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Injectable chitosan- *Ulva lactuca* polysaccharide hydrogel neutralizes oxidative stress in experimental diabetic neuropathy

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Abstract

Polysaccharide –based hydro gels from marine algae are attractive materials for biomedical applications which include polyfunctionality and biodegradability. There were reports of many algae based hydrogels and their applications in diabetic neuropathy. The present study aims to investigate the progressive effects of injectable Chitosan-*Ulva lactuca* polysaccharide hydrogel (C-UL hydrogel) on experimentally induced diabetic neuropathy in mice. Physicochemical properties of C-UL hydrogel were analyzed by proximate composition, FTIR, SEM, and swelling properties. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg). C-UL hydrogel treatment was started 15 days after diabetes induction and continued for 30 days. Serum levels of cholesterol profile and insulin were assessed. In liver, brain, and pancreas tissues, antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) were measured. In the liver, brain and pancreas tissues, C-UL hydrogel attenuated diabetes-induced changes in serum antioxidants and cholesterol excluding insulin. Diabetes-induced dysregulated levels of SOD, CAT, and GST were ameliorated by C-UL hydrogel. Histopathological analysis shows that C-UL hydrogel corrects the altered neuronal changes in diabetic animals. C-UL hydrogel prevents brain damage by regulating the antioxidant pathways in the mice model.

Keywords: Chitosan- *U. lactuca* polysaccharide hydrogel; Antioxidants; Diabetes mellitus; Neuropathy; Oxidative stress

1. Introduction

Neuropathic syndromes are the most common complication of uncontrolled diabetes mellitus (DM), both type I and II can lead the way to severe impairment of the central nervous system (CNS) and peripheral nervous system (PNS) [1]. These hostile conditions are functionally related to the metabolic abnormality in glucose concentration, accumulation of sorbitol, increased glycosylated proteins, imbalanced oxidative/antioxidant balance within the CNS and PNS [2].

In recent years, there has been an interest in understanding the key role of oxidative stress, which is a key player in the pathophysiology of diabetic neuropathy development [3]. An increased reactive oxygen species (ROS) exhibits lethal effects on any biological molecules [4]. Over time, the brain is more vulnerable to oxidative stress associated with diabetes [5]. Recent investigation has scientifically proven that antioxidant compounds Figureht is against ROS- induced neuronal injury in CNS and PNS.

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Chitosan is a natural cationic polysaccharide [6], it has been briefly employed in industrial and biological applications due to its biocompatibility and biodegradability [7]. Several studies have been performed on the various chitosan and its derivatives for its antidiabetic and neuroprotective applications [8]. Besides, chitosan and its derivatives, there are only a few studies on chitosan-based hydrogel for its antidiabetic and neuroprotective application [9]. Reported that the addition of solvent under constant stirring produces an injectable chitosan hydrogel, application of chitosan hydrogel in antidiabetic activity was investigated and the result showed the chitosan hydrogel was an effective agent for controlling diabetic condition [10].

Sulfated polysaccharide is a natural product of the *U. lactuca,* It has been widely used, especially in Asian populations have long used *U. lactuca* as both food and medicine [11]. Recent studies have shown *U. lactuca* could significantly regulate the diabetic condition and enhance the regulation by inhibiting key enzymes [12]. However; the poor water solubility of *U. lactuca* polysaccharide had limited its further applications. To overcome these issues, several strategies including nanoparticles, beads, hydrogels, etc., were developed to improve the bioavailability of *U. lactuca* polysaccharide.

In the present study, we combined using chitosan hydrogel with *U. lactuca* polysaccharide to develop a C-UL hydrogel and investigate its neuroprotective potency in experimental diabetic conditions. The administration of C-UL hydrogel might speed up the neuroprotection by modulating the activation of antioxidants due to its great capability in antioxidant applications.

2. Material and methods

2.1. Chemicals and Kits

Chitosan (9012-76-4) and Streptozotocin (18883-66-4) were purchased from Himedia laboratories. Insulin (Actrapid Insulin, Neutral 40) were purchased from a pharmaceutical company, Bangalore, India.

2.2. Extraction of *U. lactuca* polysaccharide

U. lactuca was collected from mandapam coast of south India. *U. lactuca* was cleaned with distilled water to remove impurities without damaging and dried at room temperature.

The method of sulfated polysaccharide isolation from *U. lactuca* was according to the method described by Kolsi *et al.*,[13] with minor modifications. 60g of dried algal powder was depigmented with acetone for 3h and again depigmented with ethanol for 1h followed by 2% HCL extraction at 60°C for 5h. Collected extraction was filtered through Whatman No.3 filter paper; extraction was concentrated to 1/4 of the original volume and precipitated with five volumes of 98% ethanol for 3 days at 4°C. The precipitate was collected by centrifugation at 10,000 for 20 minutes and washed three times with ethanol and lyophilized to get a dried crude *U. lactuca* polysaccharide.

2.3. Determination yield

The yield of crude *U. lactuca* polysaccharide was determined by taking the dry weight of macro-algae before treatment and dry weight of isolated polysaccharide.

2.4. Chemical composition of *U. lactuca* polysaccharide

2.4.1. Estimation of Protein content

Protein content was estimated using the method of Bradford [14]. Various concentrations of standard and polysaccharide were added to test tubes and the volume was made up to 1ml with distilled water. To each tube, 3ml of Bradford reagent (50ml of Coomassie brilliant blue G250 in 95% ethanol and 100ml of 85% phosphoric acid in 1000ml of distilled water) was added and incubated at room temperature for 10 minutes. The absorbance was read at 595nm and the protein content of *U. lactuca* polysaccharide was determined from the standard graph.

2.4.2. Sulfate groups

Sulfate content was determined by the benzidine method [15] using sodium sulfate as the standard. Various concentrations (20, 40, 60, 80, 100µg/ml) of standard and sample were dissolved in 25% formic acid at a concentration of 1mg/ml and 1ml of each was transferred to a fresh tube. To each tube, 1ml of glacial acetic acid, 3ml of acetone/ethanol (1:1) and 1ml of benzidine (0.5% in 94% ethanol) were added and left overnight at 4ºC. The reaction mixture was centrifuged at 5000g for 30 minutes and the pellet was washed in acetone/ethanol (1:1) and resuspended

in 1.5ml of 1M. The tubes were shaken well and allowed to stand at room temperature for 30 minutes followed by the addition of 1ml of distilled water and 0.5ml of 0.1M sodium nitrite. After 5 minutes, 2.5ml thymol solution (0.5% in 2M NaOH) was added and the absorbance was read at 505nm.

2.4.3. Estimation of Uronic acid

The glucuronic acid assay was performed with the previously described method Cesaretti *et al.,* [16]. The polysaccharide was dissolved in distilled water at a concentration of 1mg/ml. D-glucuronic acid lactone was used as the standard. Various concentrations of the standard (10, 20, 30, 40, 50µg) and the polysaccharide (50µg) were added to separate wells in a microtitre plate and the volume was made up to 50µl with distilled water. To each well, 200µl of sodium tetraborate (25mM in sulfuric acid) was added and incubated for 10 minutes at 100ºC. After cooling, 50µl of carbazole (0.125% in absolute ethanol) was added and incubated for 10 minutes at 100ºC. The absorbance was read at 550nm and the uronic acid content was calculated from the standard graph.

2.5. Preparation of Hydrogel

C-UL hydrogel was prepared as described previously Wang *et al*., [17]. Chitosan and crude *U. lactuca* polysaccharides were dissolved in 1% acetic acid and distilled water respectively, to obtain separate solutions at a final concentration of 2 % W/V. Then, sodium chloride at the concentration of 1.2 mol/l was added to the *U. lactuca* polysaccharide solution. The chitosan solution was added drop-wise into the *U. lactuca* polysaccharide solution under constant stirring, pH was adjusted to 7.4. The acquired product was incubated in a water bath at 60°C for 1 hour and stored in the refrigerator.

2.6. Characterization of C-UL hydrogel

2.6.1. FTIR

UV Spectra from 4000 cm^{-1} to 400 cm^{-1}were scanned on spectrophotometer Spectrum Two (Perkin Elmer) in KBr pellets at room temperature.

2.6.2. Agarose gel electrophoresis

The purity of the *U. lactuca* polysaccharide sample was determined using agarose gel electrophoresis. About, 5µg of the sample was loaded on to a 0.5% agarose gel in 0.05M1,3-diaminopropane/acetate buffer (pH 9.0) for 1 h at 110V. Subsequently, the gel was fixed with 0.1% N-acetyl-N, N, N trimethylammonium bromide solution. The staining was carried out after 12h using 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5 v/v).

2.6.3. Scanning Electron Microscopy

The surface structure of chitosan and C-UL hydrogel were observed using Scanning Electron Microscopy (SEM) (CAREL ZEISS, EVO -18).

2.6.4. Swelling study

Initially weighed (Wi) lyophilized C-UL hydrogel was introduced in the vial containing 1ml of phosphate-buffered saline (PBS) and distilled water for different intervals (1h, 2h and 24h) at 37^0C. At the end of incubation, hydrogels were dried using filter paper. The wet weight of the sample was recorded as Wf. The swelling ratio of the hydrogels was calculated using the following formula Niranjan *et al.,* [18].

$$\text{Swelling ratio} = Wi - Wf / Wi$$

2.7. Biological evaluation

2.7.1. DPPH assay

The scavenging activity of the 1, 10 -Diphenyl-2-picrylhydrazyl (DPPH) free radical assay was assessed according to the method of Sudharsan *et al.,*[19]. The C-UL hydrogel was compared to a known antioxidant, ascorbic acid (A_c). DPPH solution (0.1mM in absolute methanol) was mixed with the hydrogel sample at different concentrations (50 – 250µl). The reaction mixture was incubated for 30 minutes in the dark. The reduction of the DPPH radical was estimated by measuring the absorption at 517 nm (A_s). The scavenging activity was calculated using the following equation.

$$\text{Scavenging effect (\%)} = [(A_c - A_s)/A_c] \times 100$$

2.7.2. Hydroxyl radical scavenging activity

The hydroxyl radicals activity was assessed according to the method of Sudharsan *et al.,*[19] . 3ml of sodium phosphate buffer (150mM, pH 7.4) and different concentration ofC-UL hydrogel (50 – 250µl) (A_s) were incubated at 37^0C for 1h, in the control, sodium phosphate buffer replaced H_2O_2 (A_c). The presence of the hydroxyl radical was estimated by measuring at 510nm. The percentage of scavenging the hydroxyl radical was calculated using the following equation.

$$\text{Scavenging effect (\%)} = [(A_c - A_s)/A_c] \times 100$$

2.8. Antidiabetic and neuroprotective effects

2.8.1. Generation of neuron damage mice model and drug treatment

The experiment was performed following the regulation specified by the Institutional Animal Ethics Committee (Reg. No: AU-IEAC/1186/1/18), Annamalai University. Healthy Male mice (30-35g) were used in the current study. Animals were fed on a standard diet with water ad libitum. Diabetic neuropathy model was generated as described previously [20]. Diabetes was induced by a single dose of Streptozotocin (60 mg/kg), and blood samples were collected from the tail vein 48h after administration. Mouse with blood glucose level of more than 250mg/dl was considered diabetic. C-UL hydrogel treatment was started 15 days after diabetes induction and continued for 30 days, control animals received PBS alone.

2.8.2. Experimental design

Animals were randomly assigned into five groups with five animals each, Group I: Normal Control or animals that were left undisturbed, Group II: Control animals treated with C-UL hydrogel at a dosage of 150mg/kg body weight, Group III: Diabetic control mice, Group IV: Diabetic mice treated with C-UL hydrogel (150mg/kg body weight), and Group V: Diabetic mice treated with insulin (6 U/kg body weight).

2.8.3. Antioxidant parameters

The method of antioxidant superoxide dismutase (SOD), catalase (CAT), glutathioneS-transferase (GST) analysis were according to the previous method with minor modifications of Saravanan and Pari [21].

2.8.4. Haematological analysis

Haematological analyses like total cholesterol (TC), triglycerides (TG), Low-density lipoprotein (LDL), High-density lipoprotein (HDL), and insulin were analyzed with the help of an automated analyzer (RX Monaco).

2.8.5. Histopathology

Formalin-fixed brain tissues were processed and stained with Cresyl violet and hematoxylin and eosin (H and E) based on the protocol described by Karthick *et al.,* [22] with slight modifications.

2.9. Statistical analysis

All statistical analysis was performed using Graphpad Prism (Version 6.0). The data were represented as mean ± SEM. Group comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests. The statistical difference was $p < 0.05$, ns – non – significant.

3. Results and discussion

3.1. Total yield and composition of polysaccharide

The polysaccharides obtained from *U. lactuca* were found to be 2.5% on a dry weight basis. Protein content (<1 % dry weight) was lower than that of sulfated polysaccharide from other resources [23], sulfate (7.8 %) and uronic acid (8.6%) was lower than others [24]. Therefore, be concluded that the marine origin, nature of the seaweed, extraction procedures, and seasonal variations are determining factors for variations in the biochemical composition [25].

3.2. Agarose gel electrophoresis

Agarose gel electrophoresis demonstrated the purity of the crude *U. lactuca* polysaccharide (Figure.1). Crude *U. lactuca* polysaccharide shows only a single band in the gel, showing purified polysaccharides when compared with standard heparin sulfate 15kDa and chondroitin sulfate 12kDa [26].

Figure 1 Agarose gel electrophoresis. Lane: 1 Heparan sulphate; lane: 2 U. *lactuca* polysaccharide; lane: 3 chondroitin sulfate

3.3. Characterization of C-UL hydrogel

3.3.1. FTIR

The IR spectrum of *U. lactuca* polysaccharide in Figure.2A represents the basic peaks at 3355; 1217; 1173 cm-1 that confirmed the polysaccharide [27]. The spectrum of chitosan shows the peaks at 3207; 2883; 1644 and 1394 cm^{-1} (Figure.2B) confirming the characteristic feature of chitosan and showing the occurrence of deacetylation [28]. From the IR spectrum of C-UL hydrogel, it is observed that the OH stretching groups are shifted to 3431 cm^{-1}. The absorption band of *U. lactuca* polysaccharide and chitosan is shifted to 1622; 1384; 1194 and 1103 cm^{-1} (Figure.2C). So the FTIR analysis showed that the *U. lactuca* polysaccharide was linked with chitosan to form the hydrogel.

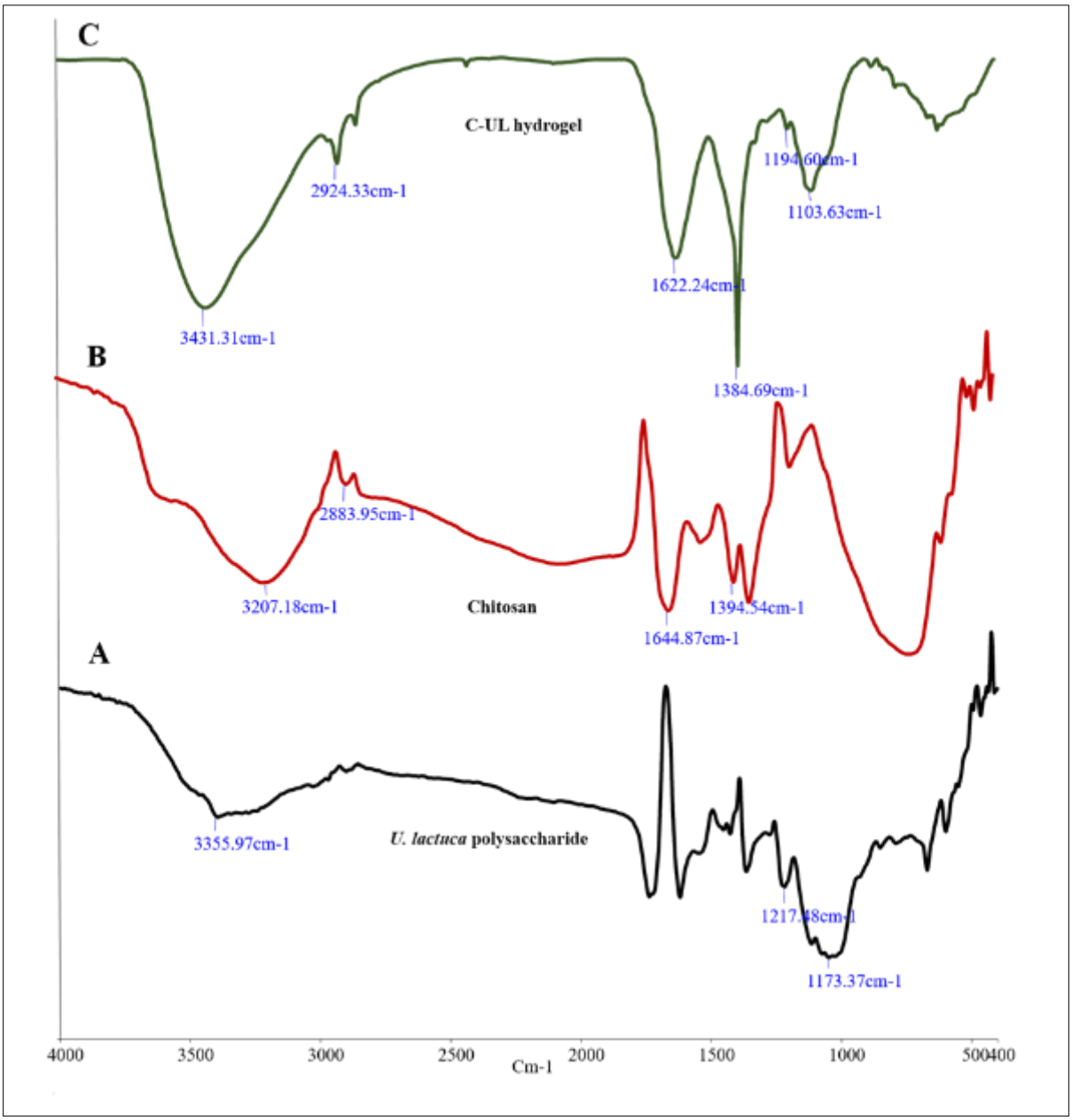


Figure 2 FT-IR spectrum of (A) *U. lactuca* polysaccharide (B) Chitosan and (C) C-UL hydrogel

3.3.2. SEM analysis

The SEM image displays the surface morphology of the chitosan. The images showed non-uniform and non-smooth exterior as shown in Figure.3A. The lyophilized C-UL hydrogel had a 3D mesh structure under a scanning electron microscope. The micro-sized *U. lactuca* polysaccharides with non-smooth surface were eventually distributed on the hydrogel surface (Figure.3B).

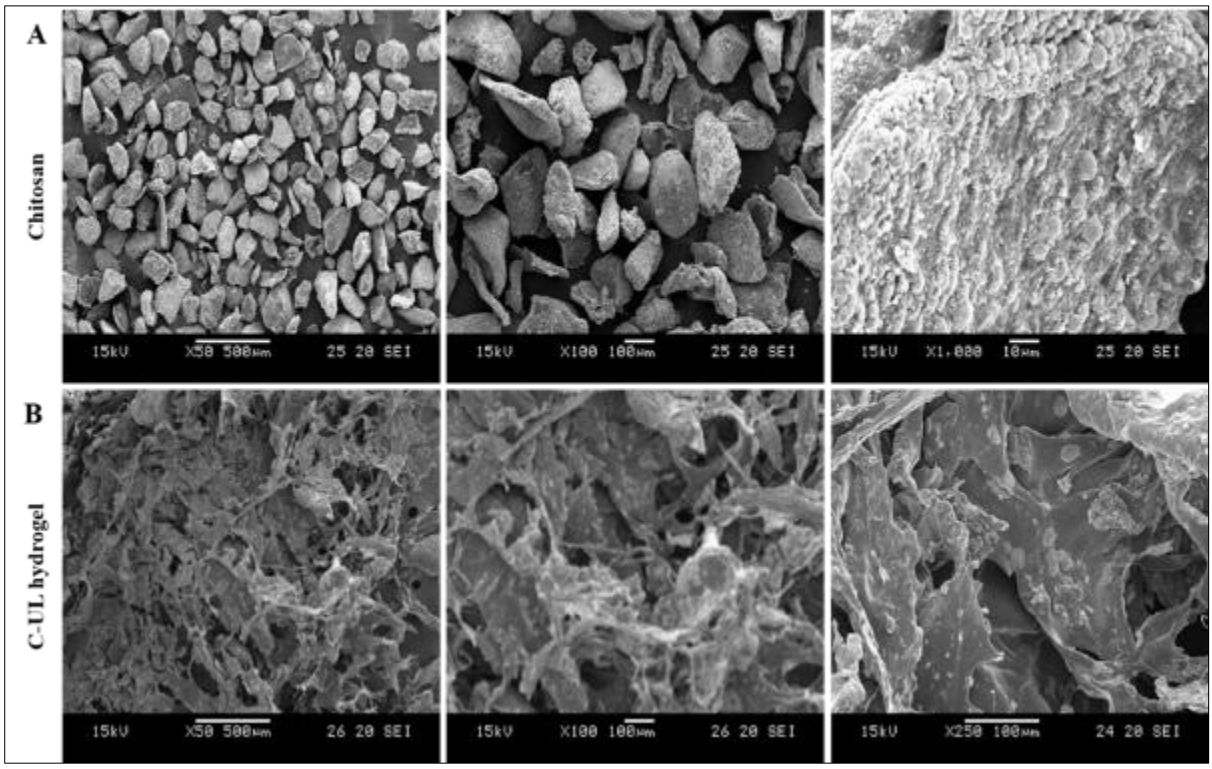


Figure 3 SEM images of (A) Chitosan and (B) C-UL hydrogel at different magnifications

3.3.3. Swelling study

The swelling behavior is an important characteristic future of the hydrogels to be considered for applications such as absorbents, drug delivery or filtration/separation systems, soil conditioners, etc., [29]. The swelling ratio of the C-UL hydrogel was checked in PBS with a pH 7.4, C-UL hydrogel sample swelled quickly, in about 1 minute. In Figure.4, we have shown the prepared C-UL hydrogel swelling ratio at the end of 1h incubation period. On the increasing, the incubation period to 2 and 24h the same pattern of swelling was observed. Higher swelling ratio probably arose from the increased degradation of the hydrogel during the swelling period. Higher degradation results in a significant loss of weight and leads to an increased swelling ratio [30]. The higher swelling capacity of the C-UL hydrogel with lower crosslinking capability resulted from the higher flexibility of free chitosan chains because of the protonation of amino groups [29, 30].

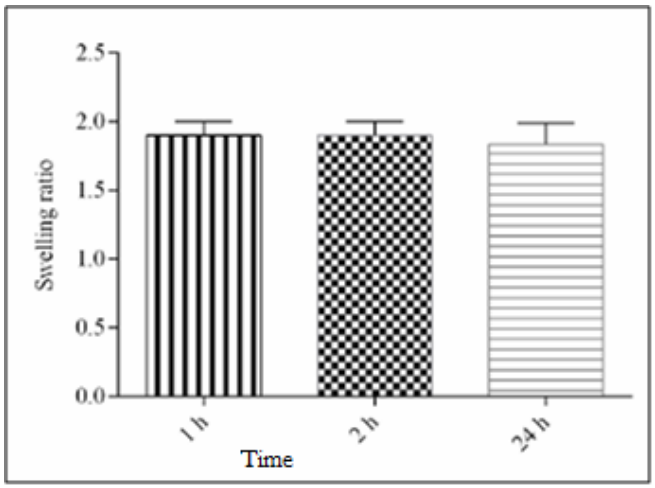


Figure 4 Swelling ratio of C-UL hydrogel in phosphate buffered saline (pH 7.4) at 1 h, 2 h and 24 h incubation period

3.4. Biological evaluation

3.4.1. Antioxidant activity of C-UL hydrogel

Enormous free radicals would exist in the diabetic condition and these radicals will result in oxidative stress and several cellular changes [31, 32]. Chitosan and *U. lactuca* polysaccharide have excellent antioxidant properties to scavenge free radicals and protect tissues from oxidative damage [33, 34]. There was an obvious decrease in the intensity of the DPPH absorption peak after adding C-UL hydrogel (Figure.5A), indicating that these hydrogels had good antioxidant capacity. The prepared C-UL hydrogel showed concentration depended on the activity at the different concentrations tested like 50 – 250µl with the inhibition of 4–82 %. Also, higher sulfate group content of polysaccharides indicated a stronger scavenging activity [35]. The hydroxyl radical is a reactive oxygen species and causes damage to the biomolecules such as lipids, proteins, and nucleic acids., which leads to aging, chronic inflammation, neurodegeneration and cancer [19, 36]. Recent studies have evidenced that seaweed polysaccharides and chitosan can stabilize the hydroxyl radical scavenging activity [37]. The C-UL hydrogel showed hydroxyl radical activity of 17% at 100µl and 82% at 500µl concentration tested. Further, in the present study, the hydroxyl radical scavenging activity of C-UL hydrogel was found to increase in dose-dependent manner (Figure.5B).

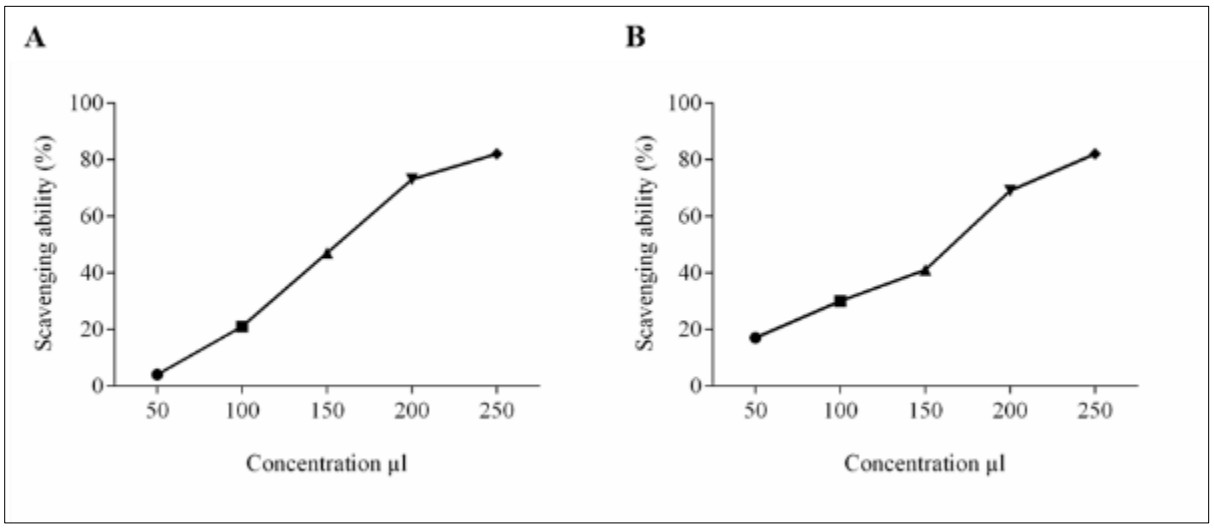


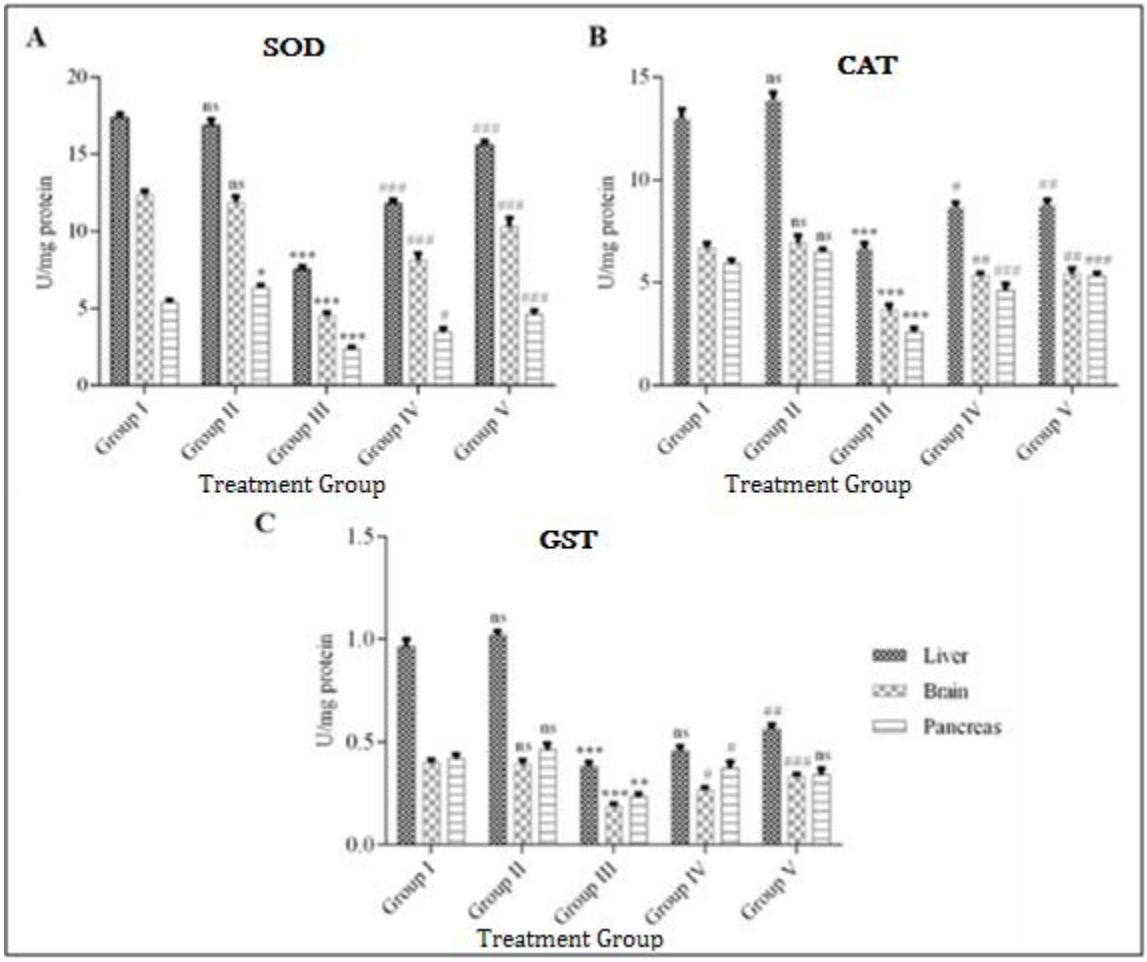
Figure 5 Scavenging effect (%) on superoxide anion radical (A), DPPH radicals and (B) hydroxyl radical of C-UL hydrogel

3.5. Ant diabetic and neuroprotective effects

3.5.1. Antioxidant parameters

As shown in Figure 6 the levels of enzymatic antioxidants such as SOD, CAT and GST were significantly (***p <0.001) reduced in the diabetic control group as compared with the normal control and C-UL hydrogel treated control group. Treatment with C-UL hydrogel to diabetic mice significantly increased the level of antioxidants as compared to the diabetic control group. The administration of C-UL hydrogel showed itself to be equally effective with that of the insulin-treated diabetic group. From the present study, the antioxidant property of C-UL hydrogel was well-proved, where C-UL hydrogel could regulate antioxidants like SOD activity (Figure. 6A), CAT activity (Figure. 6B), and GST activity (Figure. 6C) in a diabetic animal model.

That evidence indicates that the antioxidant property of C-UL hydrogel is responsible for its beneficial effects on impaired organ functions in diabetic mice. The decreased SOD and CAT activity might result from the inactivation by glycation of the enzymes [38]. The C-UL hydrogel treated diabetic animals showed an increase in the activities of SOD and CAT to near normal. These data's revealed that C-UL hydrogel contains a free radical scavenging activity. In diabetic animals, the activity of GST is significantly decreased. The decreased level of GST in the tissues represents an increase in the utilization of GSH due to oxidative stress induced by the diabetic condition. The C-UL hydrogel treated diabetic animals increased the level of GST in the liver, brain and pancreas. These results suggested that the C-UL hydrogel contains an activity of increasing the synthesis of GSH or free radical scavenging activity.

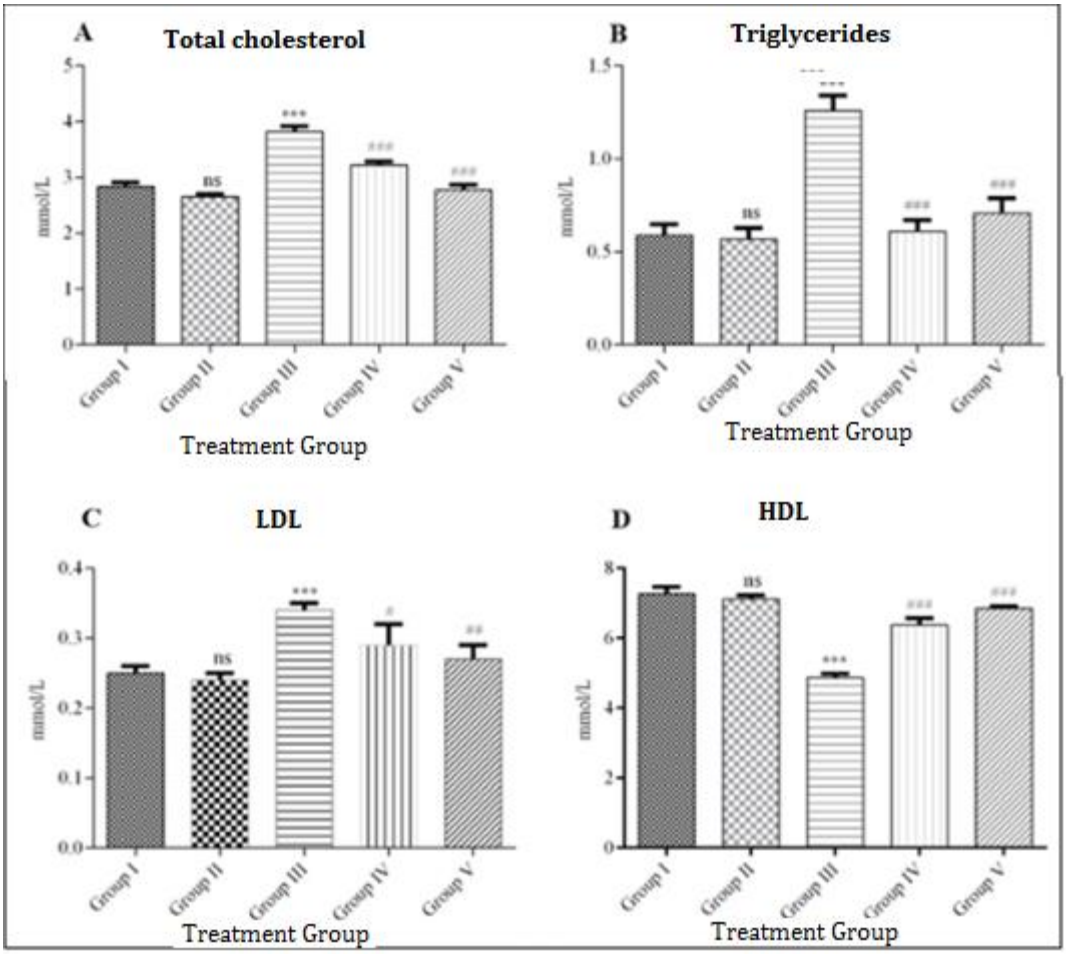


Values were presented as mean ± SEM, statistical significance was performed by one- way ANOVA followed by Tukey's multiple comparison test. Values are statistically significant at *p < 0.05; **p < 0.01; ***p < 0.001; ns – non significant. *p < 0.001 vs normal control, #p < 0.05; ##p < 0.01; ###p < 0.001vs diabetic control.

Figure 6 Effects of C-UL hydrogel on antioxidant levels in control and experimental mice. (A) Superoxide dismutase, (B) catalase, (C) Glutathione S-transferase in liver, brain and pancreas

3.5.2. Cholesterol profile

Anticholesterolemic effects of the C-UL hydrogel were analyzed by its TC, TG, LDL and HDL. From the fundamental point of view, cholesterol is involved in chemical reactions that can produce free radicals, which leads to chain reactions that may damage neurons [39]. Our results were similar to the study of Renard *et al*., [40] Reported that insulin-treated diabetic mice fed with cholesterol-rich food lead to elevate in the levels of VLDL and TG and this may contribute to inflammation. Figure.7 shows the anti-cholesterolemic activity of C-UL hydrogel. There was a significant increase in the total cholesterol, triglycerides, low-density lipoprotein and a reduced level of high-density lipoprotein was evidenced in diabetic control mice (***p <0.001). After treatment with C-UL hydrogel in diabetic mice, the level of total cholesterol, triglycerides, low-density lipoprotein were significantly reduced and high-density lipoprotein was significantly increased when compared with the diabetic control mice. There are many reasons for the lower cholesterol profile. One specific reason was the controlled release of chitosan and *U. lactuca* polysaccharides from C-UL hydrogel and anti-cholesterolemic properties influenced by Chitosan and *U. lactuca* polysaccharides [41] . The existence of the chitosan can have ways to reduction on cholesterol levels [41] and *U. lactuca* polysaccharide modulates the dysregulation in cholesterol pathways [42]

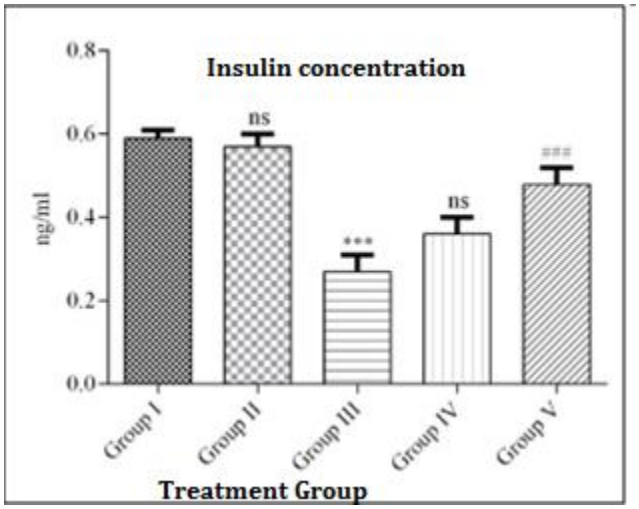


Values were presented as mean ± SEM, statistical significance was performed by one- way ANOVA followed by Tukey's multiple comparison test. Values are statistically significant at *p < 0.05; **p < 0.01; ***p < 0.001; ns – non significant. *p < 0.001 vs normal control, #p < 0.05; ##p < 0.01; ###p < 0.001vs diabetic control.

Figure 7 Effects of C-UL hydrogel on cholesterol profile in normal and experimental mice. (A) Total cholesterol, (B) triglycerides, (C) low-density lipoprotein (LDL), (D) high-density lipoprotein (HDL)

3.5.3. Insulin concentration

Insulin biosynthesis is a complex mechanism, controlled by multiple factors. Glucose metabolisms that engage blood sugar are essential for enabling effective stimulations with insulin gene transcription and mRNA translation. The transcription factors within a region spanning ~ 400 base pairs (bp) play a critical role in the specific expression of insulin [43]. Insulin analysis is useful to investigate the concentration of insulin that helps the regulation of blood sugar. Literature suggests that certain seaweed polysaccharides can increase insulin concentrations [44]. In the present study, we tried to determine the capability of C-UL hydrogel for insulin synthesis in type 1 diabetic mice. Where diabetic control mice showed significantly (***p <0.001) decreased the level of insulin concentration (Figure.8). Treatment with C-UL hydrogel cannot increase the level of insulin in diabetic mice. No significant changes were observed between control and chitosan/polysaccharide hydrogel alone treated groups.



Values were mean ± SEM (n = 5), statistical significance was performed by one way –ANOVA and multiple comparison were done by using Tukey's multiple comparison test. ***p < 0.001 vs normal control; ###p < 0.001vs diabetic control.

Figure 8 Displays the effects of C-UL hydrogel on insulin concentration of normal and experimental mice

3.5.4. Protective effects of C-UL hydrogel in various regions of the mice brain

Previous studies have suggested that the involvement of multiple factors including hyperglycemia, the formation of advanced glycation end products, dyslipidemia, free fatty acids, oxidized and glycated LDLs, oxysterols and insulin resistance can contribute to neuronal damage in diabetic conditions [45] . The current study supports the hypothesis that controlled release of chitosan and *U. lactuca* polysaccharides may decrease oxidative damage in the brain via the regulation of antioxidant pathways. To determine the neuroprotective effects of C-UL hydrogel in diabetic mice, cresyl violet stained brain regions of all groups were investigated. Firstly we examined morphological changes in the prefrontal cortex, where the regions of the frontal lobe are highly involved in the cognitive functions [46]. Damages in this region may cause severe difficulties in memory [17]. CV-stained sections of the frontal cortex revealed the normal morphology of the C-UL hydrogel treated diabetic group (Figure.9). Though, compared with normal control we were able to observe slight morphological changes in the diabetic control group, these morphological differences were similar to the earlier study which explains the pathological changes in the brain in STZ induced diabetic mice [47]. The above-described results collectively demonstrated that during diabetic conditions cortex regions were disturbed. The positive effects of C-UL hydrogel might be due to the free radical scavenging activity of *U. lactuca* polysaccharide.

Processed information in the frontal cortex is carried forward to the parahippocampal and medial entorhinal cortex to complete the memory process [48]. In the hippocampus, primary input regions in the dentate gyrus (DG) are also involved in several functions like learning, memory, and spatial coding [49]. Studies reported that diabetic conditions could lead to pathological changes in DG [50]. With this background, we intended to analyze the protective effects of C-UL hydrogel in the suspected region. The present study revealed diabetes-associated pathological changes in DG (Figure.10A) with slightly decreased neuronal density, shrunken cells and darkly stained neurons were observed. These pathological changes in DG suggested that there may trouble in projecting the signal onto the hippocampus DG from the entorhinal cortex and also creates trouble in Cornuammonis 3 (CA3) for receiving major excitatory inputs from DG [51]. The diabetic group treated with C-UL hydrogel displayed minimal morphological changes as compared with the diabetic control; these positive reactions might be due to C-UL hydrogel treatment that enhanced neurogenesis and cognitive functions in a diabetic animal model. Our conclusion was consistent with the study of [52], where the restoration of adult neurogenesis was observed in metaformin treated diabetic mice. However, no significant pathological changes were observed between control and C-UL hydrogel treated mice.

Slight morphological changes were observed in DG that could affect the formation of the hippocampus proper. Hence, we continued our investigations of other hippocampus regions like Cornuammonis 1 (CA1 and CA3). We observed significant damage in CA1 and CA3. Pathological changes like dead cells, severe neuronal misalignments and darkly stained neurons were observed in the diabetic control group (Figure.10B and C). Similar to the current study, earlier studies have demonstrated that experimental type 1 diabetes causes neuronal death in hippocampal regions like CA1 and CA3, these changes could be directed to certain cognitive alterations. In the present study, the significant level of neuronal degeneration in diabetic mice was evaluated. Damages in the hippocampus are known to impairment of hippocampal functions such as learning and memory [17]. Therefore, abnormalities in these regions could lead to the

development of spatial memory defects. Interestingly, the present study displayed a decreased level of neuronal degeneration and misalignment in C-UL hydrogel treated diabetic animals compared to normal control animals. Thus, this study also supports the fact that C-UL suppresses oxidative stress in the diabetic animal model since the neuronal loss is more related to increased oxidative stress in diabetic conditions.

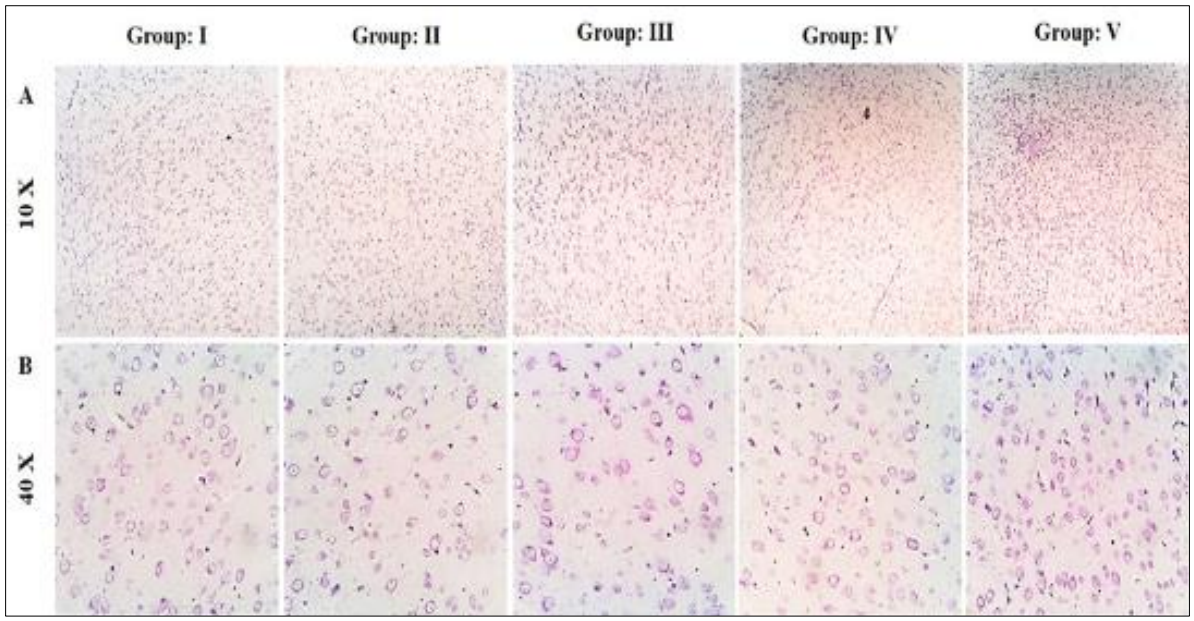


Figure 9 Histopathological comparison of frontal cortex from control and C-UL hydrogel treated control showing normal architecture. Diabetic control mice showed slight morphological changes. Diabetic mice treated with C-UL hydrogel and insulin protector effect were showed (H & E 10 X and 40 X)

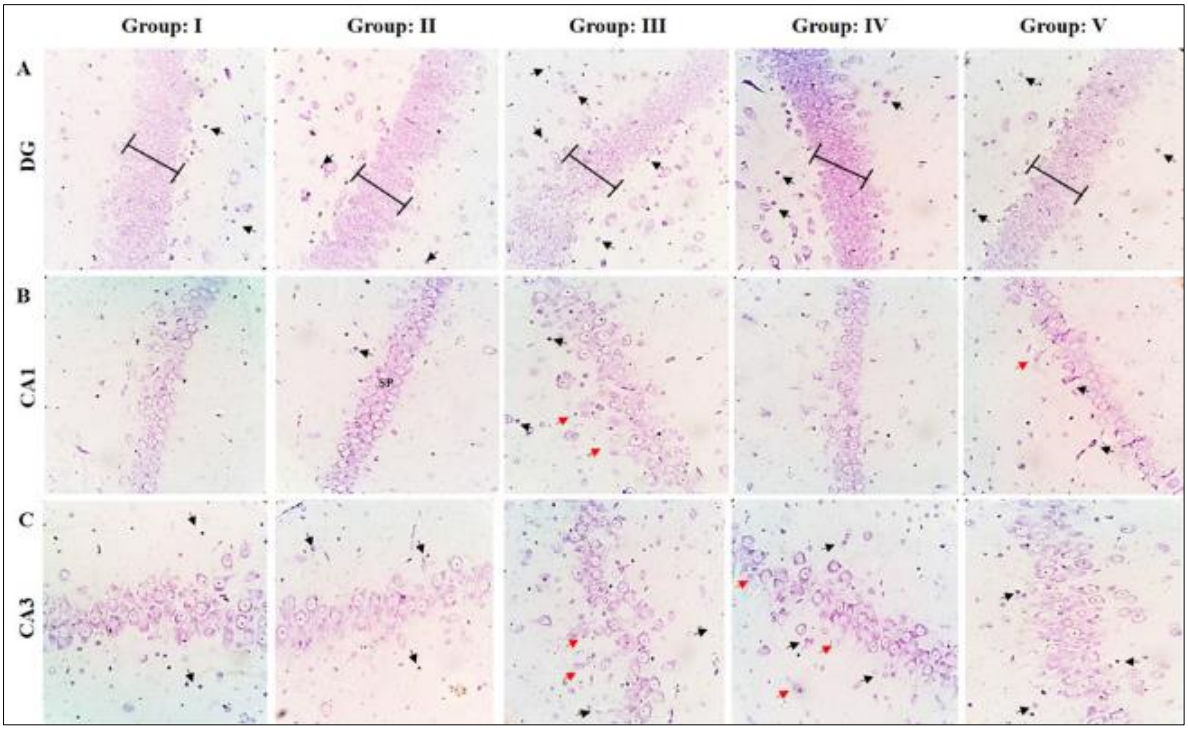


Figure 10 Microscopic photograph displays the effects of C-UL hydrogel in various sub-regions of the hippocampus. Dentate Gyrus (DG), Cornuammonis 1 (CA1) and Cornuammonis 3 (CA3), and. The pattern of the pyramidal cells in the hippocampus especially CA3 showed dark stained cells (black arrow) and misalignment and dead neurons (red arrow) in diabetic control mice's

Giribabu *et al.,* [53] reported that the diabetic condition could lead to pathological changes in the subiculum, which is an important output region that communicates a signal to PFC, EC II, Hypothalamus and amygdala (Figure.11) [54]. A significant increase in neuronal degeneration in the subiculum of diabetic control mice was observed in the microscopic studies. Whereas, the damages observed in the subiculum of C-UL hydrogel treated diabetic mice were found very less. These results collectively suggested that the hippocampal regions were highly disturbed due to oxidative stress in the diabetic condition. Fascinatingly, C-UL hydrogel treated animals showed improvement in the morphology of the damaged subiculum. Thus, this hydrogel might have chance to improve the neuronal connections between the regions of the hippocampus. However, no significant changes were observed in C-UL hydrogel treated control group.

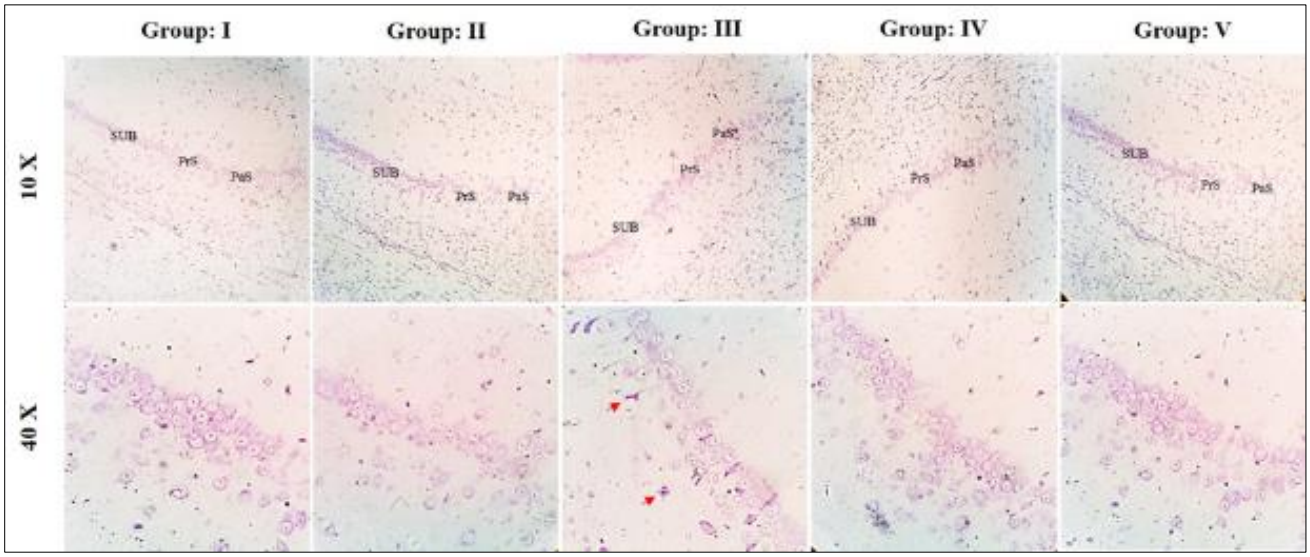


Figure 11 Photograph of CV stained coronal sections of the subiculum. 10 X and 40 X images showing the effect of C-UL hydrogel on the Subiculum (SUB), Presubiculum (Prs) and Parasubiculum (PaS) of normal and experimental groups. Neuronal degeneration (red arrow) and dark stained neurons were easily observed

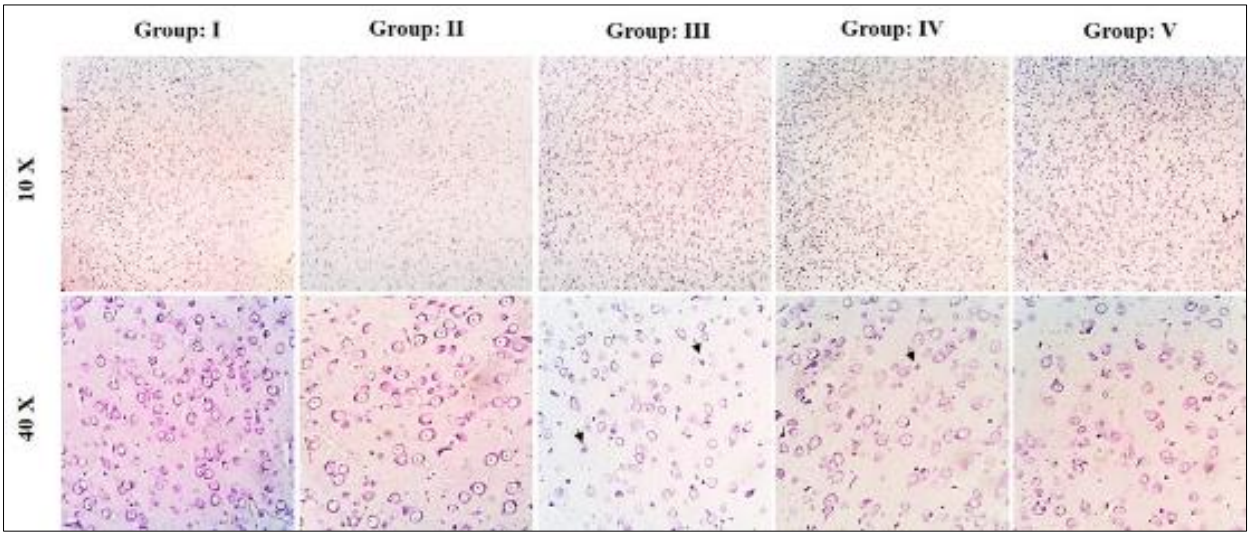


Figure 12 Histological analysis of amygdala in the control and experimental animals. Low-magnification (10 X) and high-magnification (40 X) images of amygdala show clear intact neurons and dark stained neurons (black arrow)

From the studies, it was obvious that diabetic condition causes severe pathological changes in hippocampus regions, but the pathological changes associated with other brain regions are yet to be studied. Hence, we investigated the pathological changes in the amygdala. As shown in Figure.12. Clear intact neuronal cells with the predominant strain of control and C-UL hydrogel treated control mice were evidenced. The diabetic control mice exhibited certain pathological changes such as dark-stained neurons.

Severe decrease in the level of antioxidants in the amygdala region of the diabetic model. Oxidative stress in these regions results in a disturbance of events like emotional, behavioral, and mood functions and several neuronal mechanisms. These results are similar to the conclusion reported by [55]. As we discussed earlier C-UL hydrogel normalized the pathological events in the amygdala and this could improve amygdala activity in diabetic conditions.

3.5.5. Protective effects of C-UL hydrogel onthe mice liver and kidney

Oxidative stress is a crucial mechanism in the system that leads to severe complications in liver and kidney functions in diabetic conditions [56]. As shown in Figure.13A, the hepatocyte morphology was normal in both the control and C-UL hydrogel treated group, and the hepatic cells were arranged regularly with abundant cytoplasm, clear cell borders and clear intact nucleus. In the diabetic control group, slight liver damage was observed. The changes in the liver morphology were characterized by degeneration and inflammatory infiltration (Figure.13A). In the treatment groups, treatment with C-UL hydrogel and insulin showed effects in ameliorating liver damage. The changes in kidney morphology were shown in Figure. 13B, when compared with the control and C-UL hydrogel treated group, slight cellular degeneration was observed in the diabetic control mice.

After the treatment with C-UL hydrogel and insulin, these slight pathological changes were found to be diminished as evidenced by the normal morphology of glomerular and tubular structures in Figure.13B. The present study demonstrated that C-UL hydrogel treatment significantly increased the activity of SOD, CAT and GST in both liver and kidney of diabetic control animals. These heightened activities of SOD, CAT and GST are highly effective in the prevention of damages caused by oxidative stress. Similar results were also observed in diabetic mice treated with *Ophiopogon japonicus* [57].

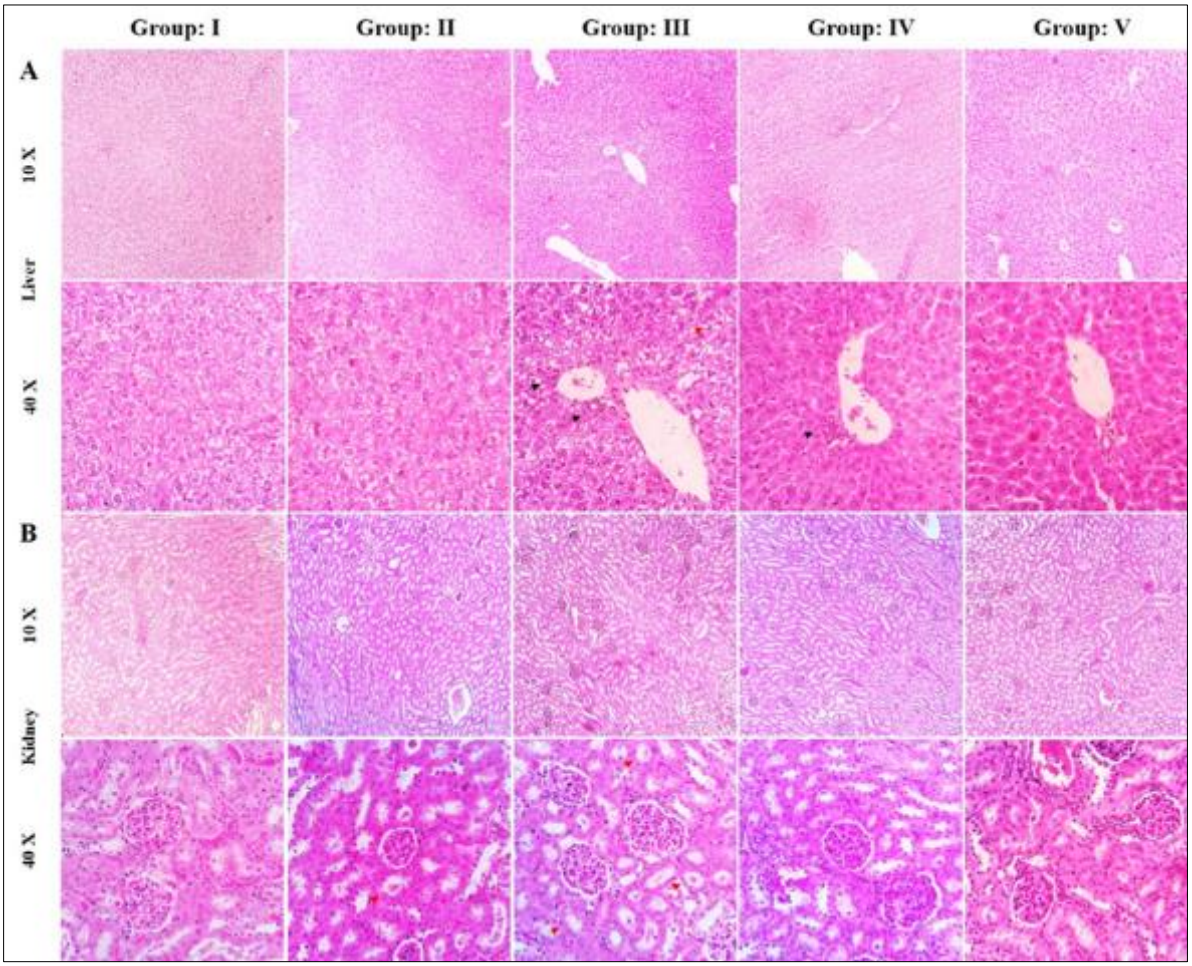


Figure 13 Effects of C-UL hydrogel on liver and kidney of the experimental groups. Microscopic photograph showing H & E stained liver and kidney (low magnification 10 X and high magnification 40 X). (A) Liver, (B) Kidney. Degeneration (red arrow) and inflammatory infiltration (black arrow)

4. Conclusion

The present study suggested that C-UL hydrogel exhibited a major role in neuroprotection against oxidative stress induced damage in diabetic mice. C-UL hydrogel prevents brain damage by regulating the antioxidant pathways in the mice model. In the future, diabetes-associated neurological complications can be treated using this therapeutic C-UL hydrogel. However, further studies are essential for a better understanding of the mechanism of action.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

TG performed experiments and analyses. TG and RS designed experiments. TG interpreted the data. TG wrote the main manuscript text and prepared Figures. All authors reviewed the manuscript.

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