Abstract
Antioxidant effects of Chitosan nano-green tea on hepatic fibrosis was studied ultrastructurally with detailed quantification comparing the percentage differences between the damaged done by CCl4 and ethanol effects and the rehabilitation by the use of Chitosan nano-green tea's antioxidant effect. Especially in demolishing the damaging effects of CCl4 and ethanol on both the cell cytoplasm and the ECM. Chitosan nano-green tea exhibited several beneficial activities. Previously, we briefly reported that Chitosan nano-green tea completely demolishes hepatofibrosis in experimental models, but it was a short account and on one selected area. However, in this report we try to give an extensive elaboration on such effect and some details regarding the various aspects of cytoplasm and organelles as well as the ECM part. The 200 to 250 nm sized chitosan encapsulated GTE particles were used targeting rat liver fibrosis after being treated with CCl4 and ethanol doses for three weeks. Our data indicates that chitosan nano-GTE induced a great change in demolishing the ECM protein fibrous materials and had left the area extremely smooth and clean. Damaged cell organelles were back to normal appearance and functions, cell cytoplasm damaged parts were highly healed up, parenchyma ECM were close to normalization with gentle removal of protein fibers which led to the smoothness of the field. This identification may explain the multiple therapeutic and anti-fibrotic activities of the nano-GTE.

Keywords: Chitosan nano-green tea; Hepatocytes; Cells; Ultrastructure

Introduction
Carbon tetrachloride CCl4 and ethanol are known to induce hepatic fibrosis in rats [1] that is similar in pathophysiology to viral hepatitis and drug-induced hepatitis in humans [2,3]. Hepatic fibrosis is a common response to chronic liver injury from many causes including alcohol and viral infection [4], CCl4, and ethanol [1]. This eventually leads to cirrhosis [5] which is often associated with a high risk of hepatocellular carcinoma [6,7]. In rats, dual treatment of CCl4 and ethanol induces liver fibrosis in just three weeks causes huge damage to hepatocytes organelles and fibrotic ECM. This has been observed in some details ultrastructurally reflecting an early phase of liver fibrosis. Previously, and in several published works we showed that the oral administration of catechin rich chitosan nano-GTE [8,9] restored levels of several liver markers and structures in rats [1]. In this work, we examined the possible anti-fibrotic effect of nano-green tea on hepatic fibrosis ultrastructurally by observing changes in the status of cell organelles and fibrotic ECM and analyzed with ImageJ software.

Materials and Methods

Group of animals under investigation
Fifty male Spargue-Dawley rats weighing 200-250 g were used in this study. They were divided into five groups according to Table 1. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.” (Standard letter of the ethical committee is enclosed).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatments</th>
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<tbody>
<tr>
<td>Control</td>
<td>GI Given Food and water ad-libitum</td>
</tr>
<tr>
<td>CCl4, Ethanol</td>
<td>GII Subcutaneous injection of 40% CCl4 followed by oral dose of 25% ethanol daily, for 3 weeks</td>
</tr>
<tr>
<td>CCl4, Ethanol-Chitosan</td>
<td>GIII Subcutaneous injection of 40% CCl4 followed by oral dose of 25% ethanol daily, for 3 weeks, then orally given chitosan alone daily for 25 days</td>
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<tr>
<td>CCl4, Ethanol-Chitosan nano-GTE</td>
<td>GIV Subcutaneous injection of 40% CCl4 followed by oral dose of 25% ethanol daily, for 3 weeks, then orally given chitosan Nano GTE daily for 25 days</td>
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<tr>
<td>Chitosan nano-GTE</td>
<td>GV Orally given Chitosan nano-GTE for 25 days</td>
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Preparation of Green Tea Extract (GTE)
Dried green tea leaves (Camellia sinensis) [100 g, Japanese-origin] were powdered in a Waring blender and extracted with double distilled water (1 L) at 85°C for 1 hour. The extract was...
filtered through a Nylon filter, and the filtrate was centrifuged at 3000g for 15 min. The clear supernatant was removed and the residual pellet was shaken with distilled water, warmed at 35°C, and centrifuged again. The supernatant was pooled, lyophilized, and the resulting material was stored at -20°C in a screw-capped bottle [6].

### Preparation of chitosan encapsulated nano-Green Tea Extract (GTE)

Synthesis of Chitosan encapsulating green tea extract (GTE) Chitosan nanoparticles were synthesized using the ionic gelation technique with pentasodium tripolyphosphate (TPP) as crosslinking agent. 9.5 ml of de-ionized water 500ul of chitosan (20mg/ml) 100ul of green tea extract (GTE; 5mg/ml) is added and stir for about an hour. After 1 hr of stirring, 100ul of TPP (10mg/ml) was added drop by drop with constant stirring. The entire solution was then sonicated for about 30second using a probe sonicator and allowed to stir for another 2 hrs (approx). After 2hrs of stirring the size of the nanoparticles was measured.

### Preparation of samples for Ultrastructural Studies

Liver samples from the four groups were fixed in 2.5% glutaraldehyde/sodium cacodylate fixative pH7.2 at 0-4°C. For two hours, then changed to a fresh fixative and left overnight. Tissues were then transferred to sodium cacodylate/sucrose buffer three changes, 20 minutes each. Then to 1% OsO4/PO4 buffer for 2 hours, and blocked in Epon in the usual manner. Semithin sections were cut and the desired areas were selected for ultrathin sections. Images were taken using Jeol 1200 EXII electron microscope operated at 80kV.

### Quantification of images by imageJ software

Area of ultrastructure images from defected versus normal regions of the cells from the five groups were carefully selected after treatment with different chemicals were carried out with the imageJ software package showing the threshold and the percentage of damages of each image has been carefully determined.

### Results

The ultrastructural observations of the five groups of rats under study showed distinct changes in the structure of hepatocyte cytoplasm in the form of patches or moth-eaten phenomenon as well as damaged to its organelles such as mitochondria, lysosomes and the endoplasmic reticulum (ER), both (rER and sER) as distinct from normal control group (GI) to the damaged group (GII) due to CCl4+ethanol (Figure 1A-3A) and with some improvements as in CCl4+ethanol and chitosan (GIII) with patches and several vacuoles still remaining (Figure 4A & 5A). While the magnitude of damage in these cells became much less in the G IV where CCl4+ethanol and chitosan nano-GTE were used under same conditions (Figure 6A). In many locations when examined at higher magnification cells cytoplasm together with their organelles appeared normal and healthy (Figure 7A). It’s clear that the impact of CCl4+ethanol (GII) was big; many profiles of mitochondria are either not shown or completely damaged, lysosomes were completely damaged, and the ER profiles both sER and rER were either misplaced or damaged or accumulated in a compacted manner. Vacuoles of various forms and shapes and sizes are phenomenon in the damaged cells (Figure 2A & 3A) and to a less extent in GIII when chitosan was included (Figure 4A & 5A), while in GV when CCl4+ethanol and chitosan nano-GTE was used, most organelles and cytoplasmic components were normal and no signs of such vacuoles [Figure 6A & 7A]. The amount of collagen fibers in the extracellular matrix was also enormously increased as compared to the normal control rats. In GV where chitosan nano-GTE was used the status was somehow similar to GI and GV.

**Figures 1-12 for groups (GI, GII, GIII, GIV, GV)**

A. TEM source images showing distribution of hepatocytes organelles and other cytoplasmic structures. B. same images as in A showing threshold/filtered with imageJ in order to differentiate the damaged (Red) from the undamaged regions (Blue). C. measurements of damaged and undamaged regions under study by imageJ analysis. D. Pie charts showing statistical percentage of damaged and undamaged regions in the five groups.

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Remediation of hepatic fibrosis as a result of the use of CCl4 and Ethanol by Chitosan Nano-Green Tea Extract: Quantification and Ultrastructural Studies

In all the groups (GI to GV), each group is formed of four subdivisions (A,B,C,D) as original or imagesource, threshold/filtered image, statistical analysis and pie chart. (Figure 1-9). In normal control group (GI) areas of damaged regions of the cells after treatment with CCl4 and Ethanol (GII) were carried out primarily with the software package ImageJ. The damaged regions were in the form of white patches inside the cell cytoplasm in the image source (Figure 1A), while red colour in the threshold/filtered image (Figure 1B) The degree of the damage was evaluated and confirmed by the image analysis (Figure 2C & 3D). In this aspect it shows marked differences between the group of rats their liver damaged with the dual effect of CCl4 and ethanol and the one given CCl4 and ethanol in chitosan (GIII) with the fourth group given CCl4, ethanol and nano-GTE in chitosan (GIV) and lastly in non-exposed animals as expressed by the statistical significance achieved for each group (Figure 1C-5C). During the measurements of the area of the cells, both the normal and damaged areas were calculated. The differences in the degree of damage were confirmed by ImageJ software analysis (Figure 2C & 3D).

**Image analysis by ImageJ software**

In all the groups (GI to GV), each group is formed of four subdivisions (A,B,C,D) as original or imagesource, threshold/filtered image, statistical analysis and pie chart. (Figure 1-9). In normal control group (GI) areas of damaged regions of the cells after treatment with CCl4 and Ethanol (GII) were carried out primarily with the software package ImageJ. The damaged regions were in the form of white patches inside the cell cytoplasm in the image source (Figure 1A), while red colour in the threshold/filtered image (Figure 1B) The degree of the damage was evaluated and confirmed by the image analysis (Figure 2C & 3D). In this aspect it shows marked differences between the group of rats their liver damaged with the dual effect of CCl4 and ethanol and the one given CCl4 and ethanol in chitosan (GIII) with the fourth group given CCl4, ethanol and nano-GTE in chitosan (GIV) and lastly in non-exposed animals as expressed by the statistical significance achieved for each group (Figure 1C-5C). During the measurements of the area of the cells, both the normal and damaged areas were calculated. The differences in the degree of damage were confirmed by ImageJ software analysis (Figure 2C & 3D).
damaged regions were measured in pixels and the results were put in percentage form after performing threshold and filtering of each image source (Figure 1B-7B).

At the beginning of ImageJ analysis, ‘Threshold Color’ for each image was selected. This is to make it suitable to cover the desired regions during the measurements. The first threshold selection was made in a manner that a number of cells was encapsulated and then selected using the ‘Select’ tab in the ‘Threshold color’ toolbox followed by using the ‘Measure’ option in ‘Analyse’. This led to the area measurement of the complete cells. The threshold was then subsequently changed to a point that only the damaged area of the cell was selected. This selection was subsequently judged by flipping between the original image source and the selected image for attaining the threshold. Once the final threshold selection was made, the ‘Select’ tab was used to choose the damaged region and the measurement of the area was made (as described earlier). The ratio of the measured damaged area and the whole cell, multiplied by 100 gave the percentage damage the cells had suffered under circumstances, as explained earlier.

The measurements were started with control cells completely undamaged except some minor white patches usually present in normal situations (Figure 1A). These white patches represent only ~10% of the total cell area. The average grey scale value of pixels at the different regions of the healthy cells was also estimated to reached (150±10). Then the percentage of the damage in the cells treated under different conditions was determined. The cut-off threshold value measured by ImageJ for healthy cells and damaged ones and the threshold value measured for healthy from damaged areas above which, the portions of the cells were found to be damaged, was approximately in the same vicinity. The damage was extreme in CCl₄ and Ethanol group (%29), especially regions with partial damage were widespread and at threshold (estimated from healthy cells) many partially regions were not taken into account during the area measurements. Thus a lower threshold or mostly threshold at the midpoint was used in all groups (Figure 1-9).

The results obtained for the cytoplasmic damaged ratio for each group are shown in the figures and summarized in the normal histogram and the combining 2D histogram (Figs 10). The percent of damage decreased from the fully damaged to control GI with ratios 5.3%, GII 29.7% GIII 34.7% GIV 23.2 to GV 6.8% respectively (Figure 10). The application of the ImageJ analysis to the ultrastructural analysis by TEM confirmed the ratios of damaged beyond the near naked eye observation.

Figure 9: A. Image of hepatocytes of Nano GT chitosan treated (GV) showing more details of cells with normal structure X 5000. B. Threshold/filtered image showing damaged and undamaged areas. C. and D. statistical analysis and pie chart showing the percentage of damaged area 24% damaged and 76% undamaged areas respectively.

Discussion

Enormous number of research papers show that green tea is thought to boost the immune system, elevate energy levels, and get rid of toxins in the body. It also provides a ‘helping hand’ to the body over its battle with cancer [20-22] and neurodegenerative diseases like Alzheimer’s disease (AD) and Parkinson’s disease (PD) help in protection against hydrogen peroxide induced toxicity, mitochondrial protection from amyloid toxicity [14]. Our previous work showed that green tea extract was effective in
modulating the toxic effects of certain drugs like reserpine [9,15]. Since oxidative stress (ROS and NOS) play a major role in aging and neurodegenerative diseases [13], an increase in longevity has been anticipated due to polyphenols such as epigallocatechin-3-gallate (EGCG), present in green tea which improves thiol content, decrease carbonylated proteins and TNF-alpha, with an increase in mitochondrial membrane potential in individuals [1].

Some laboratory studies have shown that extracts from green tea can stop cancer cells from growing [10-12]. The antioxidant properties of polyphenols in green tea play a big role in fighting or preventing the risk of developing mouth cancer, lung cancer, bladder cancer, and many others [13]. It is, however, also known that intake of excessive green tea has some side effects on the human body. It will be worthwhile to carry out quantitative analysis of the kind explained in this work on cancerous cells and estimate the repair in them. However, chitosan nano-GTE from our laboratory has shown more refined recovery in the remediation of hepatic fibrosis in a rat model in one of our latest published work we briefly reported on the quantification of the healing effect in hepatic fibrosis induced by chitosan nano-encapsulated green tea in rat both ultrastructurally and in a 3D SEM in order to serve certain point [8]. In this work, its clearly realized a full report study showing various kinds of damages in the hepatocyte cytoplasm as well as the cell-to-cell relationship. The images shown in Figure 1 A-D are typical representatives of the different subdivisions in each group of cells after different treatments described in Table1. The results obtained by the image software computational method to discriminate the damaged and undamaged areas were in close agreement, thus indicating that the threshold selections for the measurement of the desired regions were reliable. However, it was noted that selection of the appropriate threshold encompassing the damaged regions of a cell in this manner can still be considered subjective. To avoid this, the measurements and the calculations were carried out on multiple cells belonging to each group to validate our results. The quantification process employed in this work has another advantage; not all the cells were of the same size and damages within various organelles. The exact area measurements would not tell which cell had more damage, thus making the calculation complicated. The percentage ratio of the damaged to undamaged area and of the damaged to the whole cell area provided a clear picture in simple terms for deriving the results. It was found that chitosan nano-GTE effectively healed ~25% of the damaged cytoplasmic regions in the cells [8]. This is the second report of quantification of the healing effect of chitosan nano-GTE in hepatic fibrosis cell after our previous published report in a series of publications [8,16]. Chitosan nano-GTE was found to have a good beneficial effect on the remediation of hepatic fibrosis against the damaging effect on the liver due to CCl4 and ethanol as oxidizing agents. When compared with normal healthy cells; the “white patches” in GI1 that indicated the damaged portions covered nearly 52% of the total cell area under consideration. The effect of chitosan along with CCl4 and ethanol barely improved the damaged cell conditions 48%. The results indicate that about 3% repair of the damaged regions of the cells was possible with such a treatment i.e. with chitosan on its own, which was not a significant improvement. Interestingly, the percentage of the cellular damage decreased significantly from a maximum of 52% (GI1) to 28% (GIV) due to the effect of chitosan nano-GTE when added to CCl4+ethanol and administered simultaneously. Therefore registering a repair of approximately 25% of the cell area when compared to that of CCl4+ethanol (GII). Cell cytoplasm manifested its improvement; moth eaten occurrence disappeared, ER profiles were back to its normal locations and distribution, mitochondria retained their normal shape and distribution as well as the lysosomes. Cell boundaries had retained to their normal shape and width after being widened due to the effect of CCl4+ethanol dual action [17]. Although the widening of cell foundries and the ECM protein fibers had been fully discussed in one of our earlier publication [16-18]. This indicates that chitosan nano-GTE bio-nano particles have a significant positive impact when used as a therapeutic remedy for hepatic fibrosis and can be considered as a potential medicine for the treatment of the disease. It is strongly felt that such studies will prove pivotal in the development of potential drugs against hepatic fibrosis and cancers that originate due to cell damage [16,17]. From the observations of this work together with the information gained from our previously published works it can be confirmed that the healing effects of chitosan nano-GTE on hepatic fibrosis recovery seems to be genuine and promising.

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Ethical Approval
All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.” (standard letter of the ethical committee is enclosed).

References


