

Development, characterization, efficacy and repeated dose toxicity of nanoemulsified ethanolic extract of *Enicostemma littorale* in Streptozotocin-induced diabetes rats.

Deepa V¹, Sridhar R¹, Neelakanta Reddy P² & Balakrishna Murthy P¹

*Corresponding author:

Deepa V

¹ International Institute of Biotechnology and Toxicology, Padappai, Kanchipuram district, India.

² Bio-organic Chemistry Laboratory, Central Leather Research Institute, Adayar, Chennai, India

Abstract

Alginate nanocapsules of ethanolic extract of *Enicostemma littorale* (NEL) were prepared by emulsification, cross linking with calcium chloride and solvent removal. Based on total phenol content the loading efficiency of the nanocapsules was 89% at an optimum concentration of 2: 18 (mg ml⁻¹) for plant extract: olive oil. The photon correlation spectroscopy (PCS) revealed that the mean particle diameter of optimized formulation was 233 nm and scanning electron microscopy (SEM) showed a spherical morphology. When subjected to Fourier transform infrared spectroscopy (FT-IR) for the compatibility analysis between plant extract and sodium alginate, it revealed that the phytoconstituents were stable. The purpose of the present study was to compare the anti-diabetic activity of NEL and *E.littorale* (EL) in streptozotocin induced male rats. An oral dose of NEL (20 mg/kg b.w) and EL (2000 mg/kg b.w) showed a relatively similar antidiabetic effect, reducing the blood glucose, triglycerides, cholesterol, creatinine, ALT, AST, and ALP. Moreover, NEL is 100 times less than EL exhibiting better results within 10 days of treatment. These biochemical assessments were supported by rat biopsy examinations. In conclusion, the nanoemulsification method can be applied for poor water-soluble ethanolic herbal extracts to reduce the dosage and time.

Key words: nanoemulsion, streptozotocin, diabetics, anti-diabetic, *Enicostemma littorale*

Introduction

Diabetes mellitus, a chronic metabolic disorder affecting approximately 4% of the population worldwide, is expected to be increased to 5.4% in 2025 [1] and is estimated to afflict 5-7% of the population [2]. The insulin metabolism and impaired function in carbohydrate, lipid and protein metabolism lead to long-term complications related to deficiency in the insulin action or secretion. Major complications such as retinopathy [3] nephropathy [4] and neuropathy [5,6] lead to long-term damage, dysfunction, and failure of various organs, such as the eyes, kidneys, nerves, heart and blood vessels, creating a huge economic burden related to the management of diabetic complications [7]. *Enicostemma littorale* (Gentianaceae) is a small perennial herb used for the treatment of Diabetes mellitus. It contains six phenolic compounds, flavonoids and aminoacids, five alkaloids, two sterols and volatile oil [8]. Previous studies have demonstrated the antidiabetic activity of aqueous extract of *E. littorale* in type-2 diabetic rats [9] and alloxan induced diabetic rats [10-13]. The biochemical constituents of the plant are

important sources of natural antioxidants and the efficacy of the plant extract is more when they are consumed as a crude extract [14]. However, the major limitation is that the quantity of herbal extract required for treatment is higher due to the degradation of various plant constituents such as alkaloids, amides, prophenylphenols, steroids, hydrocinnamic acid and oxalic acid in the gastro intestinal tract and a longer duration of treatment is needed due to the poor absorption of these constituents in the intestine [15].

Recently, many studies focused on encapsulation of the plant extracts to enhance the sustained release of the constituents, in the intestine, for maximum absorption [16-19]. Apart from other nanoencapsulation methods, nanoemulsification is more advantageous since the targeted drug is usually packed in an inner phase which is released in a sustainable manner for a longer period and kept away from direct contact with body and tissue fluids [20] due to two immiscible liquids oil-in-water (O/W), water-in-oil (W/O), oil-in-water-oil (O/W/O) and water-in-oil-water (W/O/W) being dispersed as small spherical droplets in the other [21]. Their size ranges from 0.1-100 nm [22].



In the present study, nanoemulsification of *Encostemma littorale* by emulsioncoacervation method was prepared and characterized by using scanning electron microscopy (SEM), photon correlation spectroscopy (PCS) and Fourier transform infrared spectroscopy (FTIR). Our aim was to evaluate repeated dose toxicity and to compare the antidiabetic effect of nanoemulsified ethanolic extracts of *E. littorale* (NEL) and *E. littorale* (EL) in streptozotocin induced diabetic male rats.

Materials and methods

Extraction and preparation of nanoemulsified ethanolic extracts using sodium

alginate

The leaves of *E. littorale* were collected, washed thoroughly with several changes of sterile water followed by 70% ethanol, blotted between fields of filter paper (Whatman No.1), shade dried and homogenized. The homogenate was extracted in 99% ethanol for 8 h in Soxhlet distillation and the solvent was evaporated at room temperature (28-30°C).

The residue was lyophilized (Christ lyophilizer, German) and stored in -20°C until further use. The authenticity of the plant species were verified by Botanical survey of India, (Coimbatore, India).

Nanoencapsulation was performed using a three step procedure - emulsification, crosslinking with calcium chloride and solvent removal [23, 24]. Dispersion of ethanolic plant extract in aqueous sodium alginate (molecular weight of 80,000-120,000 Da, Sigma Chemicals, St Louis, MO, USA containing Tween 80®) solution caused immediate formation of micelles with an oil core. The alginate emulsion was cross-linked with analytical grade calcium chloride and solvent was removed by evaporation under pressure. Nanocapsules containing plant extract were obtained as dispersion in aqueous phase.

Variation in proportion of plant extract and olive oil

The optimal plant extract: olive oil mass ratio for nanoemulsification was determined using varied proportions (0.005:0.195, 0.01:0.190, 0.02:0.180, 0.03:0.17, 0.06:0.14 and 0.15:0.05) dissolved in 10 ml of ethanol to obtain a final concentration of 2% and mixed with 10 ml of aqueous alginate (0.6 mg ml⁻¹) (1:1 ratio) at pH 7.0 containing 1% (w/v) Tween 80®. After sonication for 15 min, the emulsion was combined with 4 ml of 0.67 mg ml⁻¹ CaCl₂ solution and continuously stirred for 30 min. The plant extract-loaded alginate nanocapsule suspension was then equilibrated overnight prior to removal of ethanol by rotary evaporation at 40 °C for 20 min. Finally, the alginate nanocapsules containing plant extract were obtained as dispersion in aqueous solution. Based on the turbidity, the maximum emulsion formed was visually monitored. Then the emulsion was washed with sterile water and filtered in a 0.45 µm

size filter. The total phenolic content was calculated for ethanolic plant extract, sodium alginate emulsion without plant extract and the emulsion formed at varying concentrations, before and after filtration by using Folin-Ciocalteu reagent method [25] using gallic acid as a standard. This method was employed to evaluate the phenolic content of the samples. 100 µl of the sample was dissolved in 500 µl (1/10 dilution) of Folin-Ciocalteu reagent and 1000 µl of distilled water. The solutions were mixed and incubated at room temperature for 1 min. After 1 min, 1500 µl of 20% sodium carbonate (Na₂CO₃) solution was added. The final mixture was shaken and then incubated for 2 h in the dark at room temperature. The absorbance of all samples was measured at 760 nm using Orbit Technology UV-Vis spectrophotometer. The results were expressed in mg of gallic acid (GEA) per mg of plant extract
Total phenol content before filtration
Loading Efficiency (%) = x 100
Total phenolic content after filtration

Determination of particle size

Particle size was determined using photon correlation spectroscopy (PCS) (MalvernS4700PCSSystem, Malvern,UK). For particle size analysis, the nanoemulsified plant extract was first suspended in 100ml of filtered water (0.2 µm filter, Ministart, Germany) and then subjected to sonication for 30s and vortex mixing for 10s before analysis.

Characterization of nanocapsules

The shape and surface morphology of nanoemulsions were examined using scanning electron microscopy (SEM) (JSM-T20, Tokyo, Japan). An appropriate sample of polymeric nanoparticles was mounted on metal stubs, using double-sided adhesive tapes.

The samples were gold coated and observed for morphology, at an acceleration voltage of 15 kV.

Chemical stability of the nanoemulsified ethanolic extracts

Lyophilized samples mixed with potassium bromide at a ratio of 1:100 (w/w) were laminated using a pellet mold in Fourier transform infrared (FT-IR). Spectra of ethanolic extract, encapsulated extract and extract released from alginate beads were obtained using FT-IR spectrometer (Shimadzu, Japan) by scanning between 4000–400cm⁻¹.

Storage Stability of the nanoemulsified ethanolic extracts

The physical stability of the plant extract loaded nanocapsules was determined by the assessment of average size after storage at 4°C and 28°C for two months. The encapsulation efficiency was determined at weekly interval based on the turbidity. The

release profile was microscopically observed. Based on the total phenol content the loading efficiency was calculated for the release of phytoconstituents at 28 and 4°C for the nanoemulsified ethanolic herbal extracts.

***In vitro* release of nanoemulsified extracts at acidic pH (1.2) and alkaline pH (7.2).**

To study the release kinetics, the nanocapsules were treated separately with two solutions an acidic (simulated gastric fluid, pH 1.2) and simulated intestinal fluid pH 7.2 (phosphate buffered saline, pH 7.5) at 37°C. The release profile of the phytoconstituents at different hours from the nanoemulsion was calculated based on total phenol content (TPC) (before and after filtration) and converting into the loading efficiency as mentioned above.

Test animals and treatment pattern

Male albino rats of Wistar strain (*Rattus norvegicus*) weighing 100-120 g were used for the study. The animals were housed in a group of 3 rats per cage under wellcontrolled conditions of temperature (21±2 ° C), humidity (50-60%) in 12h/12h light-dark cycle. Animals had free access to diet (Tetragon Chemie Pvt. Ltd., Bangalore, India) and water ad libitum. Diabetics was induced with STZ (sigma Ltd, USA) 50 mg/kg administered as a single intraperitoneal injection to overnight fasted animals. Animals were checked for the glucose levels after 5 days of STZ injection. Animals showing glucose (>250 mg/dL) were considered as diabetic and they were divided in to 5 groups each group containing 6 animals. The study protocol was approved by the Institutional Animal Ethics Committee.

Group 1: (negative Control) sterile water was orally dosed during entire duration of the study

Group 2: (diabetic control) Diabetic induced with STZ were orally dosed with sterile water during the entire duration of the study

Group 3: (Plant extract, 2000 mg/kg body weight) Diabetic induced with STZ was administered orally with plant extract (obtained by ethonolic extraction using Soxhlet, ethanol was evaporated by lyophilisation and stored) mixed with corn oil, which has already been proved in literature for anti-diabetic activity

Group 4: Diabetic induced with STZ was treated orally by administering with unnanotised plant extract (20 mg/Kg body weight)

Group 5: Diabetic induced with STZ was treated orally by administering with nanoemulsified plant extract (20 mg/kg body weight)

Group 6: (Reference drug, Insulin) 1 U/kg through intraperitoneal route of administration

Group 7: Plant extract 2000 mg/kg body weight was administered orally for 20 days.

Group 8: Nanoemulsified plant extract of 20 mg/kg body weight was administered orally for 20 days.

Clinical signs and body weight

All the animals in the study were observed twice daily for overt signs of toxicity, morbidity and mortality during the entire study period. At the beginning of the treatment, and at every 3rd day, thereafter, body weight was measured, i.e., on the 4th, 7th, 10th, 13th, 16th, 19th, 22nd and 25th day after treatment.

Blood sample collection and analysis

Blood was collected on the 5th, 10th, 15th, 20th and 25th day after treatment from orbital sinus of all the animals. Heparinized plasma used for biochemical analysis was obtained by centrifugation at 1500 x g for 20 min. The following clinical biochemistry parameters glucose, total cholesterol, triglycerides, creatinine, aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were analyzed by using fully automated biochemistry analyzer (Humastar 300, Human GmbH, Germany). For all determinations, Human (Human GmbH., Germany) reagent kits were used.

Gross Necropsy

All the animals were sacrificed at the end of the treatment period (Day 25) using CO₂ and were subjected to a full, detailed gross necropsy which included careful examination of the external surface of the body, all orifices, the cranial, thoracic and abdominal cavities and their contents.

Histopathology

A piece of the pancreas, liver and kidney of all the test animal were fixed with 10% neutral buffered formalin, embedded in paraffin wax, sectioned at a thickness of approximately 3-5 microns and stained with hematoxylin-eosin. A detailed histopathological examination was performed on the tissues (Pancreas, liver and kidney) from all the animals of the G1-G8 groups. The following grading system was used for histopathologic evaluation in the study: 1 – Minimal, 2 – Mild, 3 – Moderate, 4 –Marked, 5 – Severe

Statistical analysis

The data is expressed as mean ± standard deviation. For statistical analysis, the experimental values were compared with the non-diabetic control. Data were analyzed by Modified Leven's test for homogeneity and subjected to one-way analysis of variance (ANOVA). A comparison of treated with non-diabetic control groups were done using Newman-Keuls Multiple-Comparison Test. If data was found to be heterogeneity, it was subjected to non-parametric test (Kruskal – Wallis Multiple-Comparison Z-Value Test). The alpha level at which all tests were conducted is 0.05 and NCSS 2007 software was used for analysis.



Results

Nanotisation of plant extracts using sodium alginate with variation in parameters. The total phenol content of the original plant extract was 0.18 ± 0.02 mg GEA/ mg of ethanolic extract of *E. littorale*. Based on the total phenol content (before and after filtration) for NEL the loading efficiency was calculated. Different concentrations were tried to calculate the encapsulation efficiency, in some concentrations the encapsulation efficiency was less due to aggregation which will not form the nanocapsules. This can be only by monitoring the phenol content before and after filtration. At lower concentration of the extract there was no difference in the total phenol content before and after filtration. However at higher concentrations of the extract the phenol content is more in the filtrate showing the possible less of phenols indicating the reduction in the encapsulation efficiency.

This suggested that the optimum ratio of 2% of [plant extract (2 mg ml⁻¹): olive oil (18 mg ml⁻¹)] dissolved in ethanol and mixed with sodium alginate (0.6 mg ml⁻¹) at a ratio of 1:1 and cross linked with 0.2 ml of calcium chloride for 1ml of total mixture (0.67 mg ml⁻¹), the loading efficiency was 89% for NEL at pH 7.0 (Figure 1A, B). The optimum encapsulation efficiency was attained at 0.02:0.180 mg/10 ml of ethanol. This was significantly different from other concentrations tested. The polydispersity index value less than 0.3 is of narrow distribution and more than 0.3 is of broad distribution. The mean particle diameter is 213 ± 4.4 nm, which indicated that the NEL showed an excellent narrow distribution (Figure 2A) and microscopically, the nanocapsules were spherical in shape (Figure 2B, C). The FT-IR spectrum of sodium alginate nanocapsules crosslinked with CaCl₂, plant extract of *E. littorale* without nanoencapsulation and with nanoencapsulation where shown in (Figure 3A, B, C). The fingerprint characteristic vibrations bands of sodium alginate nanocapsules appear at 3320 (OH- stretching vibrations) 2924 (C-H stretching), 2852 (C-H stretching), 2362 (C-H stretching), 1747 (C=O stretching), 1103 (ester C-O group) and 952 (aromatic CH bending). FT-IR of *E. littorale* showed peaks at 3439 (OH- stretching vibrations), 2920 (C-H stretching), 2850 (C-H stretching), 2366 (C-H stretching), 1747 (C=O stretching), The bands at 1568, 1432, 1381 and 1329 are due to C-O stretching and 899 (aromatic CH bending). The common peaks that appear both in sodium alginate nanocapsules and plant extract *E. littorale* were 3439, 2922, 2852, 2357, 1342, 1037 and the common peaks that appear between plant extract *E. littorale* and the nanoemulsified *E. littorale* were 1384, 1452, 1643, 1643. From the FT-IR profile it is confirmed that all functional groups of *E. littorale* were present after interaction with the polymer. The FT-IR determined for the chemical stability of the original plant extract, nanocapsules with plant extract and for sodium alginate nanocapsules without plant extract showed that the characteristic bands of chemical groups remain unchanged for *E. littorale*.

Storage stability of nanoemulsion

The effect of storage temperature (4°C and 28°C) on the total phenol content of nanocapsules containing ethanolic plant extract [(plant extract/olive oil ratio of 0.02:0.190 mg ml⁻¹); alginate ratio =1:1] was evaluated. The total phenol content and loading efficiency analysis revealed that the nanocapsules were fairly stable when stored for 60 days at 4°C. The loading efficiency was significantly similar ($p > 0.05$) until 8 weeks of storage for *E. littorale* (Figure 4A, B), which was different on 8th week of storage at 4 °C ($p < 0.05$). However, nanocapsules stored at 28 °C released the total phenol content within a period of 3 weeks (Figure 4C, D) for *E. littorale*.

In-vitro release of nanoemulsion at alkaline and acidic pH

When studied in *in-vitro* conditions of using simulated gastric fluid (pH 1.2), the total phenols (Figure 5A) were released slowly till 4 h of incubation, and maximum release was observed after 4 h of incubation. Based on the total phenol content (GEA mg/ml of extract) the loading efficiency was calculated for different periods of incubation at acidic pH (1.2) and the total phenol content from the nanoemulsion was not released until 3 h of incubation at pH(1.2). Statistical difference did not exist in the loading efficiency until 3rd h of incubation and from 4th - 8th h which is statistically different from 1st h *-($p < 0.05$, ANOVA) (Figure 5B) for *E. littorale*. At alkaline pH (7.2), 98% of nanocapsules total phenol content were released within 4 h of incubation (Figure 5C) for *E. littorale*. Based on the total phenol content (GEA mg/ml of extract) the loading efficiency was calculated for different periods of incubation at acidic pH (1.2) and the total phenol content from the nanoemulsion was not released until 4th hour of incubation at pH (7.2). Statistical difference did not exist in the loading efficiency until 3rd h of incubation and from 4th - 8th h which is statistically different from 1st h *-($p < 0.05$, ANOVA) (Figure 5D) for *E. littorale*. In this study with the presence of sodium alginate, at acidic and alkaline pH, the emulsion was formed and loading efficiency was comparatively good but the polyphenol content was very less because it was sensitive to pH.

Morbidity/mortality and Clinical signs

No morbidity/mortality was observed in the animals from G1 (negative Control), G2 (diabetic Control), G3 (streptozotocin induced diabetics treated with plant extract, 2000 mg/kg), G4 (streptozotocin induced diabetics treated with unnanotised plant extract, 20 mg/kg), G5 (streptozotocin induced diabetics treated nanoemulsified plant extract), G6 (Reference drug -insulin), G7 (treated only with plant extract, 2000mg/kg for 20 days), and G8 (treated only with nanotized plant extract, 20mg/kg for 20 days). None of the animals in G1, G7, and G8 showed any clinical signs of toxicity throughout the observation period. However, diabetes

induced animals in G2 and G4 showed clinical signs like polyuria, polydypsia, change of body coat color and piloerection throughout the observation period whereas the above clinical signs remained in G3 until the 15th day, and in G5 and G6 until the 7th day. (Table 1A, B).

Body weight

All the animals in G1, G3, G5, G6, G7 and G8 showed constant body weight gain throughout the observation period (Table 2). However, mean body weight of animals in G2 and G4 showed significant decrease when compared to mean body weight of animals in G1. There was no significant difference in mean body weights of animals in G3, G5 and G6 when compared with mean body weight of animals in G1. This suggests that there was no constant body weight gain in diabetic induced animals in G3, G4, G5 and G6 when compared with bodyweight gain of non-diabetic animals (G1) throughout the observation period.

Plasma profile

Significant changes were observed in biochemistry parameters such as fasting glucose, triglycerides and ALP in animals belonging to G2, G3, G4, G5 and G6 on Day 5, Day 10, Day 15, Day 20 and Day 25 compared to non-diabetic control (G1). The group G5 showed a decrease in the plasma fasting glucose, triglycerides and ALP on 10th day when compared with other treated groups G3, G4 and G6. The creatinine, AST and ALT showed no significant difference to non-diabetic control (Figure 6A, B, C, D, E, F).

Urinalysis

Urinalysis performed on day 25, showed no abnormal findings in G1, G3, G5, G6, G7 and G8. However, significant increase in glucose and ketone as well as decrease in specific gravity was observed in G2 and G4 when compared to G1 (Table 3).

Histopathology for the STZ induced diabetics in rat Pancreas

Based on stained tissue sections, there were no notable changes in pancreas histology in normal rats (Figure 7A, B) In contrast, STZ administration showed shrunken pancreas, number of islets was reduced profoundly, along with vacuolar degeneration of islet cells and fibrosis focally (Figure 8A, B, C). Administration of EL (2000 mg/kg b.w) (Figure 9A, B), showed restoration of damaged islets and significantly reduced injuries to the pancreas by restricting further damage. Administration of EL (20 mg/kg b.w) (Figure 10A, B), showed no improvement in the histopathological changes when compared with the treatment groups of EL (2000 mg/kg b.w), NEL and insulin. Administration of NEL (20 mg/kg bw) (Figure 11 A, B) and Insulin (Figure 12A, B) showed a better protective effect in the numbers, size and shape of islets and in vacuolar degeneration,. The rats treated

only with EL (2000 mg/kg b.w) and with NEL (20 mg/kg b.w) showed normal histology like control rats, 2-3 islets in a view, normal round Shape and size (Figure 13).

Histopathology for the STZ induced diabetics in rats of kidney

Based on stained tissue sections, there were no notable changes in kidney histology showing normal cortex and pelvis (Figure 14 A, B) normal tubules and glomerulus (Figure 15 A, B) in normal rats. In contrast, STZ administration resulted in severe injury to the kidney causing mesangial proliferation in glomerulus, reduced bowman's space and glomerular hemorrhage resulting in glomerulopathy. Additionally the diabetic rats exhibited tubular dilation in cortex and dilated pelvis (Figure 16A, B) and (Figure 17). Administration of EL (2000 mg/kg b.w) (Figure 18) showed moderate protective effect. Administration of EL (20 mg/kg b.w) (Figure 19A, B), showed no improvement in the histopathological changes when compared with the treatment groups of EL (2000 mg/kg b.w), NEL and insulin. Administration of NEL (20 mg/kg bw) (Figure 20 A, B) and Insulin (Figure 21A, B) showed a better protective effect for tubular dilatation, bowman's space, mesangial proliferation reduced remarkably thus reducing the severity of glomerulopathy.

Histopathology of the kidney treated with EL (2000 mg/kg b.w) and NEL (20 mg/kg b.w) showed normal histological appearances like control rats indicating no toxicity to the rat kidney due to the *E.littorale* (2000 mg/kg b.w) and NEL.

Discussion

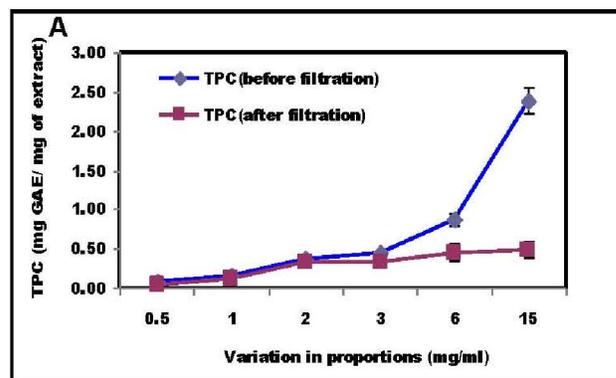
To date, there have been no investigations on nanoemulsified ethanolic extracts of *E.littorale* (NEL) for antidiabetic activity. In the present study, ethanolic extract which contains almost all phytoconstituents and which has poor water solubility, was confined in nanocapsules by emulsion coacervation method. Then the efficacy of NEL was compared with EL for the antidiabetic activity in STZ induced diabetic male rats. When the STZ was injected to adult rats, loss of body weight, polydypsia, change of body coat color and piloerection, characteristics of diabetes mellitus were caused, which is similar to the report [26]. The loss of body weight could be due to dehydration and catabolism of fats and proteins [27, 28]. Type I diabetes is characterized by an absolute deficiency of insulin secretion, associated with auto-immune destruction of pancreatic beta-cells. The increased activities of ALT, AST and ALP in the serum of diabetic rats may be primarily due to the leakage of these enzymes from liver cytosol into the blood stream as a consequence of the hepatotoxic effect of STZ [29-32]. Surprisingly, we found that the required dose of NEL was 100 times lower (20 mg/kg b.w.) than that of EL (2000 mg/kg b.w.) to decrease the glucose levels and other biochemistry parameters within a period of 10 days and significantly increase the body weight of the STZ treated animals. Based on stained tissue sections, there were no notable changes in the pancreas

histology of normal rats. In contrast, STZ administration resulted in severe injury to the pancreas, such as decreasing of the islets cells' number, vacuolation, fibrosis, degeneration diminishing of the diameter of the pancreatic gland. Administration of NEL (20 mg/kg bw), EL (2000 mg/kg b.w) and Insulin showed low expansion of islets and significantly reduced the injuries to the pancreas. These results were supported by diabetes arising from destruction of the beta islet cells of the pancreas, causing degranulation or reduction of insulin secretion [33]. The mechanism of glucose transport and insulin secretion is that glucose transporters carry the glucose into the beta cells which are then metabolized in glycolysis /Krebs cycle and produce large amount of ATP/ADP in the cytoplasm, which leads to closing of (KATP channels) due to ATPsensitive potassium channels. Consequently, the cell membrane is polarized which activates the Ca^{2+} channel. As a result, there is more influx of Ca^{2+} into the cytoplasm which stimulates insulin secretion. In case of EL the mechanism of insulin secretion as reported by [11], is due to beta cell damage by STZ (streptozotocin), the insulin secretion is stopped, thus, the glucose is not metabolized by glycolysis/krebs cycle. Therefore there is an increase in the glucose and there is no ATP production. So the KATP channel is not closed. When *E. littorale* is dosed, the glucose lowering effect is due to potentiation of glucose-induced insulin release through K^{+} -ATP channel dependent pathway but did not require Ca^{2+} influx. But the number of islets was not increased although it is treated with NEL, due to the glucose lowering effect which is due to the KATP channel and not

because of the increase in the number of islets (Figure 22). In contrast, STZ administration resulted in severe injury to the kidney causing glomerulopathy, mesangial proliferation, reduced bowmans space, tubular dilation cortex, glomerular hemorrhage and dilated pelvis in the diabetic rats when compared with normal rats. Administration of NEL (20 mg/kg bw) showed moderate effect; EL (2000 mg/kg b.w) and Insulin showed mild effects on the injuries to the kidney when compared to the diabetic control. These results supported by glomerular expansion have been reported to be a characteristic of experimental diabetic animals and humans with diabetes mellitus [33-36]. This is highly improved due to the nanoemulsion mechanism. Oil-water emulsions are effectively phagocytosed in the liver and stomach which enhances the absorption of the phytomolecules [37] and showed very good effect when compared with the unnanotised ethanolic extract of *E.*

littorale. Sodium alginate polymer used for nanoemulsion which reduces the release of polyphenol content in the acidic pH (1.2) of stomach and a little rapidly in the alkaline pH (7.2) of intestine and there is an maximum absorption in the intestine. Aqueous

extract of *E. littorale* blume (1g/kg b. wt.) and swertiamarin (50mg/kg body wt.), p.o daily for 3 weeks in the type 1 diabetics nephropathy induced with streptozotocin (45 mg/kg) in Sprague-Dawley rats, showed a decrease in serum urea, creatinine and lipid profile and also showed an improvement in the histopathology of the glomerular function [38]. The 1.5 g dry plant equivalent extract of *E. littorale* /100 g body wt. dosed for 20 days showed significant decrease in glycosylated haemoglobin, liver glucose-6-phosphatase activity and significant increase in serum insulin levels of the diabetic rats and toxicity was not observed and concluded that it is a potent antidiabetic agent without any toxic effect at this particular dose [12]. The aqueous extract of *E. littorale* (2 g/kg p.o.) dosed daily for 6 weeks in neonatal non-insulin dependent diabetes mellitus (NIDDM) rats induce with a single dose injection of STZ (70 mg/kg; i.p.) was given to the 5-day-old pups. The oral glucose tolerance test (OGTT) showed decrease in AUC glucose and AUC insulin values, Insulin sensitivity (KITT) index, elevated cholesterol, triglyceride and creatinine in NIDDM treated group [9]. Methanolic extract of whole plant of *E. littorale* blume has been studied to assess its protective effects in alloxan induced diabetic neuropathy in male Charles foster rats. The protective effect of *Encostemma littorale* blume against neuropathy was accredited to controlling hyperglycemia and reducing oxidative stress [39]. The chief problem in using plant extracts for treatment is the high dose that has to be administered in order to deliver the desired therapeutic potential. This issue can be potentially resolved using the nanoemulsification method. The results of the present study are evidence for this. In addition to using a low dose, the nanotized extract lowered the blood glucose levels in a short span of 10 days, which encourages the use of nanotized preparations for therapy.



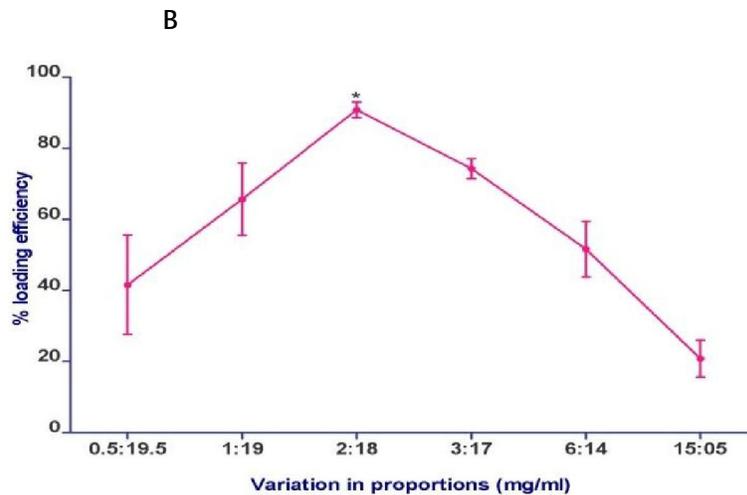
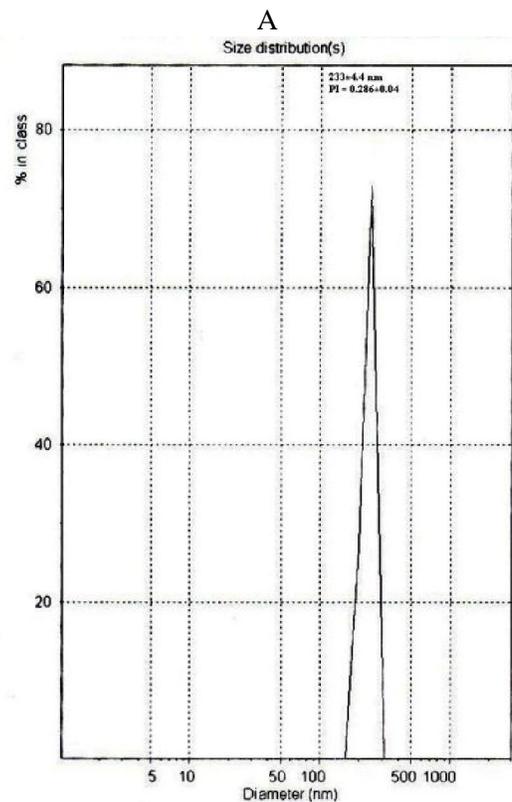


Figure:1 Variation in proportion for (A) Total phenol content and (B) loading efficiency for *E. littoral*. Based on the total phenol content (GEA/mg of extract) the loading efficiency was calculated and the maximum was observed at 2:18 (mg/ml) for *E. littoral* extract:olive oil, which is statistically different from other proportions *-($p < 0.05$, ANOVA)



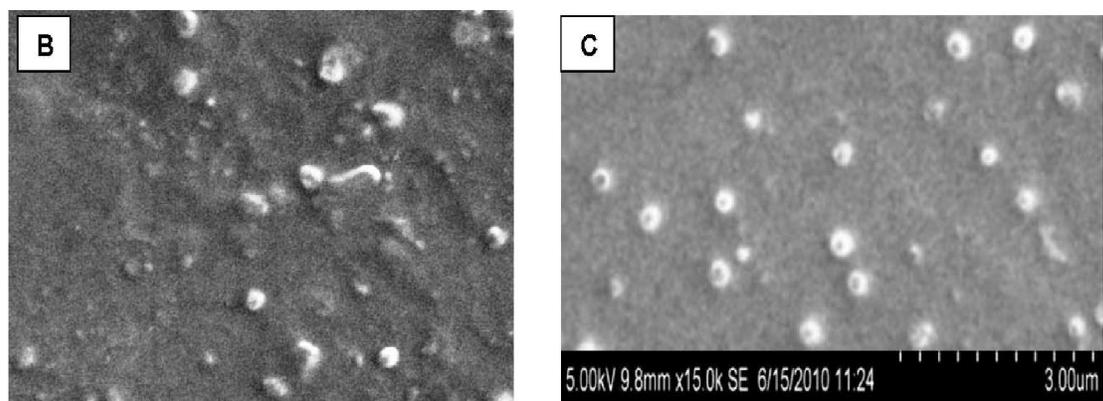


Figure 2. (A) Photon correlation spectroscopy of nanoemulsified *E. littorale* (B, & C) Scanning electron microphotograph of nanoemulsified ethanolic plant extracts of *E. littorale*

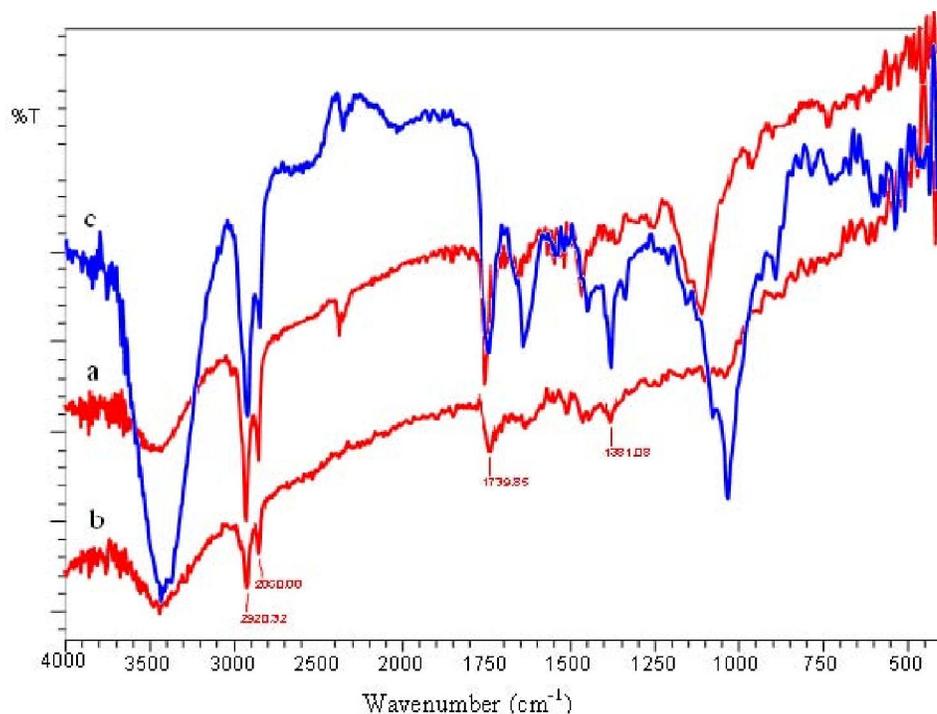


Figure3. FT-IR profile for identification of functional groups. (a) Calcium alginate beads (b) crude plant extract of *E. littorale* and (c) nanoemulsified plant extract with sodium alginate crosslinked with calcium chloride.



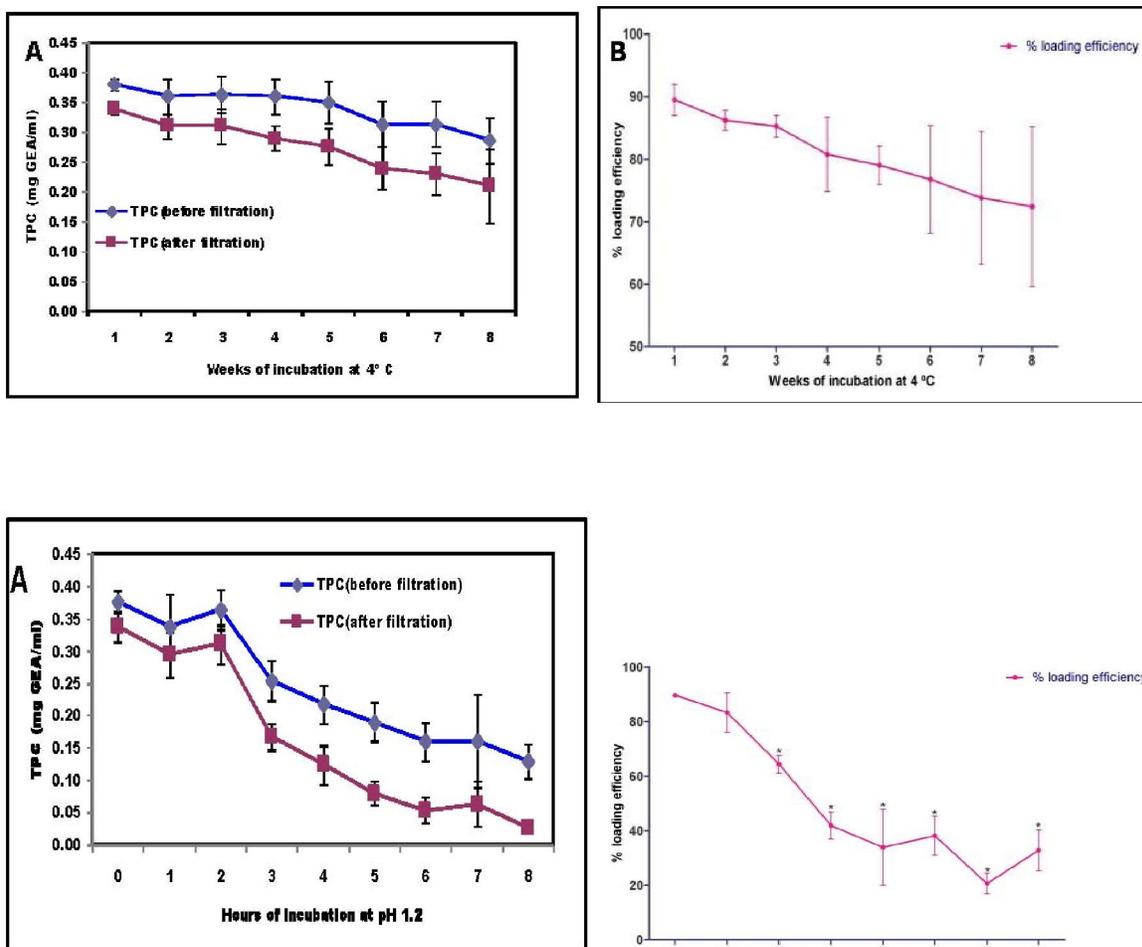


Figure 4. Effect of temperature at (4°C) and (28°C) for (A & C) Total phenol content and (B & D) loading efficiency of *E. littorale*. *-(p<0.05, ANOVA) .



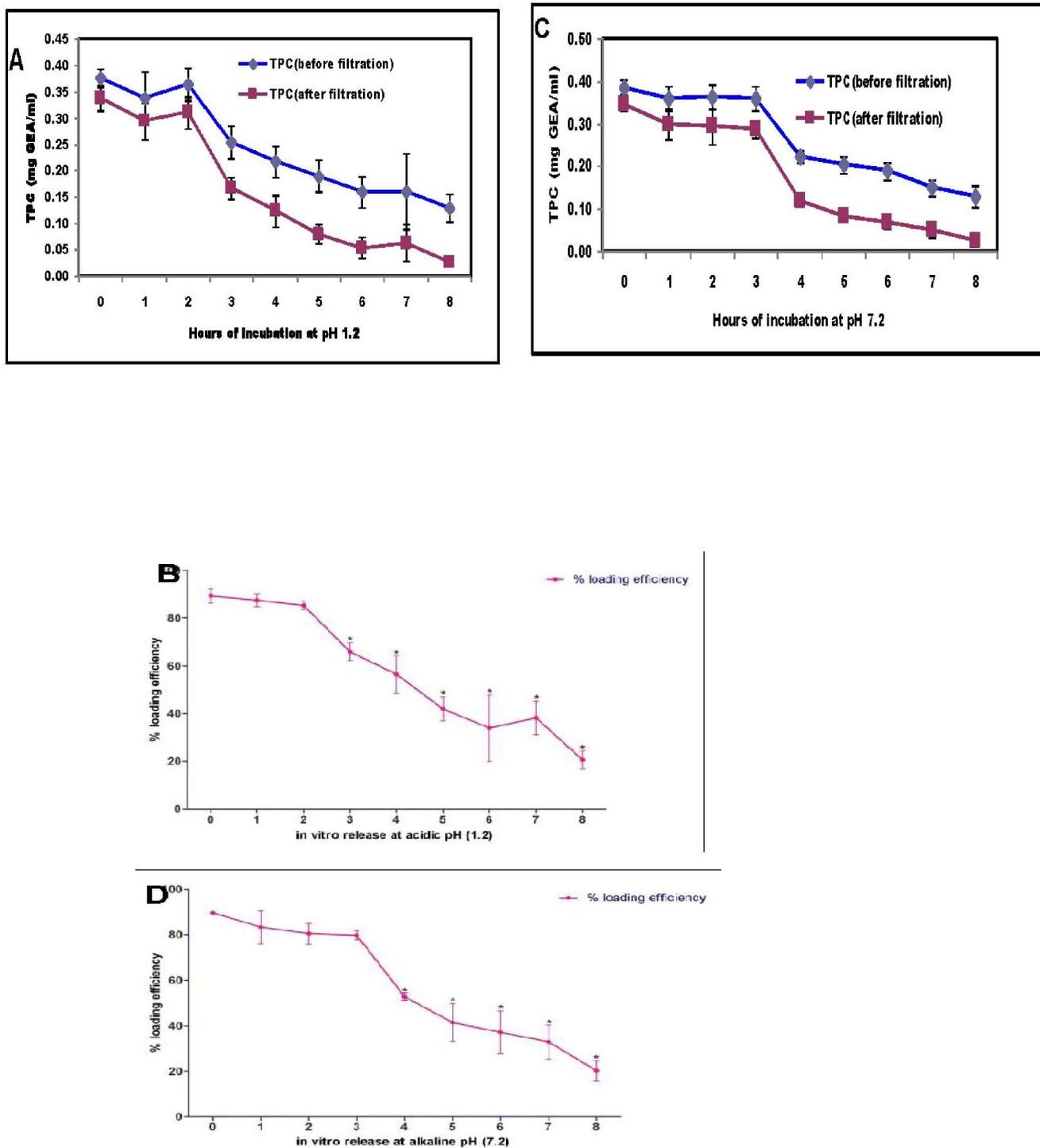


Figure 5. *In vitro* release at acidic pH (1.2) and alkaline pH (7.2) (A & C) Total phenol content and (B & D) loading efficiency of *E. littorale* *-($p < 0.05$, ANOVA).



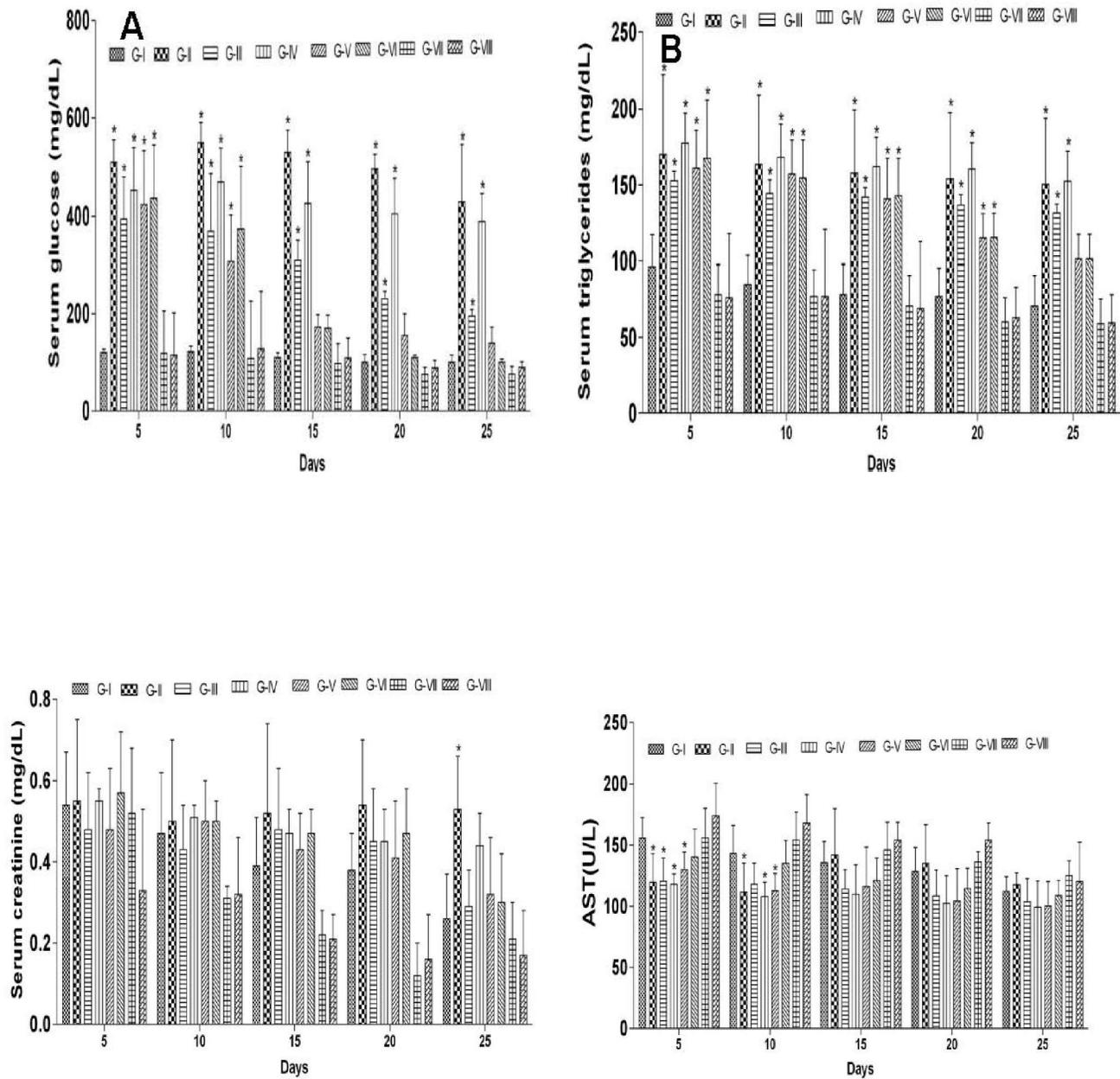


Figure 6. Effect of nanoemulsified ethanolic extract of *E.littorale* and *E.littorale* treatment on (A) Serum glucose in STZ (B) Triglycerides (C) Aspartate transferase (AST) (D) Creatinine (E) Alkaline phosphatase (ALP) (F) Alkaline transferase (ALT) induced type 1 diabetic rats during different days of blood collection. Each bar represents mean \pm S.E.M. number of animals in each group n=6. G1- Non diabetic control, G2-STZ induced diabetic control, G3- ethanolic plant extract (2000 mg/kg b.w), G4 – unnanotised plant extract (20mg/kg b.w), G5 – nanotised plant extract, (20 mg/kg b.w), G6- Insulin (1U/kg), on day 5 blood was collected after STZ induced diabetics, Day 10, 15, 20 and 25 after treatment with nanoemulsified ethanolic plant extract, G7- treated only with plant extract (2000 mg/kg), G8 - Treated only with nanotised plant extract (20mg/kg). * - ($p < 0.05$, ANOVA) Significantly different from non-diabetic control.



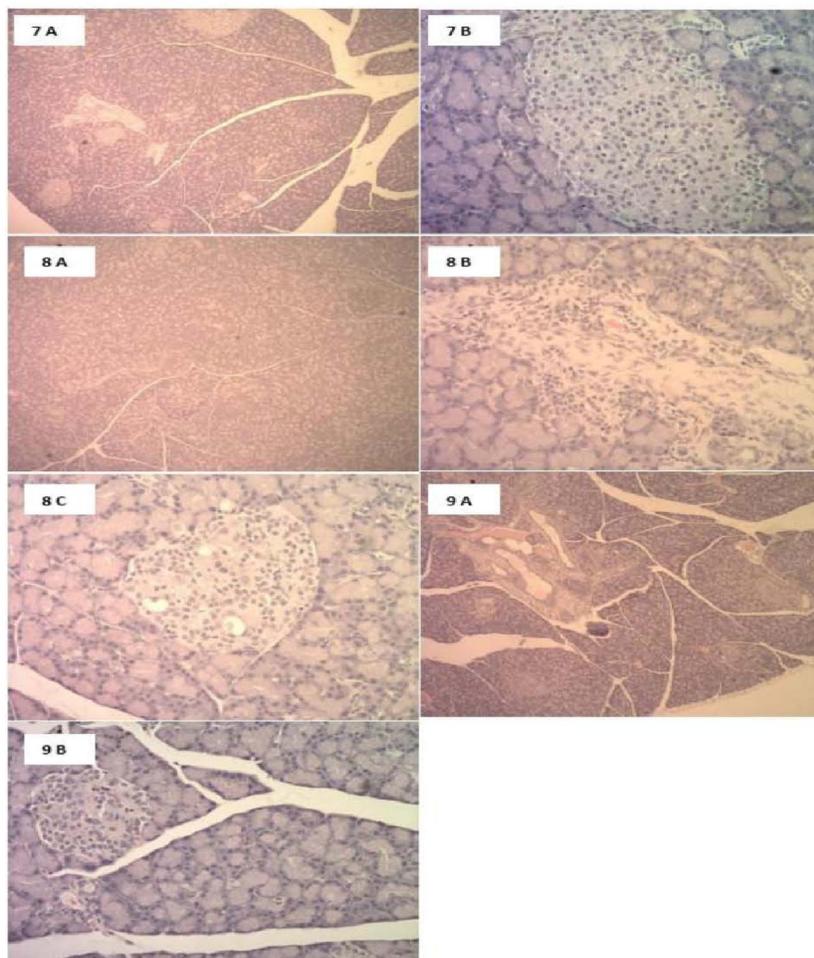


Figure 7. Microphotograph of histopathology of Pancreas of control rat showing (A) 4-5 islets in a view, H&E (40X) (B) Shape, size and numbers of beta cells were normal. H&E (200X).

Figure 8. (A & B) Microphotograph showing histopathology of the pancreas of diabetic rats induced with STZ (A) Number of islets were very less and small sized (H&E, 40X) (B & C) Vacuolation, fibrosis and degeneration in islets. (H&E, 200X) (C) Microphotograph showing histopathology of the pancreas of diabetic rats induced with STZ (B & C) Vacuolation, fibrosis and degeneration in islets. (H&E, 200X)



Figure 9. Microphotograph showing histopathology of the pancreas of diabetic rats induced with STZ +treated with EL (2000 mg/kg b.w) (A) Showed little protective effect on pancreatic islets in regard to number, shape, vacuolation, fibrosis and degeneration when compared with the diabetic control (A) H&E, 40X (B) H&E, 200X

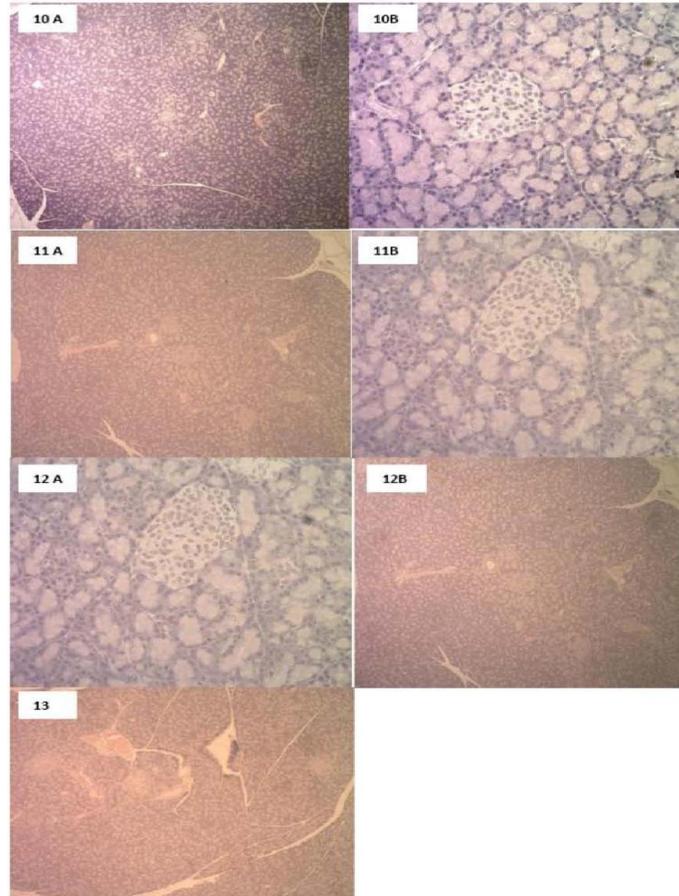


Figure10.

Microphotograph showing histopathology of the pancreas of diabetic rats induced with STZ +treated with EL (20 mg/kg b.w) (A & B) Showed no effect of EL treatment on pancreatic islets when compared with diabetic control. (A) H&E, 40X (B) H&E, 200X

Figure 11.
Figure 12.

Microphotograph showing histopathology of the pancreas of diabetic rats induced with STZ +treated with NEL (20 mg/kg b.w), Showed little more effect to protect the pancreas, increase in number and size of islets, when compared with the STZ induced diabetics + EL of 2000 mg/kg. (A) H&E, 40X (B) H&E, 200X
Microphotograph showing histopathology of the pancreas of diabetic rats induced with STZ +treated with insulin (1 U/kg b.w)), Showed little more effect to protect the pancreas, (A) increase in number and size of islets, when compared with diabetic control. (A) H&E, 40X (B) H&E, 200X



Figure 13.

Microphotograph showing histopathology of the pancreas treated with EL (2000 mg/kg b.w) and with NEL (20 mg/kg b.w) showed normal histology like control rats, 2-3 islets in a view, normal round Shape & size, H&E, 40X.

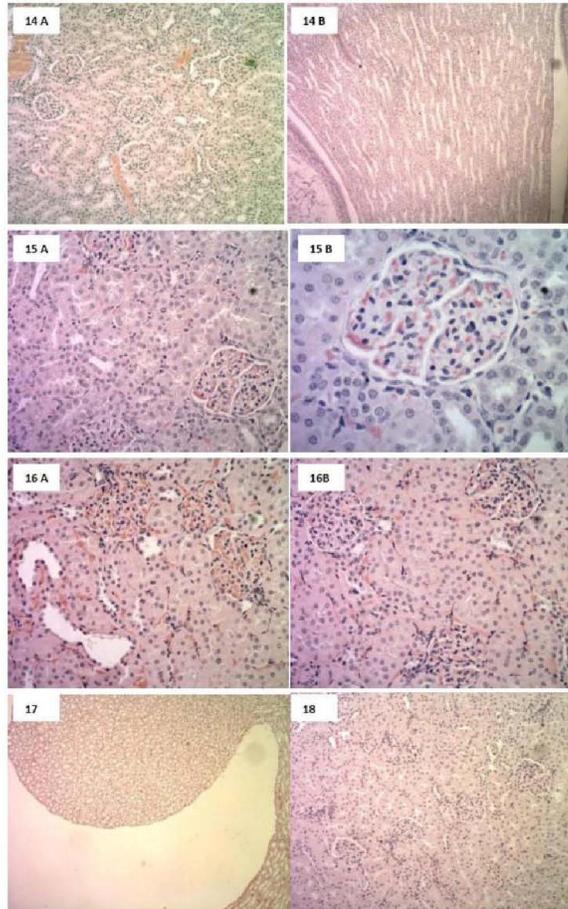


Figure 14.

Microphotograph showing normal histology of the control rat of kidney (A) cortex, H&E, 100X (B) pelvis area, H&E, 40X

Figure 15.

Microphotograph showing normal histology of the control rat of kidney (A) tubules and glomerulus, H&E, 200X and (B) glomerulus H&E, 400X

Figure 16.

Microphotograph showing histopathology of the diabetic rats kidney induced with STZ (A) Tubular dilatation cortex, glomerular hemorrhage , H&E, 200X (B) Glomerulopathy, mesangial proliferation, Reduced bowman's space, H&E, 200X

Figure 17.

Microphotograph showing histopathology of the diabetic rats induced with STZ. Dilated pelvis H&E, 40X.

kidney



Figure 18.

Microphotograph showing histopathology of kidney of the diabetic rats induced with STZ and treated with EL (2000 mg/kg b.w) showed little

protective/ameliorating effect on histological architecture as compared with the diabetic control H&E, 100X

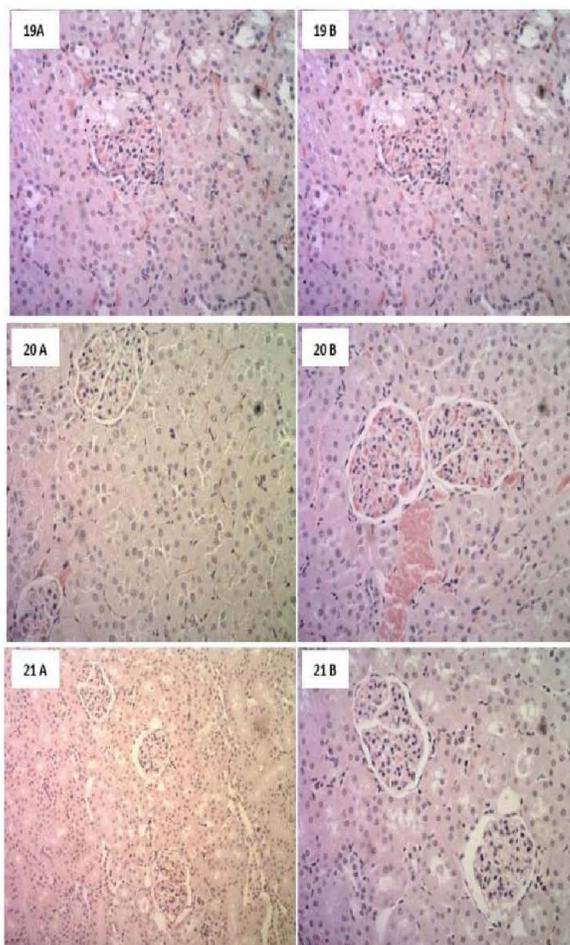


Figure 19.

Microphotograph showing histopathology of the kidney of diabetic rats induced with STZ +treated with EL (20 mg/kg b.w) showed no protective effect when compared with diabetic control (A) Glomerulopathy, mesangial proliferation, Reduced bowman's space H&E, 200X (B) Tubular dilatation, cortex, H&E, 200X.

Figure 20.

Microphotograph showing histopathology of the kidney of diabetic rats induced with STZ +treated with NEL (20 mg/kg b.w), Showed pronounced effect to protect the kidney (A) Glomerulopathy, mesangial proliferation, Reduced bowman's space H&E, 200X (B) Tubular dilatation, cortex, H&E, 200X

Figure 21.

Microphotograph showing histopathology of the kidney of diabetic rats induced with STZ +treated with insulin (1 U/kg



b.w)). Showed pronounced effect to protect the kidney (A) Glomerulopathy, mesangial proliferation, Reduced bowman's space H&E, 200X (B)

Figure 22. Mechanism of insulin secretion from beta cells and the effect of STZ (streptozotocin) for the insulin secretion and treatment with *Encostemma littorale* (EL) and nanoemulsified *Encostemma littorale* (NEL).

Table -1A: Signs of toxicity for STZ induced diabetics treated with NEL and EL from 0 to 12 days.

Groups	Treatment	Observation days												
		0	1	2	3	4	5	6	7	8	9	10	11	12
G1	Negative control	N	N	N	N	N	N	N	N	N	N	N	N	N
G2	STZControl	N	N	N	20	20	20	20	20	20	4,19,20	4,19,20	4,19,20	4,19,20
G3	STZ induced diabetics+2000 mg/kg bw EL	N	N	N	20	20	20	20	20	20	4,19,20	4,19,20	4,19,20	4,19,20
G4	STZ induced diabetics+unnano 20 mg/kg bw EL	N	N	N	20	20	20	20	20	20	4,19,20	4,19,20	4,19,20	4,19,20
G5	STZinduced diabetics+(NEL)20 mg/kg bw	N	N	N	20	20	20	20	20	N	N	N	N	N
G6	STZ induced diabetics+Reference drug (Insulin 1 U/kg bw)	N	N	N	20	20	20	20	20	N	N	N	N	N
G7	Plant extract 2000 mg/kg bw EL	N	N	N	N	N	N	N	N	N	N	N	N	N
G8	NEL (20 mg/kg bw)	N	N	N	N	N	N	N	N	N	N	N	N	N



Table-1 B: Signs of toxicity for STZ induced diabetics treated with NEL and EL in male Wistar rats from 13 to 25 days

Groups	Treatment	Observation days												
		13	14	15	16	17	18	19	20	21	22	23	24	25
G1	Negative control	N	N	N	N	N	N	N	N	N	N	N	N	N
G2	STZ Control	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20
G3	STZ induced diabetics+2000 mg/kg bw <i>E.littorale</i>	14, 19,20	14, 19,20	14, 19,20	N	N	N	N	N	N	N	N	N	N
G4	STZ induced diabetics+ unnano 20 mg/kg bw <i>E.littorale</i>	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20	4,19,20
G5	STZ induced diabetics+ nano 20 mg/kg bw <i>E.littorale</i>	N	N	N	N	N	N	N	N	N	N	N	N	N
G6	STZ induced diabetics+Reference drug (Insulin 1 U/kg bw)	N	N	N	N	N	N	N	N	N	N	N	N	N
G7	Plant extract 2000 mg/kg bw <i>E.littorale</i>	N	N	N	N	N	N ^N	N	N	N	N	N	N	N
G8	Nano (20 mg/kg bw) of <i>E. littorale</i>)	N	N	N	N	N	N	N	N	N	N	N	N	N

- | | | | | |
|-----------------------------|-------------|---------------------|----------------------|-------------------------|
| 1.Alopecia | 6. Coma | 11.Erythema | 16.Nasal irritation | 21.Prostration |
| 2.Catalepsy | 7.Death | 12.Eschar formation | 17.Nostril discharge | 22.Respiratory distress |
| 3.Chromodacryorrhea | 8. Diarrhea | 13.Exophthalmos | 18.Paralysis | 23.Rigidity |
| 4.Change of body coat color | 9. Dullness | 14.Hyperactivity | 19.Piloerection | 24.Salivation |
| 5.Convulsion | 10. Edema | 15.Lacrimation | 20.Polyuria | 25.Tremor |

TABLE – 2: Mean Body Weight of streptozotocin induced diabetics treated with NEL and EL in male wistar rats

Groups	Body weight (g)									
	1	4	7	10	13	16	19	22	25	
G-1	109.17 ± 7.42	128.67 ± 4.44	134.33 ± 3.21	145.67 ± 2.55	154.83 ± 4	165 ± 3.56	176.67 ± 3.19	181.17 ± 11.68	192.67 ± 6.72	
G-2	113.5 ± 6.35	107.17 ± 5.76 *	102.67 ± 5.22*	117 ± 2.95*	122.17 ± 3.21*	127.17 ± 4.53*	133.17 ± 4.28*	137.83 ± 7.79*	140.17 ± 7.83*	
G-3	114.17 ± 5.76	106.5 ± 2.49*	119.17 ± 7.62*	128 ± 3.58*	133 ± 2.7*	138.5 ± 5.22*	143.33 ± 4.38*	149.33 ± 3.13*	154.17 ± 4.97*	
G-4	112 ± 7.5	103.17 ± 3.94*	109 ± 6.88*	116.83 ± 8.43*	126.33 ± 6.11*	130.33 ± 5.27*	134.83 ± 4.38*	138.67 ± 3.08*	145.5 ± 6.31*	
G-5	111.83 ± 4.77	104.17 ± 3.85*	128.17 ± 5.18	138.5 ± 4.18*	143.67 ± 4.04*	152 ± 2.61*	161.83 ± 2.97*	172 ± 1.58	180.33 ± 3.42*	
G-6	112.83 ± 6.26	103.67 ± 4*	121.33 ± 4.56*	135 ± 1.82*	143 ± 4.34*	152.5 ± 4.64*	163 ± 7.97*	173.83 ± 4.15*	183.67 ± 2.59*	
G-7	107.8 ± 8.0	125.8 ± 3.7	130.2 ± 4.6	142.0 ± 2.6	156.2 ± 3.8	164.2 ± 3.3	177.4 ± 4.6	185.8 ± 7.0	193.8 ± 2.8	
G-8	108.4 ± 6.0	123.8 ± 4.4	133.2 ± 2.7	144.8 ± 2.4	155.6 ± 3.4	168.8 ± 3.8	179.8 ± 3.4	188.8 ± 4.8	195.2 ± 2.2	



Table-3: Urinalysis parameters in STZ induced diabetics treated with NEL and EL in male Wistar rats.

URINALYSIS PARAMETERS														
Groups	Colour	Appearance	Specific Gravity	PH	Chemical			Ketone (mg/dL)	Blood	Microscopic				
					Protein (mg/dL)	Glucose (mg/dL)	Bilirubin			WBC	RBC	E	C	CRY
G1	Y	C1	1.025	6.5	30.0	N	N	N	N	N	N	N	N	N
G2	Py	C1	1.010	6.5	Tr	2000	N	5.0	N	N	N	0-1/HPF	N	N
G3	Y	C1	1.030	6.0	N	N	N	15.0	N	N	N	N	N	N
G4	Py	C1	1.020	5.0	30.0	2000	N	5.0	N	0-1/HPF	N	N	N	N
G5	Y	C1	1.030	6.0	30.0	N	N	N	N	N	N	N	N	N
G6	Y	C1	1.025	6.5	30.0	N	N	N	N	N	N	N	N	N
G7	Y	C1	1.030	5.5	30.0	N	N	N	N	N	N	N	N	N
G8	Y	C1	1.025	6.0	30.0	N	N	N	N	N	N	N	N	N

STZ – Streptozotcin, EL-*E. littorale*, NEL-Nanoemulsified *E. littorale*

Py - Pale Yellow, Cl- Clear, Cry-Crystals, Hc- Hyaline Casts, RBC- Red Blood Cells, Dy - Deep Yellow, T –Turbid, C – Casts,Gc - Granular Casts, WBC - White Blood Cells, Y – Yellow, Ap-Appear, S – Small, Sp- Spermatozoa, TP - Triple Phosphate, N – Negative, Tr – Trace, L – Large, M- Moderate, E - Epithelial Cells

Conclusion

Nanocapsules containing ethanolic extract of *Encostemma littorale* were prepared by emulsion coacervation method using sodium alginate. An oral dose of *E. littorale* nanocapsules that is 100 times less than the ethanolic extract of the herb could exhibit similar anti-diabetic effects. Thus, NEL rapidly ameliorates increased glucose levels in diabetic conditions due to sustained release than its unnanotized counterpart. In addition, the preparation did not invoke any toxic response in the

experimental animals, thereby proving its safety. Hence, nanoemulsification can be efficiently utilized for ethanolic extracts with high therapeutic potential such as *E.littorale*. The efficiency and stability of the extracts are enhanced by this method. Furthermore, this method can be efficiently utilized for other plant extracts which are poorly soluble in water.

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