4.0 RESULTS AND DISCUSSION

Cancer is one of the leading causes of adult deaths worldwide. In India, the International Agency for Research on Cancer estimated indirectly that about 6,35,000 people died from cancer in 2008, representing about 8% of all estimated global cancer deaths and about 6% of all deaths in India. The absolute number of cancer deaths in India is projected to increase because of population growth and increasing life expectancy. Rates of cancer deaths are expected to rise, particularly, from increases in the age specific cancer risks of tobacco smoking, which increase the incidence of several types of cancer. India is a culturally diverse country, with huge regional and rural-to-urban variation in lifestyles and in age-specific adult death rates. (Ferlay et al, 2010).

Bladder cancer is the 10th most common cancer worldwide, with the highest rates reported in Europe, North America and Australia and accounting for an estimated 2,61,000 new cases diagnosed and 1,15,000 deaths each year, by comparison, relatively low rates are found in the Far Eastern countries. In Europe, bladder cancer is the 5th most commonly diagnosed cancer type and the 9th leading cause of cancer mortality. It affects men more frequently than women. (Larsson et al, 2008).

Chitosan receives a lot of attention because of its numerous desirable qualities and that it is produced from chitin, which is the second most abundant
biopolymer in the world. The growing interest in chitosan is due to its biocompatibility, biodegradability, antibacterial properties, affinity for many proteins, and anti-oxidative properties (Liu et al., 2012). It can be used as a flocculent, clarifier, chromatography column matrix, gas-selective membrane, plant disease resistant promoter, anti-cancer agent, wound healing promoting agent and antimicrobial agent (Ocloo et al., 2011).

The present study was undertaken to screen the antioxidant, antimicrobial and antiproliferative efficacy of chitosan and its pharmaceutical preparations on T24 urinary bladder cancer cell line. The study was also carried out in benzidine induced urinary bladder cancer in swiss albino mice to evaluate the antioxidant and antimicrobial effect of chitosan in vivo. The result of the present study was discussed under the following phases.

4.1. PHASE I – Screening of invitro antimicrobial and antioxidant efficacy of chitosan and its pharmaceutical preparations.

4.1.1. Film forming property of chitosan based pharmaceutical preparations

Minimal environmental impact and efficient utilization of natural resources must be essential criteria in the development of new materials for food packaging. Biodegradable/compostable materials are usually based on polysaccharides, proteins and lipids, which are generally non toxic and effective barriers to oxygen and carbon dioxide, so they can be used as protective
materials to maintain food quality and at the same time, reduce the environmental impact of packaging wastes. Bio-based materials appear to be more environmentally friendly than petroleum based materials when their origin and biodegradability are compared (Álvarez-Chávez et al., 2012).

Chitosan owes its film and fiber forming abilities to its linear structure which allows for strong inter and intra molecular hydrogen bonding (Demarger-Andre and Domard, 1994).

The film forming ability of chitosan based pharmaceutical cream preparation was assessed as per Ha, (1999).

The film forming properties of chitosan based and chitosan non based formulations in the various categories of cream like antibacterial cream (Fusidic acid cream, Calcium mupirocin), antifungal cream (Ketoconazole, Miconazole and terbinafine Hcl ) was carried out in the present study to support the antimicrobial property. The present study demonstrate the more pronounced film forming ability of chitosan based semi solid preparations when compared to chitosan non based formulations. (Figure - 5)
FIGURE - 5 FILM FORMING ACTIVITY OF CHITOSAN

(a) Standard market product - Fusidic acid cream

(a) Chitosan base Fusidic acid cream

(b) Standard market product - Calcium Mupirocin

(b) Chitosan base Calcium Mupirocin cream

(c) Standard market product - Framycetin sulphate cream

(c) Chitosan base Framycetin sulphate cream
(d) Standard market product - Silver sulphadiazine cream

(e) Standard market product - Ketoconazole cream

(f) Standard market product - Miconazole nitrate cream

(d) Chitosan base Silver sulphadiazine cream

(e) Chitosan base Ketoconazole cream

(f) Chitosan base Miconazole nitrate cream
Results of the present study is in agreement with Kozen et al., (2008) who reported that improve homeostasis and promote granulation by enhancing the function of inflammatory cells such as leukocytes, fibroblasts and macrophages.

Chitosan films are tough, flexible, transparent, and resistant to lipids, suggesting potential value as a food coating or packaging material. It was shown in vivo that chitosan can limit the adsorption of lipids and is therefore used as a dietary supplement and for weight loss (Tharanathan, 2003).

Salleh and Muhamad (2013) proved that Antimicrobial starch-based film incorporated with chitosan and lauric acid was prepared successfully by casting technique. Antimicrobial starch-based film exhibited good film forming property due to the presence of high density of amino groups and hydroxyl groups and inter and intra molecular hydrogen bonding.

Martínez-Camacho et al (2010) stated that chitosan is used as film forming material as well as anti fungal activity. The results of their study suggest that even in plasticized or non-plasticized films, chitosan shows fungistatic activity and antimicrobial activity which makes possible the development of active packaging based on mixtures of chitosan, with good thermal stability.

4.1.2. Antimicrobial Activity of chitosan based and chitosan non based pharmaceutical preparations

The antimicrobial activity of chitosan and its derivatives or oligomers has been recognized and is considered to be one of the most important properties,
corresponding directly to their possible biological applications wei et al (2003); Xia WS (2003); Zhao et al (2006).

In the present study, antibacterial activity of chitosan (CS), Fusidic acid (FA), Chitosan based Fusidic acid cream (CSF) and chitosan non based Fusidic acid cream (FC) was tested against four strains staphylococcus aureus, pseudomonas aeruginosa, Bacillus spizizenii and Escherichia coli in Muller Hinton (M–H) broth and results were shown in figure 6 and 7.

Figure 6 - Antimicrobial activity and zone of inhibition of chitosan base and chitosan non base raw material (in mm)
The highest zone of inhibition of chitosan (CS) against staphylococcus aureus, pseudomonas aeruginosa, Bacillus spizizenii and Escherichia coli were found to be 21.42 mm, 8.89 mm, 9.02 mm and 15.33 mm respectively at the dose of 1400 µg/well. The highest zone of inhibition of Fusidic acid (FA) against staphylococcus aureus, pseudomonas aeruginosa, Bacillus spizizenii and Escherichia coli were found to be 20.97 mm, 8.58 mm, 9.2 mm and 10.87 respectively at the dose of 1400 µg/well.

**Figure - 7 Antimicrobial activity and zone of inhibition of chitosan base and chitosan non base Fusidic acid cream (in mm)**

The highest zone of inhibition of chitosan based Fusidic acid cream (CSF) against staphylococcus aureus, pseudomonas aeruginosa, Bacillus spizizenii
and Escherichia coli were found to be 19.45 mm, 10.67 mm, 13.5 mm and 21.25 mm respectively at the dose of 70 µg/well.

The highest zone of inhibition of chitosan non based Fusidic acid cream (FC) against staphylococcus aureus, pseudomonas aeruginosa, Bacillus spizizenii and Escherichia coli were found to be 18.65 mm, 5.94 mm, 10.89 mm and 11.99 mm respectively at the dose of 70 µg/well.

From this Fusidic acid cream alone acts against the bacteria but the addition of the chitosan in fusidic acid cream has got enhanced antimicrobial functions when compared with Fucidin cream.

Allan and hadwiger (1979), first reported chitosan and its derivatives had broad-spectrum antimicrobial effects. Chitin and chitosan have been investigated as an antimicrobial material against a wide range of target organisms like algae, bacteria, yeasts and fungi in experiments involving in vivo and in vitro interactions with chitosan in different forms (solutions, films and composites).


The electrostatic interaction of chitosan having greater the number of cationized amines, the higher will be the antimicrobial activity Masson et al (2008); Yalpani et al (2002).
Several authors indicated that chitosan may directly affect cell membrane function. The poly cationic chitosan available to bind to a charged bacterial surface causes in leakage of intercellular constituents and tends to form cluster of molecular aggregation. The more adsorbed chitosan would result greater change in structure and in the permeability of cell membrane (Leuba and Stossel 1986).

The applications of chitosan to use as antimicrobial material for food have been widely reported in literatures. In fruit and vegetables (Badawy and Rabea 2009), bread (Ahn et al., 2003), seafood (López-Caballero et al., 2005), meat (Rao et al., 2005) and sausage (Soultos et al., 2008).

Antibacterial activity of the water soluble N-alkylated disaccharide chitosan derivatives against Escherichia coli and Staphylococcus aureus was investigated by Tsui-Chu Yang et al (2005). Chitosan may also have an effect on the type of bacteria living in the intestines or on the action of these bacteria. A small human study found that taking 3-6 grams per day of chitosan for two weeks reduced indicators of putrefaction in the intestines, change that might help prevent diseases such as colon cancer (Tsui-Chu Yang et al.,2005).

The antibacterial activity of chitosan was tested against two strains of Staphylococcus aureus and Salmonella paratyphi by Islam et. al.,( 2011). They found that the highest zone of inhibition against Salmonella paratyphi and Staphylococcus aureus.
Karthick Raja Namasivayam and Allen Roy (2013) demonstrated the synthesis of metallic silver nanoparticles by chemical reduction method followed by stabilization using biocompatible polymer chitosan which was found to exhibit enhanced antibiofilm activity. This study can further be used to prevent or minimize bacterial infections leading to the development of new generation of antimicrobial agents.

The present study demonstrates the presence of chitosan in the semi-solid preparation of fusidic acid cream enhanced the antimicrobial activity as compared to Fucidin cream.

4.1.3. Wound healing property of chitosan based and chitosan non based pharmaceutical preparations

Slow healing and non-healing wounds, such as ulcers, as well as wounds caused by major or minor injuries, surgery, or burns, represents the most widespread treatable conditions encountered by humans and animals. Wound repair is a well highly coordinated process that involves a series of overlapping phases, inflammation cell proliferation, matrix deposition and tissue remodeling, underlying repair is a complex dynamic series of events including clotting, inflammation, granulation tissue formation, epithelialization, neo-vascularization, collagen synthesis and wound contraction [Singer et al, 1999]. Briefly, the wound healing process consists of three major stages. First inflammatory cells from the surrounding tissue move towards the lesion site. Subsequently, fibroblasts
appear and begin to produce collagen connective fibers that impart tensile strength to the regenerating tissue. Simultaneously, numerous capillaries begin to form to supply the site with nutrients and oxygen, while the epithelial cells at the edge of the wound start filing in the area under the scab. In the third and final phase, the new epithelium forms and the wound considered healed (Wang et al, 2008).

The study was carried out in albino Wister rats to support the use of chitosan in pharmaceutical preparations like cream, lotion and chitosan coated bandages for healing of wounds.

The present study on excision wound healing rat model reveals that both the groups showed decreased wound area from day 0 to day 13. On 12th post wounding day, animals treated with chitosan non based fusidic acid cream (Fucidin) showed 84.5% of healing whereas animals treated with chitosan based Fusidic acid cream showed 93.5% healing. The results were presented in the tables 5 and 6.

Table 5 - Percentage of wound contraction in group treated with chitosan non based and chitosan based Fusidic acid cream

<table>
<thead>
<tr>
<th></th>
<th>Days</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan Non based Fusidic acid cream</td>
<td>Mean Area (mm²) ±SEM</td>
<td>521±12.9</td>
<td>416±11.7</td>
<td>342±11.6</td>
<td>283±13.3</td>
<td>155±11.7</td>
<td>118±13.4</td>
<td>72±11.4</td>
</tr>
<tr>
<td>% Contraction</td>
<td>-</td>
<td>18.8</td>
<td>32.37</td>
<td>43.8</td>
<td>67.9</td>
<td>74.9</td>
<td>83.5</td>
<td></td>
</tr>
<tr>
<td>Chitosan based Fusidic acid cream</td>
<td>Mean Area (mm²) ±SEM</td>
<td>512±14.2</td>
<td>397±12.2</td>
<td>321±15.1c</td>
<td>236±12.8</td>
<td>107±13.6</td>
<td>63±11.8</td>
<td>28±12.1</td>
</tr>
<tr>
<td>% Contraction</td>
<td>-</td>
<td>21.0</td>
<td>35.6</td>
<td>51.9</td>
<td>76.6</td>
<td>85</td>
<td>92.5</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Mean ±SEM, n=6
Table 6 - Period of Epithelization

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan non based Fusidic acid cream</td>
<td>13.20± 0.258</td>
</tr>
<tr>
<td>Chitosan based Fusidic acid cream</td>
<td>12.26 ± 0.307</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ±SEM, n=6

Both control as well as the test group showed considerable increase in rate of % wound contraction and period of epithelization. The mean % wound contraction was found to be better in group treated with chitosan based Fusidic acid cream as compared to the group treated with chitosan non based Fusidic acid cream indicating that chitosan based Fusidic acid cream is better in wound repair.

The process of epithelization was observed depending on the day on which the echar falls. Faster epithelization process is indicated by decreased number of days of falling of echar. The period of epithelization of animals of treated with chitosan based Fusidic acid cream (group 2) was comparable with animals of treated with chitosan non based Fusidic acid cream (group 1).

Murakami K et al (2010) demonstrates the preparation of composite hydrogel sheet produced from blended alginate, chitin/chitosan and fucoidan powders (ACF-HS). It possesses many advantages as a wound dressing for repair of healing-impaired wounds. The application of ACF-HS significantly
stimulated repair of mitomycin C-treated healing-impaired wounds in rats. Thus, ACF-HS is a promising wound dressing for healing-impaired wound repair.

Jayakumar et al (2011) reviewed the recent progress of chitin and chitosan based fibrous materials, hydrogels, membranes, scaffolds and sponges in wound dressing. The fibrous materials based on chitin and its derivatives have the properties of high durability, good biocompatibility, low toxicity, liquid absorption, and antibacterial activity. These properties would lead to accelerate wound healing. Chitosan/collagen membrane could be used to hasten wound healing and induce cell migration and proliferation and antibacterial activity. Polypropylene- collagen-chitosan membrane could be used as a temperature-sensitive material that would function as an automatic release of the dressing material once the wound is healed. Chitin and chitosan-based hydrogels may be considered as an occlusive dressing for wound management due to their ability to accelerate wound contraction and healing.

Inas et al (2012) proved that the biomedical product chitosan was effective for tissue regeneration and showed better and faster tissue epithelization.

The present study revealed the wound healing property of chitosan based pharmaceutical preparations which might be useful in medical applications.

4.1.4. Free radical Scavenging and antioxidant property of chitosan (Invitro study)

The Free radical Scavenging and antioxidant property was evaluated in respect to the following parameters.

a) Scavenging ability of chitosan on 1, 1-diphenyl 1-2-picryl hydroxyl radicals (DPPH)

Scavenging property of chitosan on 1, 1-diphenyl 1-2-picryl hydroxyl radicals (DPPH) was studied and the results were shown in figure 8.

The present study showed highest scavenging ability of chitosan on DPPH radicals was reported at 38.03% at 1mg/ml. The DPPH radical scavenging potential of chitosan ranged from 28.37% to 38.03 at varying concentrations (0.125 to 1mg/ml) and Ascorbic acid, BHA were ranged from 11.77% to 45.10% and 38.17% to 49.03% showed moderate to high scavenging abilities respectively. The present study revealed the moderate scavenging ability of chitosan when compared ascorbic acid and Butylated hydroxyl anisole.
The result of the presented study is in accordance with Yen et al (2008) who has reported that fungal chitosan scavenged DPPH radicals by 28.4-53.5% at 10mg/ml, Yen et al (2007) reported the scavenging ability of crab chitosan C60 on DPPH radicals was 28.4% at 10mg/ml, whereas these of other crab chitosan were in the range of 46.4-52.3%. The scavenging ability of chitosan was 38.03% at 1mg/ml. However, at 1mg/ml, BHA and Ascorbic acid showed scavenging abilities of 49.03% and 45.10% respectively.

b) Hydroxyl radical scavenging ability of chitosan

Scavenging property of chitosan on hydroxyl radicals was studied and the results were shown in figure 9.
The present study showed highest scavenging ability of chitosan on Hydroxyl radicals was reported at 40.1% at 1mg/ml. The Hydroxyl radical scavenging potential of chitosan ranged from 12.2 % to 40.1 at varying concentrations (0.125 to 1mg/ml) and Ascorbic acid ranged from 15% to 45% showed moderate to high scavenging abilities respectively. The chitosan had obvious scavenging activity and exhibited a concentration-dependent inhibition of deoxyribose oxidation.

Figure - 9 Hydroxyl radical Scavenging effects of the chitosan

Chitosan exhibits high inhibition activity on linolenic acid peroxidation, 83.7% activity against hydroxyl radicals Feng T et al 2007. Xang et al. 2005 have found, that all kinds of sulfated chitosans possessed antioxidant activities and free scavenging activities. Thus chitosan and chitosan sulfates with different molecular weight have been reacted with 4-acetamidobenzenesulfony chloride to
obtain sulfanilamide derivatives of various molecule weight. The results have indicated that the sulfanilamide group increased not only the water solubility but also antioxidant activity Zhong et al 2007.

Smith et al (1992) reported that molecules that can inhibit deoxyribose degradation are those that can chelate iron ions and render them inactive or poorly active in a Fenton reaction.

c) Superoxide anion radical scavenging ability of chitosan

The inhibitory effect of the chitosan on superoxide anion radical was studied and the results shown in the figure 10.

Figure - 10 Superoxide anion radical Scavenging effects of the chitosan
The inhibitory effect of the chitosan on superoxide anion radical was ranging from 15.20% to 32.10% for the concentration between 0.125-1 mg/ml. However, the scavenging effect of Ascorbic acid (0.125-1 mg/ml) was found to be higher than the chitosan and the range was from 19.00% to 45.00%.

The present study is similar with Dahl et al (1978) reported that superoxide is a relatively weak oxidant, it decomposes to form stronger reactive oxidative species, such as single oxygen and hydroxyl radicals, which initiate peroxidation of lipids.

Bloknina et al (2003) also demonstrates the Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. It is an initial free radical formed from mitochondrial electron transport system in presence of chitosan.

d) Reducing power of chitosan

The reducing capability of chitosan was assessed based on the measurement of Fe3+-Fe2+ transformation was studied and results shown in figure 11.
The reducing power of chitosan ranging from 15.47% to 19% at 0.125 to 1.0 mg/ml, whereas ascorbic acid ranging from 34.5% to 98.7%. In present study chitosan showed less reducing power when compared with ascorbic acid.

Feng et al., (2008) indicates that the reducing power of different molecular weights of γ-ray treated chitosan has determined by potassium ferricyanide reduction method showed that low molecular weight γ-ray treated chitosan exhibited high reducing power and the reducing power increased with the increases of chitosan concentration.

Ji-young kim et al (2009) based on this study, Chitosan show significant antioxidant activity. Antioxidative properties of the various chitin and chitosan extracts are of great interest in food industry, since their possible use as natural
additives emerged from a growing tendency to replace synthetic antioxidants by natural ones. Owing to its excellent protective features exhibited in antioxidant activity tests the chitin and chitosan extracts from the crustacean species could be concluded as a natural source that can be freely used in the food industry. This study identifies opportunities to develop value added products from crustacean processing by products with biological activity such as antioxidant properties.

Natthan Charernsriwilaiwat et al 2012 indicated that chitosan salts possess varying levels of antioxidant and free radical scavenging activities, including superoxide and hydroxyl radicals scavenging, metal ion chelating activity and reducing power.

The result of the phase I study demonstrates film forming ability, antimicrobial property, wound healing, antioxidant and free radical scavenging activities of chitosan.

4.2. PHASE II – Screening of invtro antiproliferative efficacy of chitosan in T 24 Urinary bladder cancer.

4.2.1. Inhibitory effect on proliferation of T24 cells (MTT assay)

The effect of Chitosan and cyclophosphamide (anticancer drug used as standard) on the cells viability was measured by the MTT assay, which reflects
the cellular reducing activity and the results were shown in table -7 and figure – 12&13.

Figure - 12 Comparative study of cell viability inhibition by chitosan and cyclophosphamide on T24 cell line

The present study indicated that chitosan and cyclophosphamide inhibited the T24 cells proliferation in a concentration and dose dependent manner. The median lethal concentration of Chitosan and cyclophosphamide was 62.5 µg/ml for T24 at 48h. After 48 hours the chitosan inhibit the cell viability was found to be 52.7 % and Cyclophosphamide 47.1 % respectively.
Table - 7 Comparative study of cytotoxic efficacy of Chitosan and Cyclophosphamide on T-24 cell line

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (µg/ml)</th>
<th>Chitosan</th>
<th>Cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Absorbance (O.D)</td>
<td>Cell viability (%)</td>
</tr>
<tr>
<td>1</td>
<td>1000</td>
<td>0.02</td>
<td>3.9</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>0.10</td>
<td>19.6</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>0.19</td>
<td>37.2</td>
</tr>
<tr>
<td>4</td>
<td>125</td>
<td>0.23</td>
<td>45.0</td>
</tr>
<tr>
<td>5</td>
<td>62.5</td>
<td>0.27</td>
<td>52.9</td>
</tr>
<tr>
<td>6</td>
<td>31.2</td>
<td>0.34</td>
<td>66.6</td>
</tr>
<tr>
<td>7</td>
<td>15.6</td>
<td>0.39</td>
<td>76.4</td>
</tr>
<tr>
<td>8</td>
<td>7.8</td>
<td>0.42</td>
<td>82.3</td>
</tr>
<tr>
<td>9</td>
<td>Cell control</td>
<td>0.51</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure - 13 Anticancer effects of Chitosan and cyclophosphamide on T-24 cell line

(a) Normal T24 cell line

(b) T24 cells treated with 1000 µg/ml of chitosan
(c) T24 cells treated with 125 µg/ml of chitosan
(d) T24 cells treated with 62.5 µg/ml of chitosan

(e) T24 cells treated with 31.2 µg/ml of chitosan
(f) T24 cells treated with 1000 µg/ml of cyclophosphamide
(g) T24 cells treated with 125 µg/ml of cyclophosphamide

(h) T24 cells treated with 62.5 µg/ml of cyclophosphamide
(i) T24 cells treated with 31.2 µg/ml of cyclophosphamide

Kim SK and Rajapakase, (2005) indicates the antitumor activity of chitosan has been claimed by inhibition of the growth of tumor cells mainly due to an immune stimulation effect. Jeon and Kim, (2002) have found that chitosan oligomers possess antitumor activities tested both in vitro and in vivo. Studies carried out using mice that had ingested low-Mw chitosan revealed significant antimetastatic effects of chitosan against Lewis lung carcinoma. Partially deacetylated chitin as well as chitin with a carboxymethyl group have also been effective to demote tumor progression Tsukada et al, 1990.

Ueno et al, (2001) studied the effect of chitosan on tumor growth and metastasis. The activation of macrophages by chitosan is suggested to mediate its antitumor effects in vivo, while its angiogenic inducing properties may be the harmful effects of chitosan, such as promotion of tumor growth and invasion.
Theresa et al, (2006) studied that chitosan treatment decreases SK-OV-3 cell proliferation and causes morphological changes in this cell line. Jiang et al 2011 proved that modified chitosan significantly inhibited cell proliferation, induced apoptosis in MCF-7 cell line.

Zakir Hossain And Koretaro Takahashi, (2008) demonstrated that modified chitosan significantly inhibited cell proliferation, induced apoptosis in HT-29 cell line.

4.2.2. Apoptosis by DNA fragmentation study

DNA was extracted from cultured T24 cells treated with chitosan, the occurrence of necrosis by agarose gel electrophoresis. The results was shown in figure – 16.

From the figure – 16, The lane 3, 4 and 5 (T24 cells treated with chitosan in different concentrations) of DNA fragmentation study revealed more degree of fragmentation when compared to lane 2 (control).

The result of the present study demonstrsted that chitosan induces DNA fragmentation in T24 cell line, which correlated with necrotic tumour cell death due to disruption of cell membrane.
Figure – 14 DNA fragmentation of chitosan on T24 cell line

1  2  3  4  5

agarose gel electrophoretic analysis of DNA isolated from 24 cells treated with chitosan 125µg/ml (lane 3), 62.5µg/ml (lane 4), 31.2µg/ml (lane 5), Marker (lane 1) and control (lane 2).

The present study is correlated with Lifeng et al, (2007) who showed that chitosan nanoparticles mainly induce the necrotic cell death resulting from the disruption of cell membrane in the treated with hepatocellular carcinoma cell line.

Lifeng et al, (2005) reported that chitosan nanoparticles mainly induce the necrotic cell death resulting from the disruption of cell membrane in the treated with human gastric carcinoma cell line.

The present study exemplify that chitosan could arrest growth of tumor cells.
4.2.3. Cell cycle distribution analysis:

The present study to evaluate the effects of chitosan on cell cycle progression in T24 cells were determined by flow cytometry and the results was shown in figure – 15.

Figure - 15 Analysis of cell cycle distribution and expression levels of cell related protein in T24 cells treated with chitosan.
Chitosan induced effects were detected by comparing the cell cycle profiles between the chitosan - treated and untreated cells. The present investigation demonstrated a significant decrease of cell in the Go / G1 Phase. Apoptotic peaks were observed and cell apoptotic incidence increased in a dose dependent manner after chitosan treatment.

The present study is in accordance with the report of Lifeng et al, (2007) who demonstrated the cell cycle progression and apoptotic incidence in human gastric carcinoma cell line.


4.3. PHASE III – Screening of in vitro antitumor efficacy of chitosan in benzidine induced bladder cancer in Swiss albino mice.

4.3.1. Lipid Peroxidation

Lipid peroxidation is viewed as a complicated biochemical reaction involving free radicals, oxygen, metal ions and a host of other factors in the
biological system. The levels of TBARS in different experimental group of mice is depicted in table 7 and figure 16.

Figure – 16 Effect of chitosan on TBARS in different experimental groups of animals in benzidine induced bladder cancer

![Graph showing effect of chitosan on TBARS in different experimental groups of animals in benzidine induced bladder cancer]

Table - 7 Effect of Chitosan on enzymic and nonenzymic antioxidants in benzidine induced bladder cancer in albino mice

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameters</th>
<th>UNIT</th>
<th>GROUP I</th>
<th>GROUP II</th>
<th>GROUP III</th>
<th>GROUP IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Super oxide dismutase</td>
<td>Units/mg protein</td>
<td>1.1±0.05a</td>
<td>0.6±0.04c</td>
<td>0.9±0.04b</td>
<td>1.03±0.02ab</td>
</tr>
<tr>
<td>2</td>
<td>Catalase</td>
<td>Units/mg protein</td>
<td>21.7±0.13a</td>
<td>15.2±0.15e</td>
<td>21.4±0.11c</td>
<td>21.58±0.06b</td>
</tr>
<tr>
<td>3</td>
<td>Glutathione peroxidase</td>
<td>µg/mg protein</td>
<td>6.3±0.08a</td>
<td>4.14±0.07e</td>
<td>5.92±0.06c</td>
<td>6.33±0.19b</td>
</tr>
<tr>
<td>4</td>
<td>Reduced glutathione</td>
<td>n moles/gm</td>
<td>8.4±0.27a</td>
<td>4.44±0.15d</td>
<td>7.25±0.11c</td>
<td>8.36±0.19b</td>
</tr>
<tr>
<td>5</td>
<td>Vitamin C</td>
<td>µg/gm</td>
<td>1.1±0.02a</td>
<td>0.72±0.04e</td>
<td>1.03±0.02c</td>
<td>1.13±0.09b</td>
</tr>
<tr>
<td>7</td>
<td>Vitamin E</td>
<td>µg/gm</td>
<td>2.61±0.13a</td>
<td>1.22±0.13e</td>
<td>2.11±0.07c</td>
<td>2.62±0.01b</td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples in each group. Row means followed by different superscript letter are significant at 1% level.
In animals treated with benzidine (Group II), there was a significant increase (P<0.01) in the level of thiobarbituric acid reactive substances (TBARS) in bladder tissue sample as compared to normal control (Group I).

Kim and Thomas, (2007) demonstrated the free radical scavenging activity of chitosan eliminate various free radicals by action of nitrogen on the C-2 position by employing the 2 thiobarbituric acid reactive substances (TBARS) and 2,2-diphenyl-picrylhydrazyl (DPPH) scavenging assays.

Je et al, (2004) reported the antioxidant activity of chitooligosaccharides based on scavenging potency on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and found that chitooligosaccharides have an antioxidant activity. Xie et al, (2001) revealed the role of NH$_2$ group in chitosan in the process of free radical scavenging abilities.

Rise in TBARS is considered to be indicator of the onset of oxidative stress from reduced species of molecular oxygen including H$_2$O$_2$, superoxide radical and reactive hydrogen radical (Anilkumar et al, 2002).

In animals treated with chitosan (group III) there was a significant decrease of TBARS when compared to group II animals treated with benzidine. This showed antiproliferative properties of chitosan.

Involvement of iron in the lipid peroxidation both invivo and invitro has been well demonstrated. Since lipids constitute nearly 60% of the components in
biomembranes any major perturbation is bound to affect structure and function of the cell. In recent years, lipids and their derivatives have been recognized as important molecules in signal transduction and lipid peroxidation is the focus of intense research in relation to its possible involvement in health and disease (Arbogast et al, 1984; Ramachandran et al, 2002).

The activities of enzymic antioxidants superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) of different groups of animals shown in table and figure

In animals treated with benzidine (group II), there was a significant decrease in the level of these enzymes as compared to normal control (group I). The decreased activity of these antiperoxidative enzymes superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) suggested increase lipid peroxidation as a result of uncontrolled generation of partially reduced oxygen (Jaya et al, 1993).

Superoxide dismutase (SOD) mainly by quenching of superoxide (\(O_2^-\)), an active oxygen radical produced in different aerobic metabolism. Catalase is a tetrametric enzyme, present in most of the cells and acts by catalyzing, the decomposition of \(H_2O_2\) to water and oxygen.

Glutathione peroxidase (GPx) is a selenium containing enzyme which catalyzing the reduction of \(H_2O_2\) and lipid hydroperoxide (\(LO_2H\), generated
during lipid peroxidation to water using reduced glutathione as substrate (Irshad and chaudhuri, 2003).

We found no significant difference (P<0.01) in the level of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in animals treated with chitosan (group IV) and normal control (group I).

The administration of chitosan in benzidine induced animals increased significantly (P<0.01) the level of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as compared to group II animals induced with benzidine alone.

The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) constitute a mutually supportive team of defence against lipid peroxidation (LPO) (Bandyopadhyay and Banerjee, 1999).

In animals treated with benzidine (group II), there was a significant decrease in the level of Glutathione, Vitamin E and Vitamin C as compared to normal control (group I). The decreased activity of these antiperoxidative agents Vitamin C and Vitamin E suggested increase lipid peroxidation as a result of uncontrolled generation of partially reduced oxygen (Jaya et al, 1993).

We found no significant difference (P<0.01) in the level of Glutathione, Vitamin E and Vitamin C in animals treated with chitosan (group IV) and normal control (group I).
The administration of chitosan in benzidine induced animals increased significantly (P<0.01) the level of Glutathione, Vitamin E and Vitamin C as compared to group II animals induced with benzidine alone.

Reduced glutathione (GSH) is the major thiol which binds with electrophilic molecular species and free radical intermediates (Ketterer et al, 1983). It plays a central role in the antioxidant defense system, metabolism and detoxification of exogenous and endogenous substances. (Meister and Anterson, 1983).

GSH is considered to be a crucial factor in maintaining the structural integrity of cell membrane largely through reactions that protects membranes against free radical formation (Cadenas, 1985). GSH has long been reported to be essential for recycling of antioxidants like Vitamin E and Vitamin C (Constantinescu et al, 1993; Mrtensson and Meister, 1991).

Decrease in the level of Vitamin E and Vitamin C might be due to excessive destruction of tocopherol by free radicals or due to decrease in the levels of ascorbic acid, since vitamin C and Vitamin E are synergistic antioxidants (Infers and sies, 1988).

Vitamin C is considered as the most important antioxidant in extracellular fluids that can completely protect lipids from detectable peroxidative damage induce by aqueous peroxyl radical. Vitamin C acts as a co antioxidant by
regenerating α-tocopherol from α-tocopheroxyl radical produced during scavenging of reactive oxygen species (Ray and Gibananda, 2002).

Vitamin E is the most important lipid soluble antioxidant and used to scavenge free radicals and to stabilize cell membrane (Burton and Traber, 1990). Vitamin E can directly scavenge reactive oxygen species. It is the major lipid soluble antioxidant present in all cellular membranes which protects against lipid peroxidation (Kimmick et al, 1997).

The findings of the present study may be attributed the free radical scavenging activity of chitosan as reported Carla Silva et al, 2011; Senthilkumar and Jeyapraksah, 2012).

The above findings unveil the free radical scavenging and antioxidant effects of chitosan in benzidine induced bladder cancer in mice.

3.3.3. HISTOPATHOLOGICAL OBSERVATIONS IN BLADDER TISSUE

**Group I : Normal control**

Bladder mucosa showed 2 to 5 layers of transitional epithelial cells. The muscularis propria appears normal (figure a)

**Group II : Benzidine treated mice**

Bladder mucosa were thrown into papillary folds. The transitional epithelial cells of the focal areas the lining epithelium was 7 to 8 cells in thickness. It showed the proliferation of the cells by induced benzidine. (figure b)
Group III : Chitosan + Benzidine treated mice

Bladder mucosa showed 2 to 5 layers of transitional epithelial cells. The muscularis propria appears normal. There was no obvious proliferation occurs. (figure c)

Group IV : Chitosan treated mice

Bladder mucosa showed 2 to 5 layers of transitional epithelial cells. The muscularis propria appears normal. (figure d)
Figure – 17 Histopathology of bladder tissue of animal groups.

(a) Normal Bladder cells
(b) Bladder of mice treated with benzidine
(c) Bladder of mice treated by chitosan
(d) Bladder of mice treated by chitosan alone