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Antitumor activity of chitosan from mayfly with comparison to commercially available low, medium and high molecular weight chitosans

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Abstract
Insects' cuticles have a potential to be evaluated as a chitin source. Especially adults of aquatic insects like mayflies (order Ephemeroptera) swarm in enormous numbers in artificially lit areas while mating in spring and then die by leaving huge amounts of dead insects' bodies. Here in this study, mayfly corpses were harvested and used for production of low MW chitosan. Dried mayfly bodies had 10.21% chitin content; mayfly chitin was converted into chitosan with efficiency rate of 78.43% (deacetylation degree, 84.3%; MW, 3.69 kDa). Cytotoxicity and anti-proliferative activity of mayfly and commercially available shrimp chitosans (low, medium, and high MW) were determined on L929 fibroblast and three different cancer types including HeLa, A549, and WiDr. Apoptosis and necrosis stimulating potential of mayfly and commercial chitosans were also evaluated on A549 and WiDr cells using acridine orange and propidium iodide dual staining to observe morphological changes in nuclei and thus to reveal the predominant cell death mechanism. The effects of chitosans have varied depending on cell types, concentration, and chitosan derivatives. Mayfly and low MW chitosans had a cytotoxic effect at a concentration of 500 μg mL^{-1} on non-cancer cells. At concentrations below this value (250 μg mL^{-1}), mayfly and commercial chitosans except high MW one exhibited strong inhibitory activity on cancer cells especially A549 and WiDr cells. Mayfly chitosan induced early and late apoptosis in A549 cells, but late apoptosis and necrosis in WiDr cells. This study suggests that dead bodies of mayflies can be used for production of low MW chitosan with anti-proliferative activity.

Keywords Mayfly · Chitosan · Insect cuticle · Anticancer activity · Cytotoxicity

Introduction

Due to their non-toxic properties, biopolymers have been generally preferred for anticancer studies instead of synthetic polymers (Almeida et al. 2015; Ferreira et al. 2015; Li et al. 2015). Chitosan is one of the biopolymers with biocompatibility and biodegradability, and many recent studies have proved the anti-proliferative activity of chitosan and its derivatives (Shen et al. 2009; Jiang et al. 2011; Park et al. 2011). It has already been acknowledged that four major properties (molecular weight (MW), degree of deacetylation, source of chitosan, and pH) of chitosan are very important on its antitumor activity (Shen et al. 2009; Chien et al. 2016; Younes et al. 2016). In the present study, the effect of chitosan source and MW on the anti-proliferative activity of chitosan samples on HeLa (human uterus cervical cancer), A549 (human lung adenocarcinoma), and WiDr (colon adenocarcinoma) cell lines were tested.

Cervical cancer is one of the most common cancer types occurring in women and it killed 200,000 women only in 2010 (Forouzanfar et al. 2011). On the other hand, lung and colon cancers are among the most commonly diagnosed other cancer types with high mortality rate in both sexes worldwide (Ferlay et al. 2015). In earlier studies, it was reported that water-soluble chitosan and diethylaminoethyl chitosan forms have anti-proliferative effect on HeLa cell lines (Je et al. 2006;

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Lee et al. 2011), but there is no study to show direct effect of chitosan on HeLa cell line. Chitosan is also used as building material for production of nanoparticles, on which chemotherapeutic drugs are decorated. For instance, chitosan modification was found to enhance in vitro cellular uptake and toxicity of negative-charged drug delivery platform against A549 cells (Yang et al. 2009).

Chitosan is obtained through deacetylation of chitin, the most abundant biopolymer after cellulose, in highly alkaline solutions at elevated temperatures. Chitosan dissolves in weak acidic solutions, e.g., acetic acid (0.5%) and becomes gelatinous. Due to its biodegradable and biocompatible properties, chitosan has wide applications in medicine, food industry, pharmacy, agriculture, and textile (Muzzarelli and Muzzarelli 2005; Rinaudo 2006). In this study, chitosan gels were preferred as an anti-proliferative agent against different cancer cell lines.

Mayflies are a group of aquatic insects in the order Ephemeroptera. In spring, adult male and female insects emerge in enormous numbers, mate in artificially lit places like bridges, and then die in a few days (Harker 1989). For example, only on the bridges over the Sakarya River (Adapazari, Turkey) thousands of kilos of dead mayflies' corpses are collected and disposed every year. This study aimed at utilizing waste of dead mayflies' corpses as a source for production of chitin and chitosan and investigating antitumor activity of mayfly chitosan. It also draws a comparison between the antitumor activity of mayfly chitosan and that of commercial low, medium, and high MW chitosans.

Materials and Methods

Chitin isolation from mayfly corpses and chitosan production Dead mayflies were collected with a gloved hand in Sakarya (Turkey) during their overbreeding time in June 2014. This overbreeding in the population occurs every year on the location as known as Sakarya Bridge in June or July. The collected samples (about 100 g) were dried at room temperature on a paper sheet for 2 wk. Fifty grams of dry sample was used for chitin extraction. Firstly, the sample was demineralized at 50°C by using 2 M HCl solution. The sample was rinsed and filtered up to neutral pH by using filter paper and deproteinized at 100°C by using 2 M NaOH solution. Following this step, the remaining sample was washed with distilled water up to neutral pH. Lastly, the sample was decolorized by using methanol and chloroform solution at the rate of 1:1 by volume. The sample was rinsed with distilled water to neutrality. The obtained chitin sample was dried in an oven at 50°C for 48 h. Chitin sample was deacetylated in 60% NaOH solution (*w*/*w*) at 150°C for 6 h. After the deacetylation procedure, the sample was washed with distilled water up to neutral pH again. Then the chitosan sample was dried in an oven 50°C for 48 h.

FT-IR analysis of chitosan samples Infrared spectra of mayfly and commercial chitosans were recorded on a Perkin Elmer 100 FT-IR Spectrometer 2.5 over the range of 4000–650 cm^{-1}.

MW determination of mayfly chitosan The viscosity-average MW of the chitosan from mayfly corpses was measured by using an Ubbelohde dilution viscometer. Five different concentrations of the chitosan were prepared using the solvent system of 0.1 M acetic acid and 0.2 M NaCl (1:1, *v*/*v*). All the experiments were carried out at 25°C. Triplicate measurements were conducted. Mark–Houwink equation was used to determine the MW of the chitosan as follows (Wang et al. 1991):

$$[\eta] : kM_v{}^{\alpha} \quad (1)$$

where $[\eta]$ is intrinsic viscosity; M_v, viscosity average MW; and k and α, Mark–Houwink–Sakurada constants. The constants used are $k = 1.81 \times 10^{-3}$ and $\alpha = 0.93$.

Deacetylation degree of chitosan samples using elemental analysis Elemental analysis of chitosan from mayflies, low MW commercial chitosan (LMWCc), medium MW commercial chitosan (MMWCc), and high MW commercial chitosan (HMWCc) was done on a FLASH-2000 CHNS-O elemental analyzer. The percentages of carbon, hydrogen, and nitrogen in the chitosan samples were determined.

Deacetylation degree (DD) of the chitosan samples was determined by the following formula given below where C is carbon and N is nitrogen:

$$DD = [(6.89-C/N)/1.72] \times 100 \quad (2)$$

All the commercial chitosan samples were purchased from Sigma-Aldrich (CAS numbers for the chitosan samples, 9012-76-4).

Cell lines L929 and three different cancer cell lines (HeLa, A549, and WiDr) were used as the first choice for evaluating cytotoxic and antitumor effects of chitosans in vitro. Non-tumor cell line, mouse L929 fibroblastic cell line (*Mus musculus*), human epithelial cervical cancer line HeLa (Sap Institute, Cell Culture Cat. ID 9006190), human lung adenocarcinoma cell line A549, and colon adenocarcinoma cell line WiDr were cultured and grown in Dulbecco's modified Eagle's medium (Biochrom AG, Berlin, Germany and Gibco, Grand Island, NY) supplemented by 10% fetal bovine serum (FBS) (Sigma-Aldrich and Gibco) and 1% penicillin–streptomycin (Sigma-Aldrich, Munich, Germany). The cells were maintained at 37°C under a humidified atmosphere with 5% CO_2. The medium was refreshed two or three times each wk.

Cytotoxicity assay and assessment of cell proliferation The cytotoxic effect and the anti-proliferative activity of chitosan on L929, HeLa, A549, and WiDr were determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay described by Mosmann (1983). The cells were separately seeded into 96-well plates at a density of 5×10^3 cells/well and incubated at 37°C in a humidified 5% CO_2 atmosphere. After 24 h, the cells were washed with 200 μL fresh medium and were treated with different concentrations of chitosan. The samples were then incubated for an additional 24 h. After 24 h of incubation, the medium was removed and 100 μL of serum-free medium containing 5 mg/mL of MTT was added to each well. After 4 h of incubation at 37°C in the dark, the medium containing unreacted MTT was removed carefully and 100 μL of isopropyl alcohol was added to each well to solubilize formazan. The plates were agitated for 10 min and absorbance was measured with a plate-reading spectrophotometer at 570 nm (Biochrom EZ Read 400 and Microplate readers BioTek). Absorbance of the treated cells was compared with that of the control, and cells exposed only to normal medium were considered 100% viable.

The percentage of cell viability was calculated using the following formula:

$$\textit{Cell viability}\ (\%) = \frac{\textit{Optical density of treated cells}}{\textit{Optical density of control cells}} \times 100 \quad (3)$$

L929 fibroblastic cell line from mouse, which was not tumorigenic, served as controls in order to analyze toxicity of chitosan samples on normal cells.

Acridine orange (AO)/propidium iodide (PI) staining To determine the apoptotic effect of the test materials on A549 and WiDr cells, AO/PI staining was performed at 24 h of the culture period. Briefly, 10^4 cells were seeded in each well of a 48-well plate in growth medium. After 24 h, growth medium was discarded and replaced with the fresh medium containing 250 mg/mL test materials. The cells were incubated with their new medium for 24 h to observe the apoptotic effects of materials. At the end of the incubation time, media were removed and cells were washed with phosphate buffered saline (PBS) (Sigma Aldrich, Germany) once. Then 25 μg mL^{-1} AO and 25 μg mL^{-1} PI stock solutions prepared previously were mixed 1:1 (*v*/*v*) in a falcon. Then, 200 μL of the mixture was applied to the cells for 30 s. Following, the dye solutions were removed from the wells and the stained cells were treated with PBS for 20 s. Then the mounting medium (glycerol/PBS; 1:1 (*v*/*v*)) was applied to the cells to be observed under inverted microscopy with FITC and Rhodamine filter (Leica DM IL Led, Netherlands). Following AO/PI staining, determination of apoptosis and necrosis-inducing potential of chitosan samples was carried out by counting cells according to the following criteria (McGahon et al. 1995; Leite et al. 1999; Ciapetti et al. 2002; Abdel Wahab et al. 2009): uniform green nucleus with organized structure, intact plasma membrane, and orange or green cytoplasm, viable cells (1); bright green areas of chromatin condensation in the nucleus, early apoptosis (2); dense orange areas of chromatin condensation, late apoptosis (3); and orange intact nucleus, necrosis (4). For each test group, three fluorescence microscope images with a magnification of ×20 were analyzed by examining and counting cells manually. The quantification of apoptotic and necrotic cells was calculated according to the following equations.

$$\textit{Apoptotic cells}\ (\%) = \frac{\textit{Total number of apoptotic cells (early or late)}}{\textit{Total counted cells}} \times 100 \quad (4)$$

$$\textit{Necrotic cells}\ (\%) = \frac{\textit{Total number of necrotic cells}}{\textit{Total counted cells}} \times 100 \quad (5)$$

Statistical analysis All the experimental data were represented as the mean ± standard deviation (SD). Data were analyzed with Student *t* test or Mann–Whitney *U* test with $p < 0.05$ and $p < 0.01$ indicating statistical significance. Experiments were performed at least in triplicates.

Results

Characterization of commercial chitosans and mayfly chitosan Chitin content of mayfly corpses was recorded as 10.21% on dry basis. Chitosan yield of mayfly chitin was observed as 78.43%. Elemental analysis results of chitosan samples are presented in Table 1. DD of the chitosan samples were measured as 84.3% for chitosan from mayfly, 82.6% for LMWCc, 72.7% for MMWCc, and 83.1% for HMWCc. MW of mayfly chitosan was determined as 3.69 kDa, indicating low MW.

In the FT-IR spectrum of chitosan, amide I (carbonyl ν(C=O)) and amide II (amine ν(NH2)) bands that are

Table 1 Elemental analysis results of mayfly chitosan and commercial LMWCc, MMWCc, and HMWCc

Chitosan samples	N (%)	C (%)	H (%)	DD (%)
Mayfly chitosan	7.14	38.81	6.12	84.3
Commercial chitosan low MW	7.01	38.32	6.00	82.6
Commercial chitosan medium MW	7.15	40.34	6.43	72.7
Commercial chitosan high MW	7.22	39.43	6.32	83.1

characteristics for chitosan appear at around 1650 and 1590 cm^{-1} (Pawlak and Mucha 2003). In this study, two absorption bands at 1647 and 1586 cm^{-1} observed in the spectrum of mayfly chitosan indicated the deacetylation of mayfly chitin and the formation of chitosan (Fig. 1*a*). In the spectra of Sigma-Aldrich chitosans, amide I (C=O–NHR) and amine group (–NH2) bands were recorded for LMWCc, MMWCc, and HMWCc at 1650, 1652, and 1649 cm^{-1}

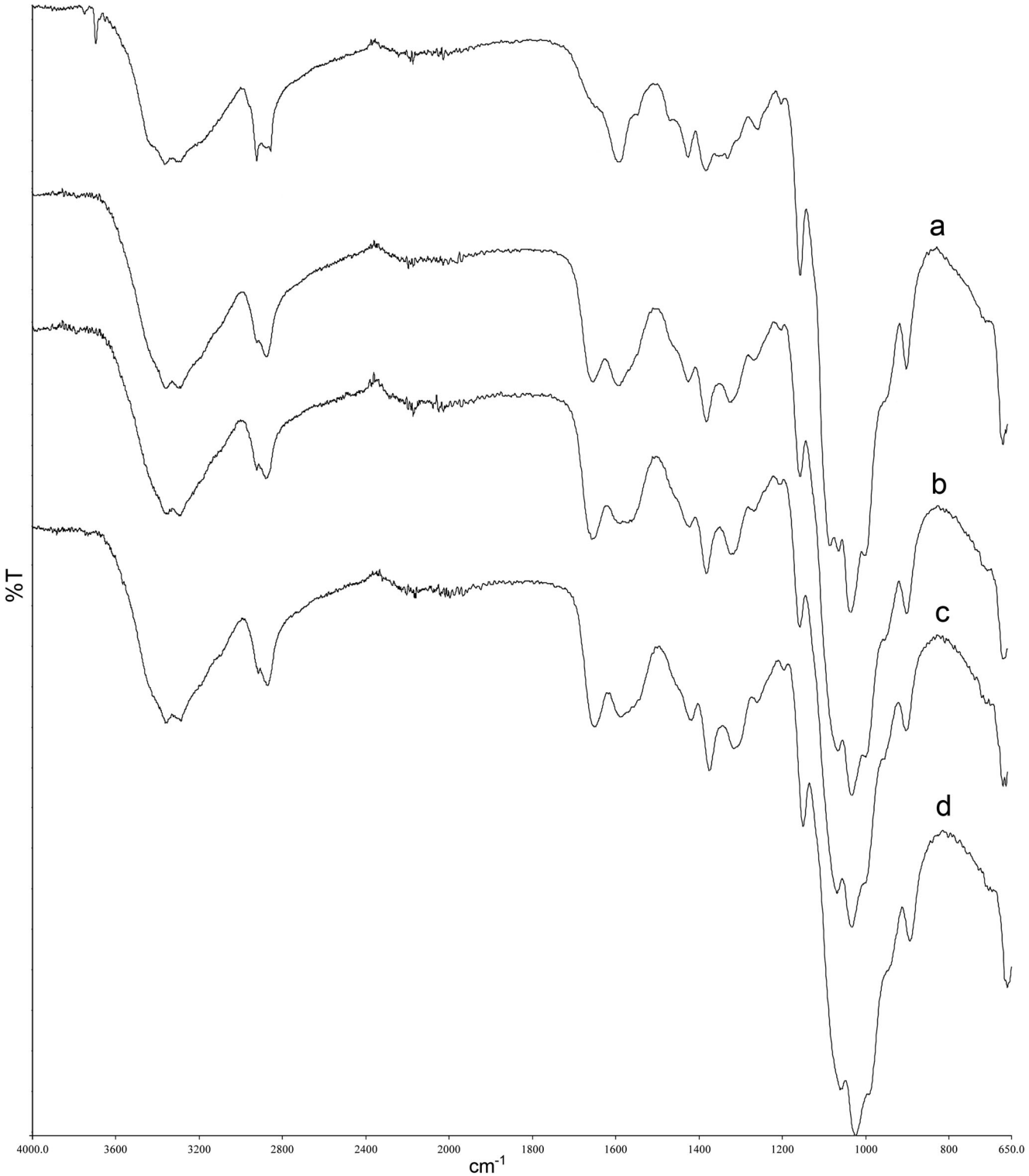

Fig. 1 FT-IR spectra of (*a*) mayfly chitosan, (*b*) low MW commercial chitosan, (*c*) medium MW commercial chitosan, and (*d*) high MW commercial chitosan.

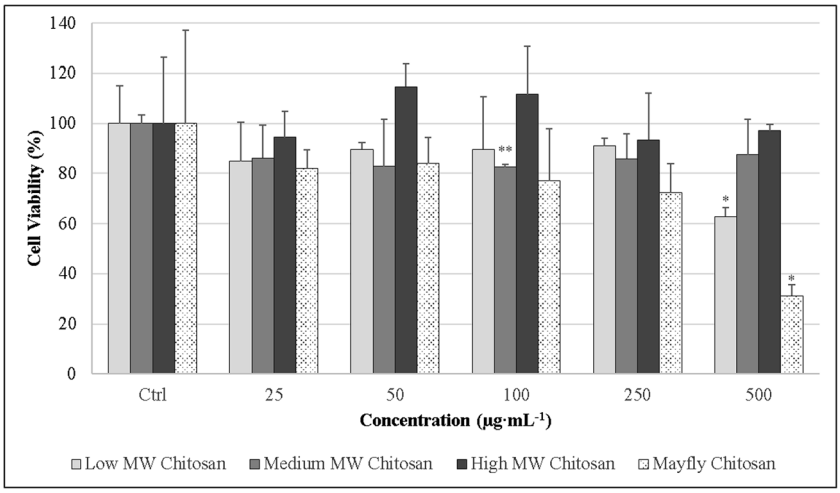

Fig. 2 MTT results of L929 cells after treatment with low, medium, and high molecular weight commercial chitosans and mayfly chitosan for 24 h. Results are shown as mean ± SD mean, derived from at least three replicates (*$p < 0.05$ and **$p < 0.01$).

and 1589, 1575, and 1587 cm^{-1}, respectively (Fig. 1*b–d*). It appears that FT-IR spectrum of mayfly chitosan is in the line with commercial chitosans.

Cytotoxicity of LMWCc, MMWCc, HMWCc, and mayfly chitosan on L929 cell line Cytotoxic effect of mayfly chitosan and LMWCc, MMWCc, and HMWCc on L929 cell line was investigated at the concentration range of 25–500 μg mL^{-1} (Fig. 2). MMWCc and HMWCc were observed to be non-cytotoxic on L929 cell line. However, mayfly chitosan and LMWCc samples exhibited cytotoxicity toward the cell line at only concentration of 500 μg mL^{-1}.

Antitumor effect of LMWCc, MMWCc, HMWCc, and mayfly chitosan on cancer cell lines Antitumor activity of mayfly chitosan and commercial chitosan samples (concentration 100 and 250 μg mL^{-1}) was tested on HeLa, A549, and WiDr cell lines (Fig. 3). Unlike the other chitosan samples, HMWCc showed a negligible antitumoral activity against all cancer cells over the concentration range studied. LMWCc

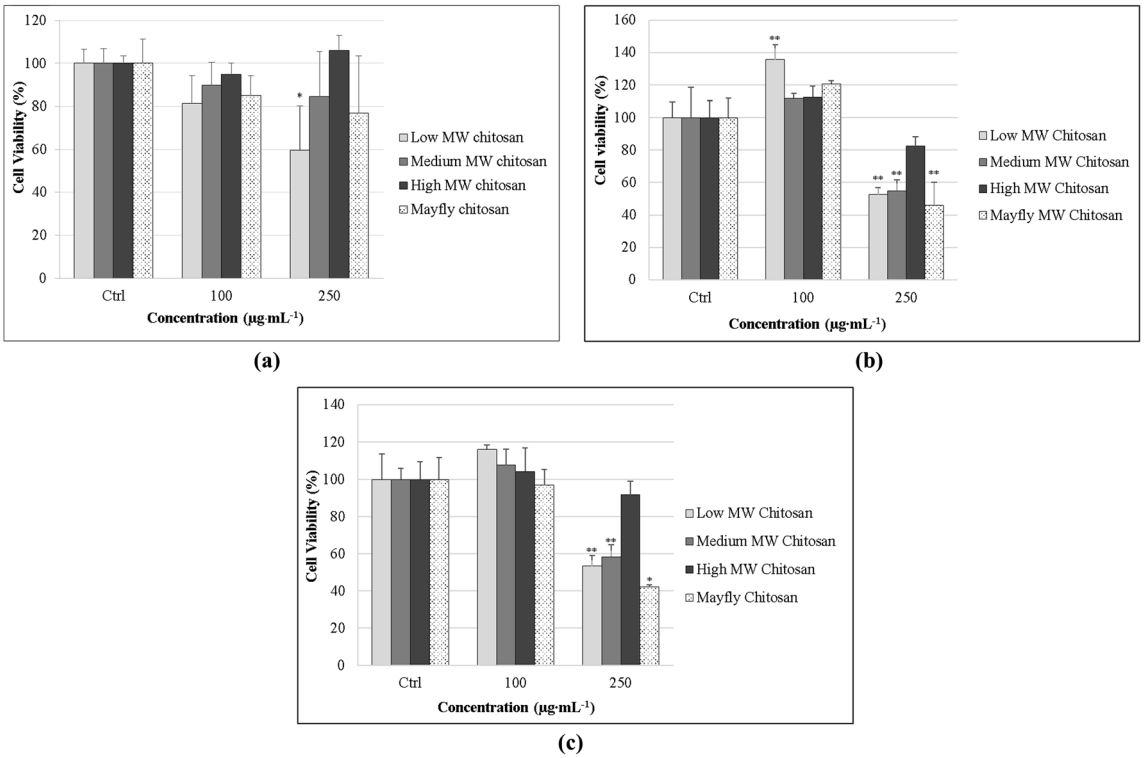

Fig. 3 MTT results of HeLa (*a*), A549 (*b*), and WiDr (*c*) cells after treatment with low, medium, and high MW commercial chitosans and mayfly chitosan for 24 h. Results are shown as mean ± SD mean, derived from at least three replicates (*$p < 0.05$ and **$p < 0.01$).

had higher antitumor activity than low MW chitosan from mayfly and MMWCc, which exhibited a moderate antitumor activity toward HeLa cell line. On the other hand, LMWCs, MMWCc, and mayfly chitosan strongly inhibited the proliferation of A549 and WiDr cells.

Apoptosis- and/or necrosis-inducing potential of LMWCc, MMWCc, HMWCc, and mayfly chitosan During apoptosis, cells undergo significant morphological changes, including cell skeleton damage, cell shrinkage, condensation of the plasma membrane, and formation of membrane swellings, nucleus condensation, and DNA fragmentation. Therefore, these important physical changes also enabled us to observe the apoptosis in cells. Morphological changes in the nucleus due to apoptosis and necrosis were examined with AO/PI. The AO/PI dual staining is widely used for detecting apoptotic cells as nuclear fragmentation and membrane shrinkage remain the hallmarks of apoptotic cells, as well as distinguishing apoptotic (early and late) and necrotic cells from each other (Leite et al. 1999; Baskić et al. 2006). A549 and WiDr cells that were treated with 250 μg mL^{-1} of different chitosan derivatives were then dual stained with cell-permeable DNA-intercalating dye AO in combination with membrane-impermeable DNA-intercalating dye PI (Figs. 4 and 5).

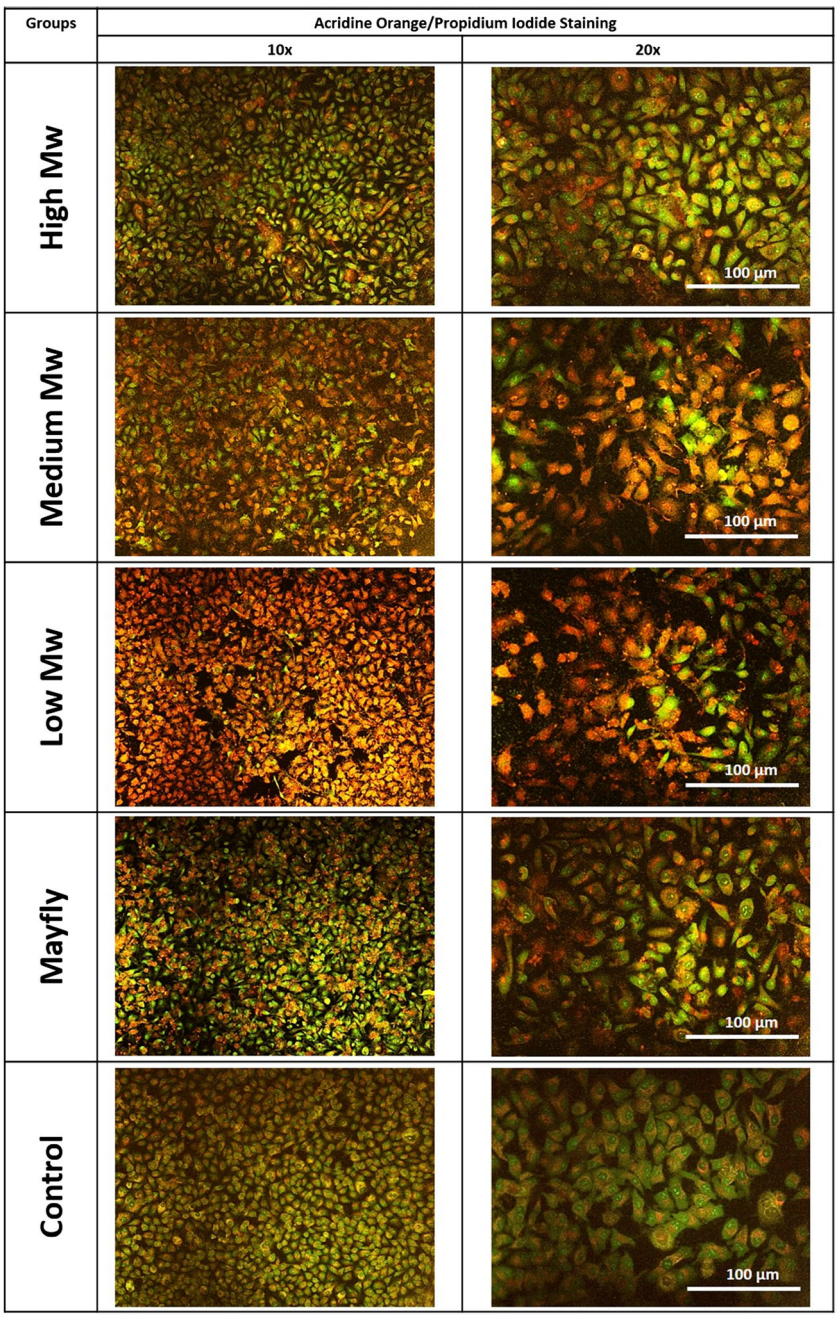

Fig. 4 AO/PI-stained A549 cells after treatment of LMWCc, MMWCc, HMWCc, and mayfly chitosans. The fluorescence images of cells are ×10 and ×20 magnifications.

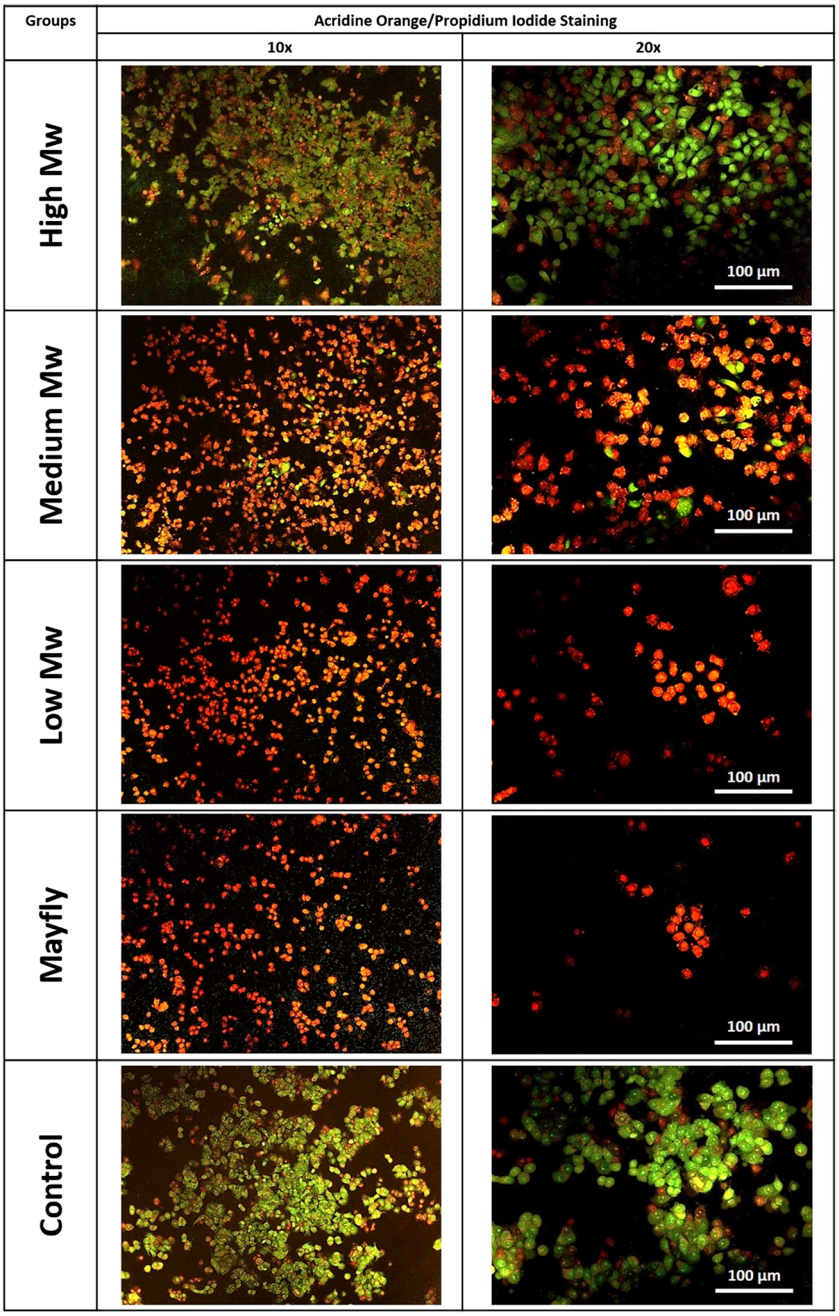

Fig. 5 AO/PI-stained WiDr cells after treatment of LMWCc, MMWCc, HMWCc, and mayfly chitosans. The fluorescence images of cells are ×10 and ×20 magnifications.

The AO is taken up by both viable and non-viable cells, and excites green fluorescence, while PI is only taken up by non-viable cells and excites orange or red fluorescence. For the AO/PI tests, concentration of 250 μg mL^{-1} was selected because this value did not lead to toxic effects in non-cancer cells. The results obtained from florescence images of A549 and WiDr cells after AO/PI dual staining are represented in Fig. 6.

AO/PI assays revealed no difference in dominant cell death mechanism between HMWCc and control group on both A549 and WiDr cells, consistent with MTT results. LMWCc and MMWCc predominantly triggered late apoptosis and necrosis in both A549 and WiDr cells. However, the cell death mechanism triggered by mayfly chitosan differed according to cancer cell lines. Mayfly chitosan caused early and late apoptosis in A549 cells, but late apoptosis and necrosis in WiDr cells.

Discussion

The findings from in vitro studies suggest that anticancer effect of chitosan samples against cancer cell lines demonstrate a decreasing trend as its MW increases. This can be attributed

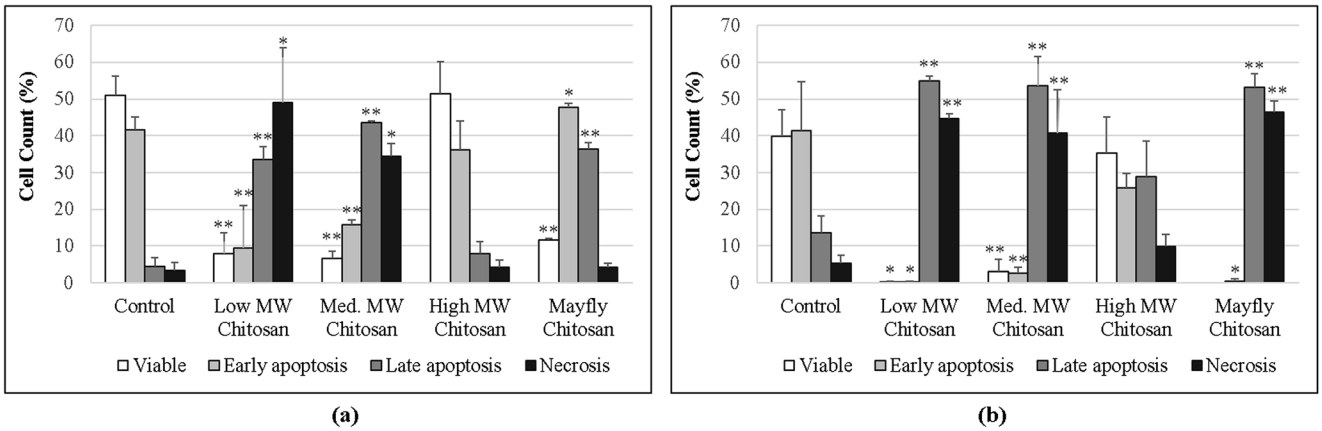

Fig. 6 The population of viable, apoptotic (early and late), and necrotic cells in A549 (*a*) and WiDr (*b*) cells treated with mayfly chitosan and commercial shrimp chitosans with low, medium, and high MW. Results are shown as mean ± SD mean, derived from at least three replicates (*$p < 0.05$ and **$p < 0.01$). All comparisons were made with respect to the control group.

to the diffusional limitations arising from the high viscosity of high MW chitosan samples. On the other hand, low MW chitosan molecules can penetrate easily through the cell membrane due to their low viscous nature and consequently show their inhibitory effect on the cell line. Interestingly, AO/PI assays have shown that LMWCc and MMWCc exhibited more stable cell death mechanism regardless of cancer cell lines studied, whereas predominant cell death mechanism induced by mayfly chitosan varied by cancer types.

Previous studies on the anticancer properties of chitosan have already revealed that especially MW, deacetylation degree, pH, and the organism used as chitin source play roles in the activity of chitosan against cancer cell lines (Chien et al. 2016; Younes et al. 2016). In this study, chitosan produced from mayfly insects had a similar deacetylation degree. In this study, the properties like MW and chitin source were dealt with. A previous study on chitosan samples with different MWs reported that MW of chitosan showed a slight anticancer activity on bladder carcinoma cell line (Younes et al. 2016). However, in some earlier studies, findings similar to the present study were reported and low MW chitosan had the highest antitumor activity on HepG2 (Chien et al. 2016). Despite these literature reports, there is still limited information available on the relationship between the antitumor activity of chitosan and its MW; it is hard to reach a general statement or concluding remark on this relationship.

Antitumor activity of chitosan also depends on its source. In a study (Chien et al. 2016), chitosan samples from crab and mushroom exhibited different activities against IMR32 and HepG2 cell lines; mushroom chitosan was more effective than crab chitosan. In the present study, mayfly low MW chitosan exhibited a similar cytotoxic activity to low MW chitosan from shrimp, despite having a similar deacetylation degree. Small variations in the anti-proliferative activity were observed in a wider concentration range.

Conclusions

In this study, waste bio-based material (mayfly insect corpses) naturally occurring in huge amounts was converted into versatile biopolymer (chitosan), which has diverse applications in biotechnology. Chitosan from mayfly was determined to be low MW and deacetylation degree of chitosan was measured as 84.3%. Anti-proliferative activity of mayfly chitosan was studied and compared to commercially available low, medium, and high MW chitosans from shrimp. Mayfly chitosan and LMWCc showed cytotoxic effect only at high concentration (500 μg mL^{-1}). Anti-proliferative activities of mayfly chitosan, LMWCc, and MMWCc were recorded higher than that of HMWCc. Also, anti-proliferative activity of HMWCc was observed to be very low. In addition, mayfly chitosan induced apoptosis in cancer cells. This study suggested that MW of chitosan plays a vital role in its anti-proliferative effect on cancer cell lines. Low MW chitosan samples had higher cytotoxic and anti-proliferative activity regardless of chitosan source. Additionally, the present study revealed that the waste mayfly specimens could be suggested as an alternative chitosan source, which could be utilized as an anti-proliferative material as well.

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