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Antioxidant and cytotoxic efficacy of chitosan on bladder cancer

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ABSTRACT

Objective: The present study demonstrated the antioxidant and cytotoxic efficacy of chitosan by evaluating cell viability in T24 human bladder cancer cell line and benzidine induced bladder cancer. The chemo preventive effects of the chitosan were evaluated in Swiss albino mice using 16 weeks medium term model of benzidine induced bladder cancer. **Methods:** Treatment of T24 cells with increasing concentration of chitosan led to a concentration dependent decrease in cell migration by MTT assay. The enzymic and non enzymic antioxidants were measured. **Results:** Bladder cancer was induced twice weekly through oral incubation of benzidine for 4 weeks. The oral administration of chitosan (100mg KG⁻¹ body wt) showed a significant increase in antioxidant enzymes like Super oxide dismutase (SOD), Glutathione peroxidase (GPx), Glutathione reductase (GR), Catalase (CAT) and non-enzymic antioxidants like reduced Glutathione (GSH), vitamin C and vitamin E when compared to benzidine treated groups. The effect is more pronounced in pretreatment regime than in the post treatment regime. The levels of lipid peroxidation were significantly decreased in the chitosan treated regimes. **Conclusions:** The present study reveals that chitosan has antioxidant and cytotoxic effects on benzidine induced bladder cancer and T24 human bladder cancer cell line.

1. Introduction

Bladder cancer is the second most common urologic malignancy and accounts for approximately 90% of cancer of the urinary Tract which is the fourth most incident cancer in male and ninth in females^[1]. In industrialized countries, more than 90% of cases are originating in the urothelial epithelial cells, (called urothelial cell carcinoma)^[2]. The carcinogens of bladder tissue damage are aromatic amines, typified by benzidine^[3]. In bladder cancer, as in most types of cancer, the transformation of a normal into a malignant cell involves a multistep mechanism. Sequentially, the expression of various classes of genes, like oncogenes, tumor-suppressor genes and DNA-repair genes are altered. These alterations involve mutations or chromosomal aberrations such as translations, insertion, amplification and deletion^[4]. Use of natural biopolymers for diversified applications in life sciences has several advantages, such as availability from replenishable agricultural or marine food resources, biocompatibility, biodegradability, therefore leading to ecological safety and the possibility of

preparing a variety of chemically modified derivatives for specific end uses. Polysaccharides, as a class of natural macromolecules, have the tendency to be extremely bioactive and are generally derived from agricultural feedstock or crustacean shell wastes. Cellulose, Starch, Pectin etc. are the biopolymers derived from the former while chitin and chitosan are obtained from the latter. In terms of availability, chitin is next to cellulose, available to the extent of over 10 giga tons annually. The application potential of chitosan is multidimensional, such as in food and nutrition, biotechnology, material science, drugs and pharmaceuticals^[5].

Chitosan, composed of β -(1-4)-linked N-acetyl-D-glucosamine (GlcNAc unit) and deacetylated glucosamine (GlcNH₂ unit) are obtained

by deacetylation of chitin, a major component of exoskeleton in crustaceans and also a cell wall component of fungi. Chitosan have various biological activities including antimicrobial activity^[6,7,8], antioxidant activity^[9,10], immuno-enhancing effects^[11] and antitumor activity^[12]. This activity was suggested mainly due to its cationic property exerted by amino groups and later it was accepted that the molecular weight also plays a major role for the antitumor activity^[14]. Recently, it was proved that strong electronic charge is an important factor for anti-cancer activity of chitosan^[15].

In addition, immune stimulation property of chitosan is also thought to be responsible for antitumor activity^[16]. Chitosan used as drug carriers as reported in cytotoxicity

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studies^[17]. Furthermore, some researchers found that antitumor effects of chitosan were due to increased activity of natural killer lymphocytes as observed in Sarcoma 180–Bearing Mice and hepatoma H22 in mice.^[18, 19] Cell death mechanisms could be distinguished by morphological criteria under microscope^[20].

Chitosan, the deacetylated derivative of chitin, is one of the abundant, renewable, nontoxic and biodegradable carbohydrate polymers. Chitosan has been applied broadly as a functional biopolymer in food and pharmaceuticals. Chitosan is known to have various biological activities including antitumor activities, immuno enhancing effects, antifungal and antimicrobial activities.^[21] Though the antitumor activity of chitosan has been studied *in vivo* and *in vitro*, the molecular mechanisms of the antitumor are still unclear. The present study has investigated the activity of chitosan against the bladder carcinoma cells (T24 cells).

2. Materials and Methods

2.1. Drugs and chemicals

Chitosan was obtained as a gift from M/s. apex laboratories, Chennai, benzidine and all of the other chemicals and reagents were obtained from Sigma Aldrich, Mumbai. MEM was purchased from Hi medic laboratories, Fetal bovine serum (FBS) was purchased from cistron laboratories, Trypsin, methyl thiazolyl diphenyl–tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals, Mumbai).

2.2. Cell culture conditions

T24 Human bladder Cancer cell line was obtained from National centre for cell sciences Pune (NCCS), India. The cells were maintained in minimal essential media supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO₂ at 37 °C.

2.3. Animals

Healthy male Swiss albino mice (6–8 weeks old) were used throughout the study.

3. Experimental Protocol

The animals were divided into five groups and each groups consisted of six animals.

Group I served as control animals and was given corn oil (vehicle) (50 ml kg⁻¹ body weight) orally for 16 weeks.

Group II animals were treated with benzidine (50 mg kg⁻¹ body weight) dissolved in corn oil orally twice weekly for 4 successive weeks to induce bladder cancer.

Group III animals pretreated with chitosan (100 mg kg⁻¹ body weight) orally for 4 successive alternative days.

Group IV animals were post treated with chitosan for 4 successive alternative days.

Group V control animals treated with chitosan alone as above.

At the end of the experimental period, the animals were fasted overnight and killed by cervical decapitation. Bladder tissues were removed from all animals and washed with

ice cold saline and used for analysis. Total protein was estimated by the method of Lowry *et al.*^[22], the level of lipid peroxides (LPO) was measured according to Ohkawa *et al.*^[23], Superoxide dismutase (SOD) was determined by Misra and Fridovich^[24], Catalase activity (CAT) was estimated by Sinha^[25], Glutathione peroxidase (GPx) was estimated by Rotruck *et al.*^[26]. Reduced glutathione (GSH) levels were measured by according to Moron *et al.*^[27], Vitamin C levels were determined by Omaye *et al.*^[28] and Vitamin E levels were determined by Desai^[29].

4. *In vitro* assay for cytotoxicity activity

The cytotoxicity of samples on T24 cells was determined by the MTT assay according to Mosman^[30]. The effect of the chitosan on the proliferation of T24 was expressed as the % cell viability, using the following formula:

$$\% \text{ cell viability} = \frac{\text{A570 of treated cells}}{\text{A570 of control cells}} \times 100\%$$

5. Statistical analysis

Statistical analysis was calculated by using statistical package for the social sciences package (SPSS). Values are mean±SD for six specimens in each group and the significance of difference between mean values were determined by Duncan multiple comparison tests. The level of significance (p value) was calculated based on 1% level.

6. RESULTS

6.1. Enzymic and nonenzymic antioxidant activity

The mice were observed for 1 h continuously and intermittently for 4 h and further for 72 h

any mortality. None of the mice showed changes and there was no mortality up to 72 h period of observation and even up to a dose of 100 mg of the chitosan. The safety of the drug was evaluated and it was found that 100 mg kg⁻¹ body weight. was found to be optimal dosage. The increased levels of enzymic and non enzymic antioxidants in Group III and Group IV animals when compared to Group II animals might be due to the cationic and antioxidant property of chitosan. The most significant with measured values were already indicated in the table 1. It represents the changes in the levels of enzymic and non enzymic antioxidants in bladder tissues of experimental animals. The enzymic antioxidants such as Superoxide dismutase, Catalase, Glutathione peroxidase, Glutathione reductase and non enzymic antioxidants such as glutathione, Vitamin E and Vitamin C were found to be significantly reduced in group 2 animals (p<0.01). In treated animals (group 3 and group 4) the antioxidant enzymes levels were significantly increased to near normal levels when compared to group 2 animals (p<0.01). No adverse effect was observed in group 5 animals.

Reduced glutathione, chemically – glutamyl cysteinyl glycine is a predominant non–protein thiol present in virtually all cell types. Glutathione is ubiquitous in animals^[31].

Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants. It fulfills a wide variety of important functions such as detoxification of electrophiles, serves as a transfer vehicle for cysteine and renders

Table 1

Effect of Chitosan on enzymic and nonenzymic antioxidants in benzidine induced bladder cancer in albino mice

Parameters	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V
Total protein	72.3±0.35	43.8±0.63	63.2±0.19	60.1±0.28	70.3±0.52
Super oxide dismutase	1.1±0.05	0.6±0.04	0.9±0.04	0.7±0.03	1.03±0.02
Catalase	1.7±0.13	1.1±0.15	1.4±0.11	1.2±0.09	1.58±0.06
Glutathione peroxidase	3.3±0.08	2.34±0.07	2.9±0.06	2.4±0.15	2.9±0.19
Reduced glutathione	10.4±0.27	5.44±0.15	7.25±0.11	7.01±0.07	8.2±0.19
Vitamin C	1.1±0.02	0.7±0.04	1.03±0.02	0.81±0.02	0.9±0.09
Vitamin E	1.6±0.13	0.66±0.13	1.1±0.07	0.91±0.01	1.26±0.01

Each value is expressed as mean ± SD for six mice in each group. Since p value is less than 0.01, there is a significant difference between groups with regard to antioxidant enzymes. Based on Duncan multiple range test, the group 2 is significant with group 3, 4 and group 1. Group 3 is significant to Group 4. Group 1 and Group 5 are not significant.

protection against ROS conjugation. The reduced glutathione in tissues keeps up the cellular level of vitamin C and vitamin E in active forms. These vitamins also exist in inter convertible form and participate in neutralizing free radicals. When there is reduction in the level of GSH, the cellular levels of vitamin C and vitamin E are also lowered. Intracellular GSH status appears to be sensitized indicator of the cell's overall health and of its ability to resist toxic challenge. Experimental GSH depletion can trigger suicide of the cell by a

process known as apoptosis[32].

The ascorbate molecules are involved in the feed back inhibition of the lysosomal glycosidases responsible for the malignant invasiveness[33]. Vitamin C protects cell membrane and lipoprotein particles from oxidative damage by regenerating the antioxidant from vitamin E[34]. Thus vitamin C and vitamin E act synergistically in scavenging wide variety of ROS.

Vitamin E is the major lipid soluble peroxy radical scavenger, which can limit LPO, terminating chain reactions initiated in the membrane lipids[35]. Decreased vitamin E content in bladder cancer bearing animals might be due to excessive utilization of this antioxidant for quenching enormous free radicals produced in these conditions. Vitamin E acts a chain breaking antioxidant by donating its labile hydrogen atom from phenolic –OH group to prorogating lipid peroxy and alkoxy radicals intermediates of LPO, thus terminating the chain in reactions[36]. The supplementation of chitosan to the experimental animals would have improved various cellular antioxidants and thiol content in tissues, which in turn reduces free radical formation during bladder carcinogenesis induced by benzidine.

Antioxidant enzymes are the main scavengers of free radicals and function as the inhibitors at both initiation and promotion or transformation stages of carcinogenesis[37]. The antioxidant enzymes SOD, CAT and GPx play an important role as protective enzymes against reactive oxygen species in tissues and also comprise the cellular antioxidant defense system[38].

The levels of lipid peroxidation in bladder mitochondria of control and experimental animals are shown in Fig. 1. There found to be an increase in LPO in group II (p<0.01) cancer bearing mice when compared with control animals (p<0.01). Chitosan treatment resulted in significant decrease in the activities of these enzymes in group III (p<0.01) and group IV (p<0.01) animals where the effect in group III is much more pronounced than group IV. However the Chitosan alone treated group V animals did not show any significant differences when compared to group I animals in the LPO

levels. The significant increase in the levels of LPO was observed in animals bearing bladder cancer.

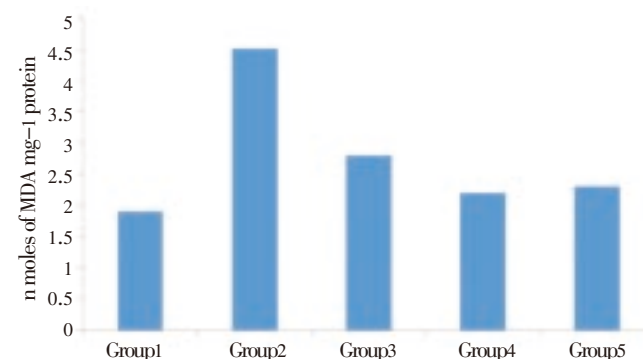


Figure 1. Levels of lipid peroxidation in bladder tissues of normal and Experimental groups of mice

6.2. Inhibitory effect on proliferation of T24 cells

Table 2

Anticancer effect of Chitosan on T–24 cell line

Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability(%)
1000	Neat	0.02	3.9
500	1:1	0.10	19.6
250	1:2	0.19	37.2
125	1:4	0.23	45.0
62.5	1:8	0.27	52.9
31.2	1:16	0.34	66.6
15.6	1:32	0.39	76.4
7.8	1:64	0.42	82.3
Cell control	–	0.51	100

Each value is expressed as mean ±SD for six mice in each group. Since p value is less than 0.01, there is a significant difference between groups with regard to lipid peroxidation.

The effect of chitosan on the cells viability was measured by the MTT assay, which reflects the cellular reducing activity. MTT assay as shown in Table –2 indicated that Chitosan inhibited the T–24 cells proliferation in a concentration and dose dependent manner. The median lethal concentration of chitosan was 62.5 µg/ml for T24 at 48h.

7. Discussion

7.1. Enzymic and nonenzymic antioxidant activity

In present study shows a reduction in the activities of SOD, CAT and GPx in bladder cancer bearing animals. All chitosan can absorb free electrons also known as free radicals and hold them. This stops further free radical damage to cells. Chitosan have shown potential as scavenging agents, due to their ability to abstract hydrogen atoms free radicals[39,40]. This ability has been reported as directly correlated with their structural properties namely that amino and hydroxyl group can react with unstable free radicals to form stable macromolecules radicals.[41,42]. Furthermore, their ready uptake by cells and the intestine, In addition with their claimed low toxicity, make chitosan very promising compounds for use as natural antioxidants [43,44].

Chakraborty *et al* 2011[45]demonstrated that modified chitosan had the antioxidant enzymic activity. Ji Young kim *et al* 2009[46]showed the free radical scavenging activity of chitosan oligosaccharides at different concentrations.

Siripatrawan *et al* 2010[47]study indicated that chitosan as a natural antioxidant incorporated with green tea extract. Elise Portes *et al* 2009[48]had showed the antioxidant activity in food packaging. O I Shevtsova, 2000[49]has shown that application of chitosan had a positive effect on the lipid peroxidation in mice liver intoxicated by tetra chloromethane. Yang Yan *et.*, all[50]has studied the treatment with chitosan, GlcNH₂, and GlcNAc significantly decrease serum creatinine and uric acid levels and inhibit lipid peroxidation in kidney homogenate.

In present study the chitosan altered these macromolecular damage mediated through free radicals thereby displaying the protective role of chitosan inhibiting free radical mediated cellular damages. The suppressive action of chitosan on bladder lipid peroxidation observed in vivo systems suggests that the drug may have a direct effect on the membranes and these may decrease the susceptibility of the membranes to lipid peroxides. The present study indicates that oral administration of chitosan significantly suppresses the LPO formation in bladder tissues of animals bearing cancer.

Chitosan has the ability to suppress the malignancy by modulating cell transformation, decreasing the degree of bladder cancer growth and controlling cell proliferation.

7.2. Inhibitory effect on proliferation of T24 cells

In recent years, naturally occurring compounds have grabbed increased attention for the prevention and or intervention of early stages of carcinogenesis and neoplastic progression before the occurrence of invasive malignant diseases, such as many are regarded as chemo preventive agents[51, 52,53]. The antitumor activities of chitosan in mice have been already reported[54]. Kun Te Shenet *et al.*, 2009 [55]demonstrated the protective effect of Chitosan on the proliferation of HepG2 cells and suppress tumor growth in HepG2-bearing SCID mice.

Chakraborty *et al* 2012[56]demonstrated that modified chitosan had in vitro cytotoxicity against HeLa cell lines in Swiss mice. Hosseinzadeh *et al* 2012[57]proved that chitosan nanoparticles had inhibition cell viability on HT–29 colon carcinoma cell line. In present study indicated that chitosan inhibited the T–24 cells proliferation in a concentration and dose dependent manner.

Conclusion

The present study reveals protective effect of Chitosan in T24 bladder cancer cell line. It also demonstrates the antioxidant and antitumor property of chitosan against benzidine induced bladder cancer. Hence, Chitosan may be considered as a promising agent for the treatment of bladder cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

References

- [1] Jemal, A., R. Siegel, E. Ward, T. Murray, J. Xu and M.J. Thun. Cancer Statistics. *CA cancer J Clin*,2007; **57**:43–66.
- [2] Parkin, D.M., F. Bray, J. Ferlay and P. Pisani, 2005. *cancer J Clin*, **55**:74–108.
- [3] ACS, 2011. Known and probable human carcinogens. American Cancer Society. <http://www.cancer.org/Cancer/CancerCauses/OtherCarcinogens/GeneralInformationaboutCarcinogens/known-and-probable-human-carcinogens?sitearea=PED>
- [4] Visapaa H, Bui M, Huang Y, Seligson D, Tsai H, Pantuck A, Figlin R, Rao JY, Belldegrin A, Horvath S, Palotie A. Correlation of Ki-67 and gelsolin expression to clinical outcome in renal clear cell carcinoma. *Urology*. 2003 ;**61**:845–850.
- [5] Prashanth, K.V.H. and R.N. Tharanathan. Chitin/chitosan: Modifications and their unlimited application potential—An overview. *Trends Food Sci. Technol*. 2007; **18**: 117–131
- [6] Choi, B.K., K.Y. Kim, Y.J. Yoo, S.J. Oh, J.H. Choi and C.Y. Kim. In vitro antimicrobial activity of a chito oligosaccharides mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. *Int. J. Antimicrobial Agents*. 2001; **18**: 553–557.
- [7] Tarsi, R., B. Corbin, C. Pruzzo and R.A. Muzzarelli. Effect of low-molecular-weight chitosans on the adhesive properties of oral streptococci. *Oral Microbiol. Immunol*. 1998; **13**: 217–224.
- [8] Tsai, G.J. and W.H. Su. Antibacterial activity of shrimp chitosan against *Escherichia coli*. *J food prot* . 1999;**62** :239–243.
- [9] Park, P.J., J.Y. Je and S.K. Kim. Free radical scavenging activity of chito oligosaccharides by electron spin resonance spectrometry. *Journal of Agricultural and Food Chemistry*. 2003; **51**:4624–4627.
- [10] Xie, W., P. Xu and Q. Liu. Antioxidant activity of water-soluble chitosan derivatives. *Bioorg. Med. Chem. Lett* .2001; **11**: 1699–1701.
- [11] Feng, J., L. Zhao and Q. Yu. Receptor-mediated stimulatory effect of oligo chitosan in macrophages. *Biochem. Biophys. Res. Commun*. 2004; **317**: 414–420.
- [12] Tsukada, K., T. Matsumoto, K. Aizawa, A. Tokoro, R. Naruse, S. Suzuki and M. Suzuki. Antimetastatic and growth-inhibitory effects of N-acetylchitohexaose in mice bearing Lewis lung carcinoma. *Jap. J. Cancer Res*.1990; **81**: 259–265.
- [13] Qin, C., Y. Du, L. Xiao, Z. Li, X. Gao. Enzymic preparation of water-soluble chitosan and their antitumor activity. *International Journal of Biological Macromolecules*; 2002; **31**:111–117.
- [14] Huang, R., N. Rajapakse, E. Mendis and S.K. Kim. Strong electronic charge as an important factor for anticancer activity of chito oligosaccharides (COS). *Life Sciences*. 2006; **78**: 2399–2408.
- [15] Suzuki, K., T. Mikami, Y. Okawa, A. Tokoro, S. Suzuki and M. Suzuki. Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. *Carbohydrate Res*. 1986; **151**: 403–408.
- [16] Xu, Y. and Y. Du. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. *Int J pharm*. 2003;**339**: 250–226.
- [17] Maeda, Y. and Y. Kimura. Antitumor effects of various

- lowmolecular- weight chitosans are due to increased natural killer activity of intestinal intraepithelial lymphocytes in sarcoma 180-bearing mice. *Journal of Nutrition*. 2004; **134**: 945–950.
- [18] Qi, L. and Z. Xu. In vivo antitumour activity of chitosan nanoparticles. *Bioorg. Med. Chem. Lett.* 2006; **16**: 4243–4245.
- [19] Rello, S., J.C. Stockert, V. Moreno, A. Gamez and M. Pacheco et al. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis*; 2005; **10**: 201–208.
- [20] Sudarshan, N.R., D.G. Hoover and D. Knorr. Antibacterial action of chitosan. *Food Biotechnol.* 1992; **6**:257–272.
- [21] Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. Protein measurement with the folin's phenol reagent. *J. Biol. Chem.*1951;**193**: 265–275.
- [22] Ohkawa, H., N. Ohishi and K. Yagi. Assay for lipid peroxidation in animal tissues by thio barbituric acid reaction. *Anal. Biochem.*1979; **95**: 351–358.
- [23] Misra H.P. and Fridovich. The role of superoxide anion in the autooxidation of epinephrine and a simple assay of superoxide dismutase. *J. Biol. Chem.* 1972; **247**:3170–3175.
- [24] Sinha, A.K.. Colorimetric assay of catalase. *Anal.Biochem.*1972; **47**: 389–394.
- [25] Rotruck, J.T., A.L. Pope, H.E. Ganther, A.B. Swanson, D.G. Hafeman and W.G. Hoekstra. Selenium: Biochemical Role as a component of glutathione peroxidase purification and assay. *Science.* 1973; **179**: 588–590.
- [26] Moron, M.S., J.W. Depierre and K.B. Manerwik. Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. *Biochem. Biophys. Acta.* 1979; **582**: 67–68.
- [27] Omaye, S.T., J.D. Tumball and H.E. Sauberlich. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Meth. Enzymol.*1979; **62**: 1–11.
- [28] Desai, I.D. Vitamin E analysis methods for animal tissues. *Method Enzymol.*1984; **105**:138–147.
- [29] Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 1983; **65**: 55–63.
- [30] Duke, R.C., D.M. Ojcius and J.D.E. Young. Cell suicide in health and disease. *Sci. Am.*, 1996; **275**: 80–87.
- [31] Cameron, E., L. Pauling and B. Leiboriz. Ascorbic acid and cancer. *Cancer. Res.*, 1979; **39**: 663–681.
- [32] Buettner ,G.R. The pecking order of free radicals and antioxidants: Lipid peroxidation,a-tocopherol and ascorbate, *Arch, biochem, biophys*,1993; **300**: 535–543.
- [33] Wiseman, H. Dietary influences on membrane fuction; Importance in protection against oxidative damage and disease. *J.Nutr. biochem.*1996; **7**:2–15.
- [34] Das, S. Vitamin E in the genesis and prevention of cancer. *Acta Oncological.*1994; **33**:615–619.
- [35] Guillaume, J., S. Kaushik, P. Bergot and R. Metailler. Nutrition and Feeding of Fish and Crustaceans. Praxis PUBLISHING, UK.,2001: 111–130.
- [36] Thirunavukkarasu, C. J.P. Singh, K. Selvendiran and D. Sakthisekaran. Effect of selenium on N-nitrosodiethylamine-induced multi-stage hepatocarcinogenesis with reference to lipid peroxidation and enzymic antioxidants, *Cell biochem. Funct.* 2001; **19**: 27–35.
- [37] Kinnula, V.L., J.D. Crapo and K.O. Raivio. Generation and disposal of reactive oxygen metabolites. *Lab. Invest.*1995; **73**: 3–19.
- [38] Huang, R., E. Mendis and S.K. Kim. Factors affecting the free radical scavenging behavior of chitosan sulfate. *International journal of biological macromolecules.* 2005; **36**:120–127.
- [39] Varum, K.M., M.H. Ottoy and O. Smidsrod. Water-solubility of partially N-acetylated chitosans as a function of PH, effect of chemical composition and depolymerisation. *Carbohydrate Polymers.* 1994; **25**: 65–70.
- [40] Je, J.Y., P.J. Park and S.K. Kim. Free radical scavenging properties of hetero-chito oligosaccharides using an ESR spectroscopy. *Food Chem. Toxicol.*2004; **42**: 381–387.
- [41] Kim, S.K. and N. Rajapakse. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review, *carbohydrate polymers.* 2005; **62**:357–368.
- [42] Chae, S.Y., M.K. Jang and J.W. Nah. Influence of molecular weight on oral absorption of water soluble chitosans. *Journal of controlled release.* 2005; **102**:383–394.
- [43] Fernandes, J.C., P. Eaton, H. Nascimento, L. Belo and S. Rocha et als. Effects of chito oligosaccharides on human red blood cell morphology and membrane protein structure. *Biomacromolecules.* 2008; **9**: 3346–3352.
- [44] Chakraborty SP, Mahapatra SK, Sahu SK, Pramanik P, Roy S. antioxidative effect of folate modified chitosan nanoparticles. *Asian Pac J Trop Biomed* 2011; 29–38.
- [45] Ji Young Kim, Kil Nam Kim, Jong Gwan Kim, Seong Chul Kim, Wook Jae Lee and Chang Gu Hyun. In vitro antimicrobial and antioxidant activities of chitosan oligosaccharides. *J. Appl. Biol. Chem.*2009;**52**(2): 84–87.
- [46] Siripatrawan U and Harte BR. Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. *Food Hydrocolloids.* 2010:770–775. (Journal homepage: www.elsevier.com/locate/foodhyd).
- [47] Elise Portes, Christian Gardrat, Alain Castellan, Veroniaue Coma. Environmentally friendly films based on chitosan and tetrahydrocurcuminoid derivatives exhibiting antibacterial and antioxidative properties. *Carbohydrate polymers.* 2009: 578–584. Journal homepage www.elsevier.com/locate/carbpol).
- [48] O I Shevtsova. Effect of chitosan on lipid peroxidation in toxic liver injury, Vladivostok, *Ukrainskii biokhimicheskii zhurnal.* 2000; **72** (2): 88–90.
- [49] Yang Yan, Liu Wanshun, Han Baoqin, Liu Bing, Fu Chenwei. Protective effects of Chitosan oligosaccharide and its derivatives against carbon tetrachloride-induced liver damage in mice. *Hepatology Res.* 2006; **35**(3):178–84.
- [50] Chen, A.S., Taguchi, T., Sakai, K., Matahira, Y., Wang, M.W., Miwa, I. Effect of chitobiose and chitotriose on carbon tetrachloride-induced acute hepatotoxicity in rats. *Biol. Pharm. Bull.*2005a; **28**: 1971–1973.
- [51] Chen, P.N., Chu, S.C., Chiou, H.L., Kuo, W.H., Chiang, C.L., Hsieh, Y.S. Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside, exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line. *Cancer Lett.* 2006a; **235**:248–259.
- [52] Chen, P.N., Kuo, W.H., Chiang, C.L., Chiou, H.L., Hsieh, Y.S., Chu, S.C. Black rice anthocyanins inhibit cancer cells invasion via repressions of MMPs and u-PA expression. *Chem. Biol. Interact.* 2006b;**163**:218–229.
- [53] Kobayashi, M., Watanabe, T., Suzuki, S., Suzuki, M. Effect of Nacetylchitohexaose against Candida albicans infection of tumor-bearing mice. *Microbiol. Immunol.* 1990; **34**: 413–426.
- [54] Kun Te Shen, Mei Huei Chen, Hing Yuen Chan, Jjiang Huei Jeng, Ying Jan Wang. Inhibitory effects of chito oligosaccharides on tumour growth and metastasis. *Food and chemical toxicology.* 2009;**47**:1864–1871.
- [55] Chakraborty SP, Sahu SK, Pramanik P, Roy S. Biocompatibility of folate modified chitosan nanoparticles. *Asian Pac J Trop Biomed* 2012; 215–219.
- [57] Hosseinzadeh H, Atyabi F, Dinarvand R, Ostad SN, Chitosan pluronic nanoparticles as oral delivery of anticancer gemcitabine; preparation and in vitro study. *Inter. J. of nanomedicine.* 2012; **7** 1851–1863.