of chitooligosaccharides (Qin *et al*., 2002).

The present study aims to study the cytotoxic and antioxidant activities of chitooligosaccharides with different molecular weights.The antioxidant results were compared with that of trolox as a reference compound. The constituent of chitooligosaccharides with low molecular weight less than 1.0 KDa were also investigated by HPLC.

MATERIAL and METHODS

Materials

Chitosan with molecular weight 300 KDa, (GlcN), $(GlcN)_2$ and $(GlcN)_4$were purchased from Merck chemical Co, Germany. ABTS and PDDH were purchased from Sigma-Aldrich.HEP-G2 (hepatocellular carcinoma cell line), MCF7 (breast carcinoma cell line) and HCT-116 (colon carcinoma cell line) cells were gifted by the National Cancer Institute. All chemicals used in this work were of analytical grade.

Preparation of chitooligosaccharides

Four chitooligosaccharides groups I, II, III and IV with different molecular weights > 100KDa, 100 to 10 KDa, 10 to 1 KDa and <1 KDa, respectively, were prepared (El-Sayed *et al*., 2016). Chitooligosaccharides were prepared by enzymatic hydrolysis of chitosan using immobilized pepper chitosanase. The reaction mixture contain immobilized chitosanase: chitosan ratio 0.95U/mg in acetate buffer, pH 5.6. It was incubated for 1.5 h at 55°C. Chitooligosaccharides were separated by cooling centrifugation. They were fractionated by successive steps to tangential flow filtration in a cross flow filtration system (Sartorius startoflow) using filters with a gradual reduction on the cut off membrane, Sartorius, polysulone. All fractions were lyophilized and stored at -4°C.

Determination of the prepared chitooligosaccharides

The prepared chitooligosaccharides concentrations were estimated by dinitrosalicylic acid (DNS) method (Miller, 1959) using D-glucosamine as standard. Equal volume of chitooligosaccharides and DNS reagent (3ml) was heated for 15 min in boiling water bath and then1.0 ml of 40% Rochelle salt solution was added. The intensity of brownish-red colour was measured using spectrophotometer at 575 nm.

In vitro anticancer assays

Cytotoxic activities against some carcinoma cell line (*in vitro*) were performed in the National Cancer Institute using SRB assay (Skehan *et al*. 1990). One mg of each dry chitooligosaccharides groups was dissolved in 0.1 ml of DMSO and 0.9 ml with distilled water. HEP-G2, MCF7 and HCT-116 cells were plated in (10^4 cells/well) for 24 h. Then different concentrations of the dry chitooligosaccharides groups from 0, 1, 2.5, 5 to 10 µg/well were added and incubated at 37°C in atmosphere of 5% CO_2 for 48 h. The cells were fixed and stained with sulforhodamine-B stain. The intensity of colour was measured in an ELISA reader. Surviving fractions was plotted against the chitooligosaccharides concentrations. The half maximal concentration (IC_{50}) of chitooligosaccharides, which caused 50% inhibition of cell growth was calculated. Doxorubicin was used as positive control.

High-performance liquid chromatography (HPLC)

Chitooligosaccharides group IV and standards (GlcN), $(GlcN)_2$ and $(GlcN)_4$ were subjected to HPLC analysis using shimadzu: SLC-LG 10 A DVP, SCL-10 AVP system controller, LC-10 A DVP pump with microflow modification, DGV-14a degasser, An equipped with auto sampler and R 3D 10 A detector. The analytical column was schodex column Sc1011 No: H706087 with a guard column Schodex sugar SC-LG No: 6703073. The mobile phase consisted of distilled water at flow rate 1.0 ml/min. and 80°C using RI detector. The injection volume for samples was 10µl. Peaks were identified by retention times and UV spectra were compared with those of standard using shimadzu class-VP v5.03 software.

Total antioxidant capacity

Antioxidant activity was measured as the ability of chitooligosaccharides to scavenge free radicals DPPH and ABTS and reducing power FRAP activity.

DPPH method

The scavenging activity of chitooligosaccharides on HPPD was found to be used mostly for the *in vitro* antioxidant activity evaluation purpose. Chitooligosaccharides samples were react with methanol DPPH solution in dark for 24 h (Brand-Williams *et al*., 1995) Then, the intensity of colour was measured using spectrophotometer at 515 nm.

Inhibition of scavenging activity percent = $(1-A/A_0)x100$,

where A is the absorbance of the test and A_0 is the absorbance of the controls (reagents). Trolox was used as a positive control. The standard curve of trolox concentration against inhibition percent (scavenging activity percent) was linear between 1-10 µg Trolox/reaction mixture (Figure 1). Results were expressed as µgTE/mg chitooligosaccharides.

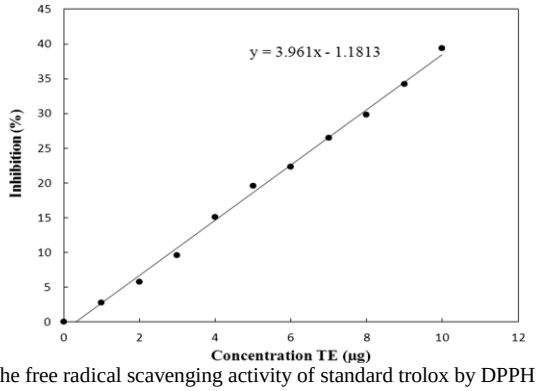

Fig. 1: The free radical scavenging activity of standard trolox by DPPH method.

ABTS radical cation decolourization assay

ABTS method was based on the ability of chitooligosaccharides to quench the long-lived ABTS (Arnao *et al*., 2001). Chitooligosaccharides were react with ABTS solution in a dark for 2 h. The intensity of the colour was measured using spectrophotometer at 734 nm. Scavenging activity was calculated as the percentage of absorbance decrease from negative control. The assay was performed in triplicate. Trolox was used as a reference compound. The standard curve of trolox, plotting between percent of inhibition versus and µg trolox/ reaction mixture, was linear between 0.25-2.5 µg Trolox (Figure 2). The relative ABTS scavenging ability for each group was expressed as µgTE/mg chitooligosaccarides.

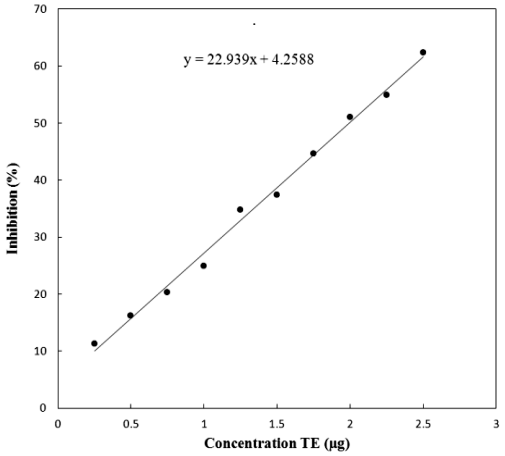

Fig. 2: The free radical scavenging activity of standard trolox by ABTS method.

Ferric reducing antioxidant power (FRAP) method

Determination of the scavenging activity of the chitooligosaccharides on FRAP was based on reduction of $Fe3^{+}$(tripyridyltriazine Fe) to$Fe2^{+}$ (Benzie and Strain 1996). Chitooligosaccharides were react with freshly prepared FRAP solution in the dark condition for 30 min. The intensity of the colour was measured using spectrophotometer at 593 nm. The higher absorbance of the reaction, the greater reducing power of the sample. The reducing power was direct correlated with the antioxidant activity (Duh *et al.*, 1999). The standard curve was linear between 2.5-25 µg Trolox (Figure 3). Scavenging activity of the chitooligosaccharides were expressed in µgTE/mg.

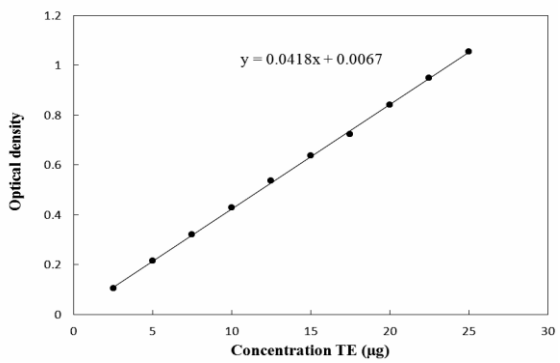

Fig. 3: The reducing power activity of standard trolox FRAP method.

RESULTS AND DISCUSSION

Chitooligosaccharides had many biological activities, useful for the industrial application. In this case, we separated four groups from the prepared chitooligosaccharides previously with different molecular weights (El-Sayed *et al*., 2016). The antimicrobial activity of the four groups against pathogenic microorganism *in vitro* was studied previously (El-Sayed *et al*., 2017). This study was undertaken to focus on cytotoxic and antioxidant effects of the separated chitooligosaccharides groups *in vitro*.

The antioxidant activity depends on many factors. It is difficult to evaluated the antioxidant activity of a compounds by of a single method. Therefore, in the present study, we used three separated methods, DPPH and ABTS radical scavenging and reducing power, FRAP assay. Chitooligosaccharides groups showed difference in the antioxidant activities (Table 1).

Table 1: Antioxidant capacity of the four chitooligosaccharides groups.

Chitooligosaccharides Groups	Antioxidant activity (ugTE/mg chitooligosaccharides s)		
	DPPH	ABTS	FRAP
I	1.84	1.99	1.44
II	8.17	10.03	8.52
III	7.78	9.12	8.24
IV	86.97	93.56	91.07

Results were expressed as µg TE/mg chitooligosaccharides.

The free radical scavenging capacity (DPPH and ABTS) and reducing power (FRAP) of the chitooligosaccharides varied between 1.84 to 86.97, 1.99 to 93.56 and 1.44 to 91.07 µg TE/mg chitooligosaccharides, respectively. Generally, group IV with molecular weight below 1.0 KDa had the highest antioxidant activity than other groups I, II and III. The antioxidant activity increased with decrease the molecular weight of

chitooligosaccharides. The difference in molecular weight and the subsequent effect towards antioxidant activity was reported. Chitooligosaccharides had high antioxidant activity as radical scavenging in vitro (Nago *et al*., 2008 and Yuan *et al.*, 2009). Chitooligosaccharides with low molecular weight exhibited better effect of scavenging free radical activity and reducing power than that with high molecular weight (Li *et al*., 2012*).* Yen *et al*. (2007) and Fernandes *et al*. (2010) showed similar results with chitooligosaccharides with the molecular weight (<3 kDa and <5 kDa) and low molecular weight chitosan by using DPPH and ABTS assays. Free radical DPPH (deep violet colour) and ABTS are scavenged by chitooligosaccharides compounds through the donation of hydrogen and forming of reducing DPPH (yellow colour) and ABTS, respectively. Antioxidant and radical scavengers substances are able to form this reaction and the degree of discoloration indicates free radicals scavenging capacities of the sample (Brand-Williams *et al.*, 1995). The reducing power(FRAP) was used to investigate the ability of an antioxidant to donate an electron. Reductants cause reduction of the Fe^{+3}/ferricyanide complex (Prussian blue) to ferrous Fe^{+2} (yellow) forms. The reducing power (donate electron) of bioactive compounds had been reported to be associated with their antioxidant activity (Dorman *et al.*, 2003).

The chitooligosaccharides content of group IV (the highest antioxidant group) was analyzed by HPLC. Identification of the compounds was carried out by comparing the eluted peaks of chitooligosaccharides retention time with the same typical retention times of monomers (GlcN), dimers $(GlcN)_2$ and tetramers $(GlcN)_4$ standards (Figure 4). The retention times of chitooligosaccharides main peaks were matched well with glucosamine, chitobiose and chitotetraose standards (Table 2). The molecular weights of IV group peaks were calculated by constructing a calibration curve, in which the logarithm of the molecular weight of three standards (D-glucosamine, chitobiose and chitotetraose) was plotted as a function of the retention time (Fig. 5).

The purity of D-glucosamine, chitobiose, chitotriose and chitotetraose in group IV were 36.7, 29.1, 1.9 and 23.5%, respectively according to the percentage of peak area in HPLC spectrum. Chen *et al.* (2003) found that chitobiose and chitotriose were more potent as antioxidant than that of glucosamine.

Table 2: HPLC analysis of the prepared chitooligosaccharides IV group.

Peak	Retention time	Matching standard	Molecular weight (Da)	Area (%)
1	6.2	-	177.4	2.716
2	6.783	Glucosamine	216	36.706
3	7.883	Chitobiose	432	29.074
4	8.733	-	565.97	1.864
5	9.733	Chitotetraose	864	23.506

*Retention time of glucosamine, chitobiose and chitotetraose were 6.783, 7.9 and 9.75, respectively. Molecular weight was calculated from the standard curve of molecular weight.

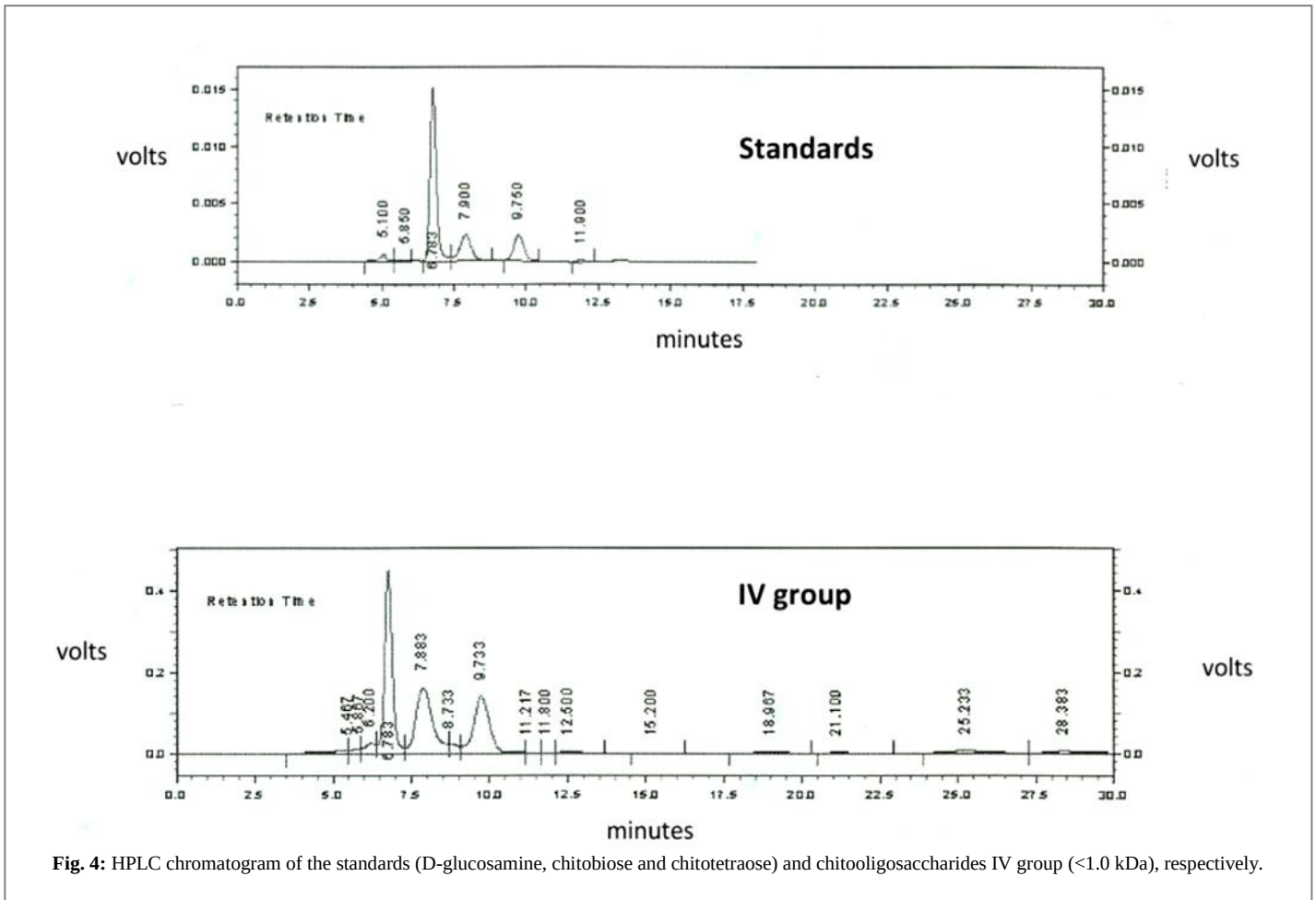

Fig. 4: HPLC chromatogram of the standards (D-glucosamine, chitobiose and chitotetraose) and chitooligosaccharides IV group (<1.0 kDa), respectively.

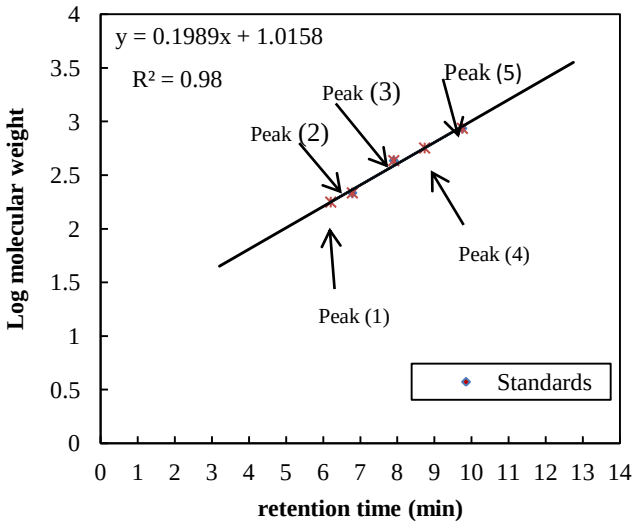

Fig. 5: The calibration curve of standards (D-glucosmine, chitobiose and chitotetraose) and chitooligosaccharides group IV sample.

In vitro cytotoxicity of the chitooligosaccharides group (I, II, III and IV) was performed against several types of tumor cell lines, hepatic carcinoma (HepG2), colon carcinoma (HCT-116) and breast carcinoma (MCF7) using sulforhodamine B (SRP) assay. We used different chitooligosaccharides concentrations (1.0-10 µg/ml) in the different cell lines. Surviving fraction was expressed as ratio of control. The IC_{50} values were calculated from the linear equation of the dose effect of the chitooligosaccharides groups against HepG2, HCT-116 and MCF7. The results showed that cytotoxicity of group II with 10 to 100 KDa was the best one that effect on the three tumor cell lines, HepG2, HCT-116 and MCF7 (Figure 6) with low IC_{50} values (1.56, 1.84 and 2.21 µg/ml, respectively (Table 3).

Table 3: Cytotoxicity of the chitooligosaccharides groups.

Chitooligosaccharides	HEPG2 (IC_{50})	HCT (IC_{50})	MCF7 (IC_{50})
Group I	-ve	-ve	-ve
Group II	1.56	1.84	2.20
Group III	12.95	11.95	-ve
Group IV	-ve	-ve	-ve

* IC_{50} (µg/ml) is the concentration of each sample required for 50% inhibition of surviving cell.

Chitooligosaccharides group III with molecular weight 1.0 to 10 KDa had cytotoxicity effect on two tumour cell lines, HepG2 and HCT-116 with IC_{50} values 12.95 and 11.95 µg/ml, respectively. While I and IV with high molecular than 100 KDa and low molecular weight than 1.0 KDa, respectively, had no effect on the three tumor cell lines. According to the resulted obtained, group II may reduce the viability of HepG2, HCT-116 and MCF7 tumor cells to about 20 %. Group III also may reduce the viability of HepG2 and HCT-116 tumor cells to about 25 %. Shen *et al*. (2009) reported a similar results by using MTT assay. HepG2 cells were the most sensitive cells to chitooligosaccharides with 23,99 kDa, as compared with stomach adenocarcinoma AGS cells and colon adenocarcinoma COLO205 cells. de Assis *et al*. (2012) prepared chitooligosaccharides by using fungus *Metarhizium anisopliae* and evaluated cell viability of these compounds toward hepatocarcinome (HepG2) and uterine carcinoma (Hela) cells. They found that chitooligosaccharides mixture ($GlcN_{1-5}$) reduced the viability of HepG2 and Hela tumour cells.

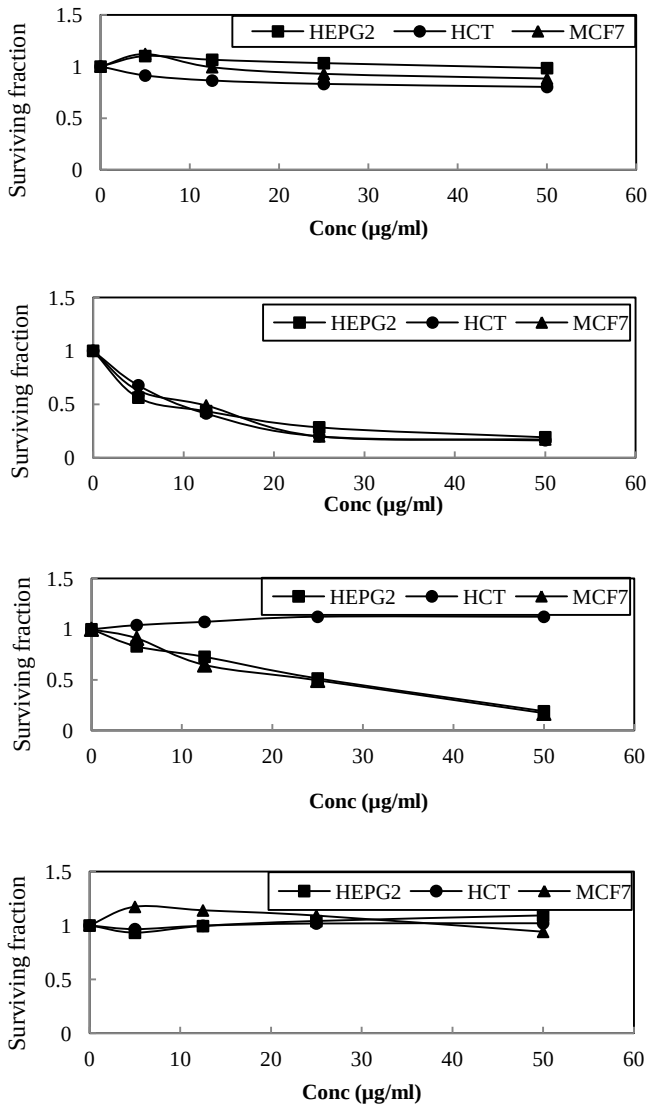

Fig. 6: Cytotoxic activity of chitooligosaccharides groups I, II, III and IV, respectively on the growth of three cell lines.

CONCLUSION

According to our findings *In vitro*, chitooligosaccharides had variable biological activities such as antioxidant (scavenging free radical, reducing power) and cytotoxicity. The solubility of chitooligosaccharides in water assists their industrial application. Their antioxidant and cytotoxic activities were related to their molecular weights. Group IV with low molecular weight lower than 1.0 KDa showed better effect as antioxidant activity than that with higher molecular weight. Chitooligosaccharides with 10 to 100 KD acted directly on HepG2, HCT-116 and MCF7 tumor cells and with 1.0 to 10 KDa acted directly on HepG2 and HCT-116. They decreased viability of tumor cell to 20-25 %.

Chitooligosaccharides may be used as a functional food ingredient in pharmaceutical and food industries in future.

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