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Original Communication

Comparative biochemical assessment of chitosan and carboxymethyl chitosan as antitumor agents

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ABSTRACT

In this study, a natural polymer, chitosan (CS) has been converted through modified procedures into a water soluble carboxymethyl chitosan (CMCS). This was fully characterized by numerous techniques, including Fourier transform infrared spectroscopy (FTIR), elemental analysis (EA) and two dimensional wide-angle X-ray scattering (2D-WAXS). The anticancer activity of the obtained CMCS was investigated in comparison with its parent compound, CS using mice bearing Ehrlich ascites tumour cells (EAC). It has been found that treatment with CMCS and CS has significantly inhibited tumour growth in a dose-dependent manner, which indicates their significant antitumor activity. However, CS showed to be more superior, as an anticancer agent, over CMCS under the same experimental conditions. Biochemical assays also revealed that treatment with CMCS and its parent polymer (CS) has led to an augmentation of the antioxidant defense system without affecting lipid peroxidation in EAC-bearing mice.

KEYWORDS: carboxymethyl, chitosan derivative, chitosan, chemotherapy, cancer, antioxidants

1. INTRODUCTION

Chitosan (CS), a cationic water insoluble polymer, is derived from chitin by N-deacetylation [1]. It has many advantageous biological properties, being biodegradable, biocompatible, non-toxic, bioabsorbable, haemostatic, bacteriostatic, fungistatic, and anticholesteremic [1, 2]. Moreover, CS itself has antacid and antiulcer activities, which can prevent or weaken drug-induced irritation in the stomach [2, 3]. CS also represents the core of a new generation of drug and vaccine delivery systems because of its ability to reduce the clearance rate and encourage the uptake of antigens by dendritic cells and macrophages [4-7]. In addition, the conjugates of some anticancer agents with CS and its derivatives showed promising anticancer efficiencies with a noticeable reduction in the unfavourable side effects of the original anticancer agent [4]. However, the poor solubility of CS in both water and common organic solvents limits its extensive use. Various approaches such as carboxymethylation have been applied for conversion of CS into a water-soluble form [8]. It has been reported that carboxymethyl chitosan (CMCS) has several desirable characteristics, including its good ability to form films, fibres and hydrogels [9, 10]. Hence, CMCS has been widely utilized in many potential applications including drug delivery [9-11]. For the proliferative responses, researchers have investigated the roles of CMCS on cell proliferation, such as mesothelium and skin fibroblast [12, 13]. The effect of CMCS on nerve regeneration was investigated for its biodegradability [14]. The CMCS tube was made up by cross-linking with carbodiimide hydrochloride, the CMCS-carbodiimide hydrochloride cross-linking tubes were demonstrated suitable for nerve autograft in tissue engineered nerves.

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Recently, El-Far *et al.*, [15, 16] showed the antitumor activity and antioxidant role of a novel synthesized water-soluble CMCS derivative-based copolymer in cancer treatment. The aim of this present study was to synthesize the water soluble CMCS and provide characterization of it. We also aimed to study the antitumor effect and antioxidant role of it against Ehrlich ascites carcinoma (EAC) tumour in mice. Furthermore, the present contribution aimed to make a comparative study between CMCS and its parent compound CS under same experimental conditions. To the best of our knowledge, this would be the first to provide such comparative biochemical assessments.

2. MATERIALS AND METHODS

2.1. Materials

Chitosan (CS) of medium molecular weight was obtained from Acros Organics (New Jersey). Monochloroacetic acid was purchased from Riedel-De Haenag Seelze (Hanover, Germany). Isopropanol, methanol, acetone, acetic acid and all other chemicals and reagents were of analytical grade and used as received.

2.2. Methods

2.2.1. Physicochemical characterization of the CS under investigation

The percentage of N-deacetylation (N_d%) of the CS used in this study was determined from elemental analysis and also using FTIR analysis using the following relationship [17]:

$$N_d\% = \left[1 - \left(A_{1655}/A_{3340}\right)\left(\frac{1}{1.33}\right)\right] \times 100 \quad (1)$$

where, A is the absorbance and the two FTIR absorption peaks at 1655 and 3340 cm^{-1} represent the amide and the primary NH_2 groups of CS, respectively. The value, 1.33 represents the ratio of A_{1655}/A_{3340} for a fully N-acetylated CS sample.

The average viscosity molecular weight (Mw) of the CS used in this study was also determined using the standard Mark-Houwink viscometry procedure [8], in a solvent mixture of 0.2 M NaCl and 0.1 M acetic acid maintained at room temperature (about 25°C). The effluent times of solvent mixture and CS solutions were measured using Cannon-Fenske Routine Viscometer (Cannon Instrument Co, State College, PA. 16801, USA). Each sample was measured three times.

2.2.2. Carboxymethylation of CS

The carboxymethylation process of CS into a water soluble derivative (CMCS) was carried out through a modified procedure reported in our previous studies [8, 15, 16, 18]. Briefly, 1 g of CS was transferred to a round-bottomed flask and suspended in 30 ml of isopropyl alcohol at room temperature for about 2 days. To the swollen CS, 50 ml of (60% w/v) aqueous NaOH solution was added, and the entire reaction mixture was refluxed at 85ºC for 2 h. Then, 50 ml of aqueous monochloroacetic acid solution (60% w/v) was added over a period of 10 min. The reaction mixture was then heated with stirring at 65ºC for further 3 h. The mixture was neutralized through dropwise addition of 4 M HCl solution. The undissolved residue was filtered, and the resulting CMCS was precipitated via adding methanol. The CMCS was collected by filtration, washed extensively with a (1:1) mixture of methanol/H_2O, dried at 40ºC under vacuum, and then stored in a desiccator until further investigation.

2.2.3. Physicochemical characterization of the prepared CMCS

The average viscosity molecular weight of the prepared CMCS was estimated using standard Mark-Houwink viscometry procedure. The CMCS was dissolved in 0.1 M aqueous NaCl, and the intrinsic viscosity was determined at 25ºC using the following equations [19]:

$$\begin{aligned} \eta_r &= \frac{t}{t_0} \\ \eta_{sp} &= \eta_r - 1 \\ [\eta] &= \frac{\left(4\eta_{sp}^{1.02} \times \ln \eta_r\right)}{C^{1.01}\left(3\eta_{sp} + \ln \eta_r\right)} \\ [\eta] &= 7.92 \times 10^{-5} M_r^{1.00} \end{aligned} \quad (2)$$

where t_0 and t are the flow times of the solvent (aqueous NaCl solution) and CMCS solutions, respectively. C is the CMCS concentration (g/ml), η_r and $[\eta]$ are the relative and intrinsic viscosities of CMCS solutions, respectively, and M_r is the average viscosity molecular weight of CMCS.

The carboxymethylation degree (Dc) of the CS was also determined using a potentiometric titration method [19]. An aqueous solution of CMCS (300 mg/100 ml) was adjusted to pH < 2 through drop wise addition of HCl solution. Then, the CMCS solution was titrated against aqueous NaOH (0.1 M) with recording the pH values simultaneously. The equivalent NaOH was determined using the second order differential method, and the Dc value was calculated using the following relationship:

$$Dc = \frac{161 \times V \times C}{m_{CMCS} - 58 \times V \times C} \quad (3)$$

where, m_{CMCS} is the mass (*g*) of CMCS, *V* and *C* are the volume and molar concentration of NaOH solution, respectively. The values 161 and 58 are corresponding to the molecular weights of the glucosamine unit of CS and the carboxymethyl group, respectively.

2.2.4. Structural characterization of CS and CMCS

The structural characteristics of the used CS and the prepared CMCS were investigated using Fourier transform infrared (FTIR). Dried samples were pressed with a spectroscopic grade KBr and their FTIR spectra were recorded on a Perkin Elmer Paragon 1000 FTIR spectrometer within the wave number range, 500-4000 cm^{-1} at room temperature. The elemental analysis for both CS and the prepared CMCS were also carried out using Carlo Erba Elemental analyzer EA 1108 with the aid of a flash combustion technique. The diffraction patterns of CS before and after carboxymethylation (CMCS) were investigated with the aid of wide angle X-ray scattering (WAXS). The analysis was performed using 2D-WAXS equipment (Rigaku Micro Max 007 microfocus imitating anode X-ray generator (Cu Kα) coupled with Osmic "Blue" confocal optics and a Rigaku RAxis (VI++) image-plate detector). The diffractograms were recorded and analysed with the Crystal Clear software (1.3.6-SPI, Pflugrath, JW, 1999, Acta Crystallogr. D50 1718-1725).

The values of the *d*-spacing for the investigated CS and CMCS molecules in the crystalline, semi-crystalline and amorphous states were determined from the scattering wave vector *q* according to the following equation:

$$d = \frac{2\pi}{q} \quad (4)$$

where, $q = (4\pi/\lambda)\sin\theta$ and the 2θ represents the scattering angle

2.2.5. Preparation of CS solution for injection

The CS (250 mg) was immersed and dissolved into 10 ml of 2% (v/v) acetic acid at room temperature for 1 h with stirring. The resulting acid solution was neutralized using 0.5 M (20g/L) NaOH through its drop wise addition with stirring until turbidity appears. Then, the solution was diluted to desired concentrations using isotonic saline. The clear slightly viscous and flowable solutions obtained were then adjusted to pH of 6.4 which is ready for injection.

2.2.6. Tumours and biochemical protocols

The prepared CS derivative, CMCS was dissolved using isotonic saline solution and diluted to the desired concentrations. All experiments were performed with adult Swiss albino mice strain purchased from Theodore Bilharz Institute, Giza, Egypt, with an average body weight of 20 to 25 g. Mice were housed in steel mesh cages and maintained for one week acclimatization periods on commercial standard diet and tap water ad-libitum. Ehrlich ascites carcinoma (EAC) line was kindly supplied from the National Cancer Institute, Cairo University, Egypt.

2.2.7. Tumour cells and transplantation of EAC cells

EAC cells were used for *in vivo* experiments. The tumor cell line was maintained in mice through serial intraperitoneal (i.p.) transplantations of (2×10^6) viable tumor cells in 0.2 ml of saline using a 25 G needle. The tumor was characterized by moderately rapid growth, while killing the mice within 3 weeks due to accumulation of ascetic fluid and showing no distal metastasis or spontaneous regression. Counting of the viable EAC cells was carried out by trypan blue exclusion using the method described [20].

2.2.8. *In vivo* anti-tumour activity and tumour volume experiments

Swiss albino female mice were divided into several groups, five mice in each cage at least. All animals

were housed in plastic cages and maintained under controlled conditions of humidity, temperature, and normal environment of light and darkness. Animals were randomly assigned to several groups according to our adopted protocol [15, 16] as follows: Group 1 (n=10) animals were injected (i.p.) with 0.2 ml of EAC cells containing 2×10^6 cells for tumor induction and then left without any treatment for 14 days (control). Group 2 (n=5), animals were injected (i.p.) with 0.2 ml of EAC cells containing 2×10^6 cells, next day animals received CS solution (i.p.) at a dose of 50 mg/kg body weight dissolved as described and then diluted to the final concentration using isotonic saline with final volume of 0.2 ml and injected every 48 h for 14 days (6 separate doses). After 18 h of the last dose administration, the mice were then sacrificed for observation of anti-tumor activity which was assessed by measuring ascetic tumor volume and then compared to that obtained in the same way in mice of control. Group 3 (n=5), animals were injected (i.p.) with 0.2 ml of EAC cells containing 2×10^6 cells, next day animals received CMCS solution (i.p.) at a dose of 50 mg/kg body weight dissolved in 0.2 ml isotonic saline every 48 h for 14 days (six separate doses). After 18 h of the last dose administration, the mice were subjected to anti-tumor activity evaluation by comparing it with the control group. Group 4 (n=5), animals were injected (i.p.) with 0.2 ml of EAC cells containing 2×10^6 cells, next day animals received CS (i.p.) at a dose of 100 mg/kg body weight dissolved as described and diluted to final concentration using isotonic saline with final volume of 0.2 ml and injected every 48 h for 14 days (6 separate doses). After the end of two weeks, the mice were subjected to anti-tumor activity evaluation by comparing it with the control group. Group 5 (n=5), animals were injected (i.p) with 0.2 ml of EAC cells containing 2×10^6 cells, next day animals received CMCS (i.p) at a dose of 100 mg/kg body weight dissolved in 0.2 ml isotonic saline every 48 h for 14 days (six separate doses). After 18 h of the last dose administration, the mice were subjected to anti-tumor activity evaluation by comparing it with the control group. The effect of the tested synthesized compound CMCS on the growth of transplantable tumor was estimated according to El-Far *et al.* [15, 16]. The anti-tumor effect was assessed by observation of changes with respect to ascetics tumor volumes as we recently described [15].

2.2.9. Estimation of biochemical parameters

After the collection of ascetic samples from the groups of mice, EAC cells were homogenized with cold saline solution using a homogenizer, and then the biochemical parameters were estimated according to previously reported protocol [15]. The levels of malondialdehyde (MDA) were estimated in cell homogenate according to Ohkawa *et al.* [21]. The level of superoxide dismutase (SOD) was also determined according to El-Far *et al.*, [15] and Dechatelet *et al.* [22].

2.2.10. Statistical significance

All values were expressed as mean ± SD. The statistical significance was determined using the one way ANOVA. Statistical package for social science (SPSS, Inc, Chicago, IL) was used for the statistical analysis. Statistical significance was considered at values of $P < 0.05$, while high significance was considered at values of $P < 0.001$.

3. RESULTS AND DISCUSSION

3.1. Physicochemical characterization of CS and CMCS

The degree of *N*-deacetylation (N_d%) of the CS under investigation was found to be 73.5% as determined by elemental analysis and FTIR [17]. Also, the average molecular weight (Mw) of the CS was determined to be about 0.32×10^6 D using the standard Mark-Houwink viscometry method [8]. The degree of carboxymethylation (D_c) of the CS into CMCS was estimated to be 0.48 as determined by potentiometric titrations. Besides, the intrinsic viscosity of the prepared CMCS in 0.1 M aqueous NaCl at 25°C was found to be 5.1 dLg^{-1}.

3.2. Structural characterization of CS and CMCS

3.2.1. Fourier transform infrared (FTIR)

The structural changes occurred upon carboxymethylation of CS were investigated using FTIR spectra as apparent in Figure 1. In the FTIR spectrum of CS (Figure 1a), a strong signal appeared at 3427 cm^{-1} which was assigned for the intermolecular H-bonds, the O-H stretching

vibration, and N-H extension vibration of the polysaccharide moieties. Also, a weak peak appeared at about 1654 cm^{-1}. This peak was attributed to the stretching vibration of the amide C=O. The FTIR spectrum of CMCS (Figure 1b) showed a strong new signal at about 1734 cm^{-1} which was assigned to the asymmetric stretching vibration of the carboxylate C=O, whereas, the peak appeared at 1384 cm^{-1} was attributed to the carboxylate C=O symmetric stretching vibration. It has been found from the FTIR spectrum of the CMCS that, the C-O absorption peak of the secondary OH groups became sharper, stronger and has been shifted to about 1089 cm^{-1}. This change tends to indicate that the carboxymethylation process occurred particularly at the C_6 position of the CS backbone. The conversion of CS into CMCS is illustrated in Scheme 1.

3.2.2. Diffraction analysis

Two dimensional wide-angle X-ray scattering (2D-WAXS) analysis of the powder CS and CMCS samples was performed using X-ray beam with a double graphite monochromator for the CuKα radiation (λ=0.154 nm). Some typical 2D-WAXS diffraction patterns for the investigated polymer molecules, CS and CMCS are illustrated in Figure 2.

The 2D-WAXS diffractogram of CS (Figure 2a) demonstrated three major crystalline bands at the

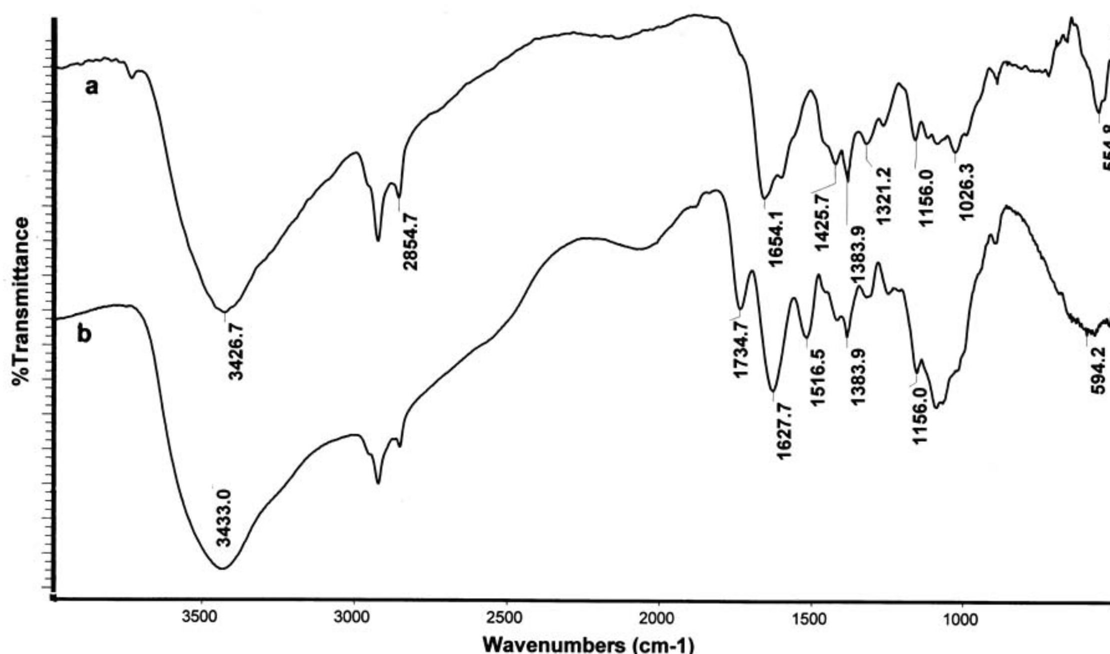
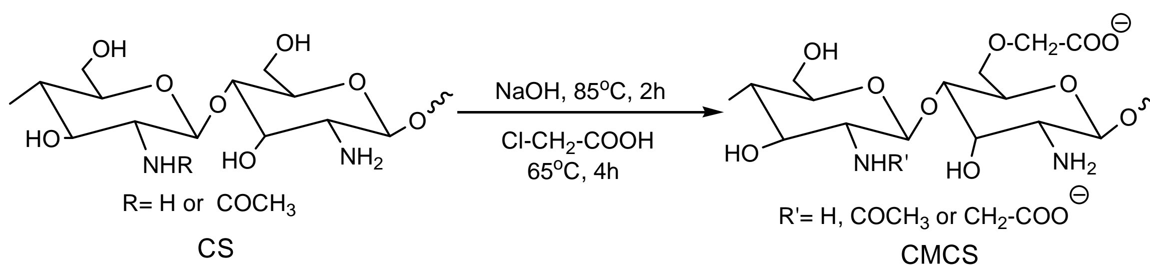


Figure 1. FTIR spectrum of (a) Carboxymethyl chitosan, CMCS as compared to (b) Chitosan, CS.



Scheme 1. Schematic illustration for conversion of chitosan (CS) into carboxymethyl chitosan (CMCS).

2θ values of 8.38, 11.49 and 18.25° in addition to several weak crystalline peaks. This 2D-WAXS diffraction pattern reflects a type of crystallinity for the CS investigated in this study. Figure 2b illustrates the diffractogram of the prepared CMCS. From this diffraction pattern, it was apparent that, CMCS demonstrated various crystalline bands in addition to two broad bands appeared at the 2θ values of about 21.43 and 26.33°. Besides, the 2D-WAXS diffractogram of CMCS kept many of the characteristic diffraction bands of the starting polymer, CS. These bands appeared mainly at the 2θ values of 10.40, 11.49, 16.10, ~18.46 and ~21.43°. The calculated d-spacing in addition to the 2θ values of the investigated CS and CMCS polymer molecules are shown in Table 1.

3.3. *In vivo* evaluation of the anti-tumour activity of CS and CMCS

The anti-tumour activity was evaluated on EAC bearing mice using an animal model. The CS sample at a dose level of 50 mg/kg showed a significant decrease in tumour volume at the end of *in vivo* experiment when compared to EAC control non-treated group at the same experimental conditions, using two millions of cells for tumour induction (Table 2). On the other hand, CS at a dose level of 100 mg/kg showed a highly significant decrease in tumour volume at the end of *in vivo* experiment when compared to EAC control non-treated group at the same experimental conditions, using two millions of cells for tumour induction. This shows that reduction of tumour volume as a measure of antitumor effect using CS is dose-dependent.

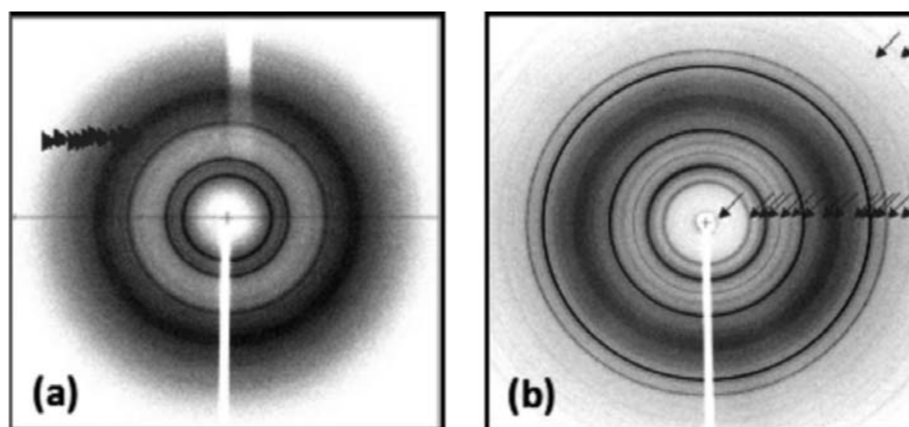


Figure 2. 2D-WAXS diffractograms, taken at T=298 K, of the (a) chitosan, CS in comparison with (b) carboxymethyl chitosan, CMCS.

Table 1. Some characteristics of the investigated chitosan (CS) before and after conversion into carboxymethyl chitosan (CMCS).

Sample Code	*Elemental analysis data*				Δc_p ($Jg^{-1}K^{-1}$)	T_g (K)	*XRD data for the investigated molecules*		
	C (%)	*N (%)*	*H (%)*	*O (%)*			*d (nm)*	*2θ (°)*	*Phase*
CS	36.11	8.06	5.24	50.59	13.51	207.2		8.38, 11.49, 18.25, 10.40, 11.49, 16.10, 18.46, 21.43	Semi-crystalline
CMCS	37.21	5.11	5.85	51.83	11.88	481.6	0.20, 2.01, 2.21, 2.93, 3.18	45, 8.50, 6.44, ~5.5, 4.41, ~3.98, 3.21, 3.04, 2.82	Semi-crystalline

The CMCS at dose levels of 50 mg/kg, and 100 mg/kg, injected to EAC bearing mice received two millions of cells for tumour induction, showed that reduction of tumour volume is also dose-dependent as compared to that of EAC control non-treated group at the same experimental conditions (Table 2). As apparent, a highly significant decrease in tumour volume was only observed at a dose level of 100 mg/kg. No changes or significant difference were found in tumour volume at a dose level of 50 mg/kg of CMCS when compared with EAC control non-treated group. So it is worth mentioning that treatment with CS exhibited better antitumor activity when compared with CMCS at the same experimental conditions as we demonstrated. Furthermore, the mortality rate of CMCS was found to be also dose-dependent. It was >35% at a dose level of 100 mg/kg of CMCS, while it was 12% at the same dose of CS. Mice that received 100 mg/kg of CMCS showed slight toxic symptoms. These include inactiveness, loss of appetite, slow movement and dizziness. Thus, higher dose of CMCS showed some symptoms of toxicity. In the case of CS, no such toxic symptoms were observed or any abnormal behaviour indicating the safety of its use as antitumor compound.

Furthermore, the CS at a dose level of 25 mg/kg injected using the same i.p protocol to EAC-bearing mice that received only one million of cells for tumour induction to each mouse, showed a significant decrease in tumour volume when compared to control untreated animals. This confirms the antitumor effect of it using lower dose.

The effect of CS and the prepared CMCS polymer on the lipid peroxidation was also investigated as illustrated in Table 2 by the determination of the levels of malondialdehyde (MDA) in EAC-bearing mice treated with both CS and CMCS. As apparent from the data, there was no significant change observed in the values and all the values were found to be within the normal range. This tends to indicate the absence of any harmful lipid peroxidation effect upon using the CMCS or CS under our experimental conditions.

Presence of oxygen is very important and vital to our life but this gas during metabolic utilization may result under certain conditions in the production of some undesirable by-product named as superoxide, a reactive oxygen species (ROS). As a matter of fact, aerobic organisms can survive the presence and existence of this harmful ROS only because they contain antioxidant defences. These antioxidants are molecules or compounds that act as free radical scavengers; they can regulate the levels of ROS to prevent oxidative stress. The superoxide radical anion ($^{\cdot}O^{-}_2$) found to be formed when oxygen acquires one electron is considered to be an initial form of metabolically produced ROS. It can be very toxic that intracellular levels above 1nm are lethal; that is why overproduction of it may cause several diseases. A recent review by Valdivia *et al.*, 2009 [23], extensively discussed

Table 2. Tumour volumes, levels of malondialdehyde (MDA), and the levels of superoxide dismutase (SOD) in mice treated with CS and CMCS (with a dose of 50 & 100 mg/Kg, day after day for two weeks) compared to control non-treated tumour bearing group using two million of cells/ mouse.

Groups	tumour volume (ml) ± SD	MDA (nmol/g wet tissue) ± SD	SOD (% of inhibition) ±SD
EAC control (n=10)	4.13 ± 0.61	31.38 ± 4.35	23.14 ± 5.11
CS 50 (n=5)	3.40 ± 0.41*	33.31 ± 4.85	32.62 ± 4.4**
CMCS 50 (n=5)	4.36 ± 0.47	32.60 ± 5.22	31.9 ± 4.5**
CS 100 (n=5)	2.56 ± 0.37**	32.38 ± 5.16	35.78 ± 4.4**
CMCS 100 (n=5)	3.26 ± 0.51*	31.21 ± 5.21	35.07 ± 5.4**

(*) significant, P<0.05; (**) highly significant, P<0.001 for effect on treated groups versus the control group. All the values in the table represent mean ± SD.

the central rule of this superoxide when present in excess to cause several diseases including cancer. They extensively showed how it is cleared successfully by SOD.

The inhibition of liver SOD activity as a result of tumour growth in EAC bearing mice was reported before [24], which supports our finding of decreased tumour target tissue SOD activity in animals bearing EAC only (control) as compared with treated animals. The administration of CS and CMCS was found to increase levels of SOD, indicating the antioxidant and free radical scavenging property of both of them. It also clearly demonstrated their potential use as an inhibitor of EAC induced intracellular oxidative stress due to superoxide.

On the other hand, the levels of SOD in target tumour tissues in mice treated with CMCS and CS revealed a highly significant increase as compared to that of the EAC control group (Table 2). The SOD is a free radical scavenger and it provides a defence against the potentially damaging reactivity of superoxide [25, 26]. From the data in Table 2, the administration of CMCS and CS has increased significantly the levels of SOD, which indicates the antioxidant and free radical scavenging characteristics of them. It is well known that the excessive production of free radicals would lead to an oxidative stress, which causes damage of macromolecules such as lipids and induces lipid peroxidation *in vivo* [27]. The MDA, the end product of lipid peroxidation, was reported to be increased in cancer tissues. Our data showed that the administration of both CMCS and CS did not increase the MDA and very significantly increased the SOD activity. This indicates that the CMCS and its parent compound, CS has a potential as anti-tumour agents with an inhibition ability of EAC-induced intracellular oxidative stress with additive antioxidant activity. However, further investigations are still needed as it is well known that CS molecular weight plays a significant role in anti-tumour and enzymatic activities.

CONCLUSIONS

The results of the current study illustrated that the synthesized CMCS has significantly inhibited the tumour growth only upon using higher dose level of 100 mg/kg. Moreover, the biochemical assays showed that the treatment with CMCS and CS led to augmentation of the antioxidant defense system without affecting lipid peroxidation in EAC bearing mice. In conclusion, the collected data revealed promising potential for CMCS as anticancer agent, but the therapeutic effect of the parent CS showed a highly significant improvement, in a dose-dependent manner, in tumour growth inhibition in comparison with that obtained of using CMCS at the same experimental conditions. Both compounds CS and CMCS augmented antioxidant defence enzyme SOD in tumour tissues which was found to play a significant role in cancer treatment.

DECLARATION OF INTEREST

The authors report no declarations of interest.

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