

Development, Characterization and Antidiabetic Potentials of *Nilgirianthus ciliatus* Nees Derived Nanoparticles

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Abstract

Nilgirianthus ciliatus Nees is used in traditional Indian system of medicine for the treatment of numerous physiological disorders. Previous investigations revealed that various bioactive compounds have been isolated from this plant for the treatment of various disorders. But these compounds may be unstable in gastrointestinal tract due to the elevated pH and harsh condition that renders the bioactive compounds ineffective. In order to improve the pharmacokinetic and pharmacodynamic properties of various drugs, nanoparticles were developed by various techniques. Nanoformulation of ethanolic extract of *Nilgirianthus ciliatus* was prepared by solvent evaporation technique using gelatin as carriers and evaluated its antidiabetic effects in L6 myoblasts and 3T3L1 adipocytes. The mean particle size of plant extract loaded gelatin nanoparticles was approximately 110 nm and they are spherical in shape on observation by transmission electron microscope. The ethanolic extract of *Nilgirianthus ciliatus* was encapsulated with 80% efficiency in gelatin biodegradable nanoparticles formulation. MTT assay was also performed to study the toxicity of nanoformulation; the nanoencapsulated form does not produce any toxicity upto 1000 µg/ml concentrations. Nanoformulation of *Nilgirianthus ciliatus* was evaluated for its antidiabetic activity by glucose uptake assay in L6 cells and anti-adipogenic assay in 3T3L1 preadipocytes. The plant extracts loaded nanoparticles showed significant antidiabetic effect in both assays in a dose dependent manner when compared to the ethanolic extract. After nanoencapsulation the aqueous dispersion is more, this may be the reason for the better antidiabetic effect. Therefore nanoencapsulated *Nilgirianthus ciliatus* could be considered as potential antidiabetic drug.

Keywords: *Nilgirianthus ciliatus* Nees; Nanoencapsulation; Gelatin; Drug release study; Antidiabetic activity; Cytotoxicity

Introduction

Diabetes mellitus is a group of chronic diseases affecting approximately 4% of the population worldwide is expected to be increased to 5.4% in 2025 [1]. 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 [2], nephropathy [3] and neuropathy [4] lead to long-term damage, dysfunction, and failure of various organs such as eyes, kidneys, nerves, heart and blood vessels, creating a huge economic burden related to the management of diabetic complications [5]. Modern medical care uses a vast array of lifestyle and pharmaceutical interventions aimed at preventing and controlling hyperglycemia. New drugs are frequently being tested and new approaches developed to prevent and treat diabetes. Some of the current therapeutic options for diabetes include diet, exercise, use of carbohydrate digestive enzyme inhibitors [6], thus limiting intestinal absorption of glucose and the use of insulin therapy, insulin mimetics [7], and insulin secretagogues [8] to enhance cellular glucose uptake. However, treatment with oral hypoglycemic agents is associated with adverse effects related to pharmacokinetic properties, hypoglycemia, gastrointestinal discomfort, skin reactions, nausea, hematological disorders, weight gain and rise in hepatic enzyme level. Treatment of diabetes without side effects and dyslipidemia is still a challenge to the medical community. As an alternative, a greater number of people are seeking herbs/natural products to prevent or treat diabetes. For thousands of years plants and their derivatives are being used for treatment of diabetes mellitus. Although, herbal medicines have long been used effectively in treating diseases throughout the world and frequently considered to be less toxic and free from side effects as

compared to synthetic ones [9,10]. 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 [11]. However, the major drawback is that the quantity of herbal extract necessary for treatment is higher due to the degradation of different plant constituents such as alkaloids, amides, phenols, steroids, and hydrocinnamic acid in the gastrointestinal tract since they are very sensitive to the acidic pH of the stomach, which promotes their destruction, and loss of the desired effect and a longer duration of treatment is needed due to the poor absorption of these constituents in the intestine [12]. Recently, many studies focused on encapsulation of the plant extracts to augment sustained release of active constituents in the intestine for maximum absorption [13,14]. Currently, nanotechnological processes involving medicinal plants have provided innovative delivery systems include controlled drug delivery to their site of action by developing into polymeric nanoparticles. The biodegradable and biocompatible polymers such as chitosan, gelatin and sodium alginate represent an option for controlled drug delivery [15]. Applying nanotechnology to plant extracts has revealed an advantageous strategy for herbal drugs including the enhancement of

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solubility, bioavailability, pharmacological activity, sustained delivery, protection from toxicity, physical and chemical degradation [16].

Nilgiranthus ciliatus Nees Brenek belongs to the family Acanthaceae. Acanthaceae family plants have got high reputation in traditional medicinal practice due to its extraordinary medicinal properties. Thorough review of ethnopharmacological, ethanobotanical and modern scientific validation data revealed that not much work has been done on this plant in terms of its bioactivity related to diabetes and nanoparticle synthesis. Since, the present study has been designed to validate the antidiabetic potential of the plant *Nilgiranthus ciliatus* Nees and herbal extract derived nanoparticles.

Materials and Methods

Nilgiranthus ciliatus Nees was collected from Kerala and taxonomically identified by Dr. P. Jayaraman, PARC, Chennai. Gelatin type A was purchased from sigma Aldrich. L6 myoblasts and 3T3L1 preadipocytes were procured from National Centre for Cell Science, Pune, India. MTT-(3-(4, 5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide was obtained from SRL chemicals. Phosphate buffered saline and antibiotics were obtained from Gibco Invitrogen. Foetal bovine serum and Dulbecco's Modified Eagles Medium were obtained from Lonza. Rosiglitazone, insulin and glucose were obtained from Sigma Aldrich, USA. Glucose oxidase-peroxidase (GOD-POD) kit was obtained from Accurex Biomedical Pvt. Ltd. All chemicals and solvents used were of analytical grade.

Preparation of *Nilgiranthus ciliatus* extract

The freshly collected plant was dried completely under control conditions without any moisture content. The dried plant was then pulverized and made into fine powder. The powdered samples were macerated with petroleum ether to remove fatty substances; the marc was further extracted with ethanol by cold maceration method [17]. The crude extract was separated by filtration and concentrated on rotary evaporator. The concentrated extract was stored in a desiccator for further use in the subsequent experiment.

Preparation of calibration curve

A 10 µg/ml solution of *Nilgiranthus ciliatus* extract in ethanol and phosphate buffered saline buffer (PBS) pH 7.4 were scanned in UV range between 200 to 600 nm (Shimadzu UV-1800 spectrophotometer). *Nilgiranthus ciliatus* showed maximum absorbance at 402 nm in ethanol and 408 nm in PBS. Various concentrations ranging from 5 to 50 µg/ml solutions were prepared in ethanol and PBS; measured the absorbance at respective nm. The calibration graph was plotted with respect to absorbances (Figure 1).

Preparation of extract loaded gelatin nanoparticles

The nanoparticles of *Nilgiranthus ciliatus* Nees extract with polymer was prepared by solvent evaporation method [18]. Briefly, the required quantity of dry extract was dissolved in 10 ml of ethanol by sonication at 20 watts for 60 seconds. This solution was acted as organic phase. The organic phase was then slowly added drop wise with a syringe into aqueous phase containing gelatin and tween 80 with continuous magnetic stirring at 1000 rpm. After 1 h, 0.01% of glutaraldehyde solution was added to cross link the gelatin and the mixture was continuously stirred for 7 h at room temperature to allow solvent evaporation and nanoparticles formation. A blank nanoparticle containing the polymer and surfactant (without extract) was also formulated for comparative studies. The nanoparticles were separated by centrifugation at 25,000 xg for 30 min; the pellet was re-suspended in MilliQ water and washed three

times with water. The resulting suspension was dried on a lyophilizer and stored at 40°C until further use.

Method optimization

The nanoformulation method was optimized by varying the concentration of polymer and the formulation (Table 1) were coded with NCF1, NCF2, NCF3, NCF4 and NCF5. The prepared nanoformulations were characterized for entrapment efficiency, particle size, Zeta potential, polydispersity index and surface morphology.

Characterization

Particle size and polydispersity index: Average particle size and polydispersity index were measured by dynamic light scattering (DLS) (Malvern nano Zs, Malvern Instruments), equipped with vertically polarized light supplied by an argon-ion laser. Particle size measurement was performed at a fixed angle of 90° at 25°C [19]. The sample was prepared by dispersing the nanoparticles in distilled water and sonicated for 6 min in an ultrasonic bath to obtain a well-dispersed suspension. All the measurements were performed in triplicate and presented as mean ± SD.

Zeta potential analysis: Surface charge of the nanoparticles was also determined by DLS (Malvern instruments). Zeta potential data's were measured through electrophoretic light scattering at 25°C, 150 V in triplicate measurements for every sample (Table 2). Zeta potential is based on the charge conductivity principle to ensure the stability of the formulation [20].

Surface morphology: The surface morphology of the plant extract loaded gelatin nanoparticles was measured by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For TEM analysis, samples were prepared by dispersing the samples in double distilled water and placing a drop of the dispersion over the copper grid by using the micro pipette, and samples were allowed to dry in order to get the water vaporized. Digital micrograph and soft imaging viewer software were used to perform the image capture analysis including particle sizing [21].

For SEM analysis, samples were prepared by placing a pinch of the sample on the carbon tape and stick to the grid, a thin gold was coated to a thickness of 100 Å then the image was captured for the analysis [22].

Fourier transform infrared spectroscopy (FTIR) technique: In order to check the integrity (compatibility) of drug in the formulation, FTIR spectra of the samples (extract, polymer and mixture of extract and polymer) were recorded in the region of 4000-400 cm⁻¹ by using SHIMADZU FTIR-8400S spectrophotometer. In the present study, Potassium Bromide (KBr) pellet method was employed. The samples

S.No.	Formulation code	Extract: polymer ratio	Extract in mg	Polymer in mg	Surfactant in %
1	NCF1	01:01	20	20	0.5
2	NCF2	01:02	20	40	0.5

Table 1: Method optimization parameters.

S.No	Formulation code	Particle size	Polydispersity index	Zeta potential	Entrapment efficiency %
1	NCF1	116.4 ± 2.81	0.210 ± 0.98	-13.4 ± 1.34	70.81 ± 0.86
2	NCF2	129.5 ± 3.30	0.221 ± 0.32	-16.4 ± 2.11	74.48 ± 1.24
3	NCF3	117.7 ± 3.64	0.143 ± 0.84	-17.2 ± 2.31	82.23 ± 1.31
4	NCF4	133.7 ± 2.42	0.126 ± 0.21	-17.9 ± 3.10	81.16 ± 1.86
5	NCF5	161.4 ± 4.20	0.207 ± 0.11	-17.7 ± 1.78	74.29 ± 1.18

Table 2: Physicochemical properties of prepared nano-formulations.

were thoroughly blended with dry powdered potassium bromide. The samples were compressed as discs by applying pressure of 5 tons for 5 min in a hydraulic press. The disc was placed in the light path of spectrophotometer and the spectrum was recorded [23]. The FTIR spectra of the formulations were compared with the FTIR spectra of the pure drug and the polymers.

Entrapment efficiency: Encapsulation Efficiency (EE) of extract loaded gelatin nanoparticles was analyzed using UV-visible spectrometry method [24]. The formulation was ultra-centrifuged at 25000 g for 30 min in a cooling centrifuge apparatus at 10°C (REMI C24 centrifuge, REMI Instruments Limited, India). The supernatant solution was diluted suitably with water to measure the absorbance from which the concentration of drug in supernatant was calculated using the standard calibration data. The entrapment efficiency was calculated using the formula-

$$\%EE = \frac{\text{Actual Drug Content}}{\text{Theoretical drug content}} \times 100$$

In vitro drug release: The drug release study was monitored by dialysis method [25]. The 5 mg of freeze dried samples were dispersed in water and kept in the dialysis bag (molecular weight cut off: 12 kDa to 14 kDa, surface area of 22.5 cm²); both the ends were tied. The sealed bag was submerged in a beaker containing release media (phosphate buffered saline) PBS buffer (pH 7.4, 70 ml) and the whole assembly was placed in an automated shaker maintained at 100 rpm and 37°C. At selected time intervals 1 ml of release media was collected and replaced with equal volume of fresh medium. Then the media was quantified using a sophisticated spectrophotometer. Aliquots withdrawn were assayed for amount of drug released at each time interval released at 402 nm using double beam UV-spectrophotometer by keeping phosphate buffer pH 7.4 as blank. Overall three trials were carried out.

In order to describe the kinetic and mechanism of drug release, the data obtained from the *in vitro* drug release study of nanoparticles were fitted with different kinetic equation like zero order [26] (cumulative% release vs. time), first order [27] (log% drug remaining vs. time), Higuchi's model [28] (cumulative% drug release vs. square root of time) and Korsmeyer-Peppas model [29] (log cumulative drug released vs. log t). The most excellent fitted kinetic model of the dissolution data was evaluated by comparing the regression coefficient (r²) values of the various models. The release exponent "n" value of the Korsmeyer-Peppas model used to characterize the different mechanisms of drug release from polymeric systems. The release exponent n ≤ 0.5 means Fickian diffusion release, 0.5 < n < 1 means non-Fickian release (anomalous) and n > 1 means zero order release.

Identification of phytochemical constituents in the nanoformulation: The freshly prepared nanoformulation containing plant extract was subjected to phytochemical screening for identifying the primary and secondary metabolites such as flavonoids, alkaloids, steroids, phenolic compounds, saponins and tannins using the standard procedure [30].

Cell culture procedure: The L6 myoblasts and 3T3L1 preadipocytes were cultured in DMEM (Dulbecco's Modified Eagle's Medium) with 10% Foetal Bovine Serum (FBS) and supplemented with penicillin (120 units/ml), streptomycin (75 µg/ml), gentamycin (160 µg/ml) and amphotericin B (3 µg/ml) at 37°C in 5% carbon dioxide atmosphere. For the differentiation of L6 myoblasts, the cells were grown in DMEM for 4 days with 2% FBS, post confluence. The differentiation level was measured by observing the multinucleated cells. For the differentiation of 3T3L1 preadipocytes into adipocytes, the cells were grown in 24

well plates for 2 days and the cells were induced by the differentiation medium containing the mixture of 0.5 mM/l of IBMX, 0.25 µM/l of DEX and 1 mg/l of insulin in DMEM medium with 10% FBS. After induction for three days, the differentiation medium was substituted with fresh medium containing 1 mg/ml insulin. After 2 days the medium was replaced again with fresh culture medium (DMEM with 10% FBS) subsequently and the degree of differentiation was established by monitoring the multinucleated cells.

Cytotoxicity assay: MTT assay is a colorimetric technique was used for measuring energetic cell metabolism by determining the activity of one oxidative enzyme [31]. It is based on the reduction of water soluble yellow tetrazole compound by mitochondrial succinate dehydrogenase enzyme into insoluble violet formazan crystals. In this assay, the L6 myoblasts and 3T3-L1 preadipocytes were seeded at a density of 1 × 10⁴ in a 96 well plate and were allowed to attach to the well plate or 24 h, cells were treated with different increasing concentrations of extract and extract loaded nanoparticles. At 48 h, following plating, the cells were incubated with 100 µl of fresh medium supplemented with MTT solution (0.25 mg/ml) for 45 min. The supernatant culture medium was aspirated and the insoluble formazan crystals were dissolved in DMSO for at least 2 h in the dark. MTT reduction was quantified by measuring the absorbance at 570 nm using a microplate reader. The effect of plant extract and its nanoparticles on cell viability was expressed using the formula:

$$\text{Percent viability} = \frac{(\text{OD of untreated cells} - \text{OD of treated cells})}{\text{OD of untreated cells}}$$

Glucose uptake assay: Glucose uptake activity was determined in differentiated L6 cells by method described by Pareek et al. [32]. The ability of plant extract and polymer loaded nanoparticles to induce glucose uptake was measured in two different ways i.e., glucose uptake in absence of insulin (extract nanoparticles alone) and in presence of insulin (extract and nanoparticles with insulin). Cells were grown on 24 well plates and washed twice with serum free DMEM and incubated with same medium for 2 h. The cells were washed three times with Krebs Ringer Phosphate (KRP) buffer and incubated with KRP buffer contains 0.1% BSA for 30 min at 37°C. The cells were treated with different non-toxic concentration of extract, nanoparticles, standard drug, insulin and added glucose (1M) and incubated at 37°C for 30 min. The glucose uptake was terminated by washing the cells thrice with ice cold KRP buffer solution. Cells were subsequently lysed by freezing and thawing thrice and an aliquot of cell lysates were used to measure the cell associated glucose. Glucose uptake was measured as the difference between the initial and final glucose content in the incubated medium by Glucose Oxidase Peroxidase (GOD-POD) method [33].

One ml of reagent was mixed with 10 µl of sample and incubated for 10 min at 37°C; within 60 min measured the absorbance of sample and standard at 510 nm against reagent blank. The time interval from sample addition to measured time must be exactly same for standard, control and sample.

Anti-adipogenic assay: 3T3-L1 preadipocyte was cultured in maintenance medium comprised of DMEM containing 10% Fetal Bovine Serum (FBS) and antibiotics. 3T3-L1 preadipocyte was cultured in maintenance medium comprised of DMEM containing 10% FBS and antibiotics. As previously described, the 3T3L1 preadipocytes differentiated into adipocytes by the combination of insulin, DEX (Dexamethasone) and IBMX (Isobutylmethylxanthine) (day 0). After 3 days of induction; the differentiation medium was substituted with 10% FBS-DMEM containing insulin (1 mg/l) for 48 h (day 5) [34].

Subsequently the medium was replaced again with fresh medium for 48 h (day 7). The quantity of differentiation was examined from day 0 by adding different concentration of extract and nanoparticles ranging from 15.6 µg/ml to 250 µg/ml, time duration of complete induction and post induction periods. On the other hand, preadipocytes were also maintained with fresh FBS–DMEM for the entire induction period. At the end of the induction period oil red O staining assay [35] was performed to monitor the degree of differentiation. For the comparison of triglyceride accumulation photo microscopic assessment was also carried out.

Oil red O staining: Briefly, the 3T3-L1 adipocytes were washed twice with phosphate buffered saline at pH 7.4 and fixed with 10% formalin for 30 min. Cells were rinsed with deionized water and then stained with oil red O (0.25% w/v in 60% Isopropanol) at room temperature for 30 min. Finally, the dye retained in the 3T3-L1 cells was extracted with isopropanol and quantified by measuring the absorbance at 540 nm using microplate reader. The relative lipid contents were calculated from (OD of sample-OD of non-differentiated control ÷ OD of untreated control-OD of non-differentiated control) × 100.

Statistical analysis: All sample results were presented as mean ± standard error mean (SEM). The significant differences in the treatments means were evaluated by one-way analysis of variance (ANOVA) test followed by Dunnet's t test. The criterion for statistical significance was considered at P<0.001.

Results and Discussion

For the past few decades there is a significant research attention in the area of drug delivery systems. Biodegradable nanoparticles have been used frequently used as drug delivery vehicles due to their high bioavailability, good encapsulation properties, and relatively lack of toxicity [36]. Different nano-sized carriers, such as nanoparticles, polymeric micelles, liposomes, surface-modified nanoparticles and solid lipid nanoparticles [37-40], have been developed and suggested for achieving these goals. As the basis for a natural encapsulation agent, gelatin is widely used in a number of formulations because of its biocompatibility, biodegradability, and low antigenicity. Gelatin nanoparticles have been used for delivery of different drugs, gene delivery, as carriers to deliver drug to lungs, and recently antibody modified gelatin nanoparticles were used to target lymphocytes, leukemic cells and primary T-lymphocytes [41,42]. Gelatin is obtained by partial hydrolysis of the fibrous, insoluble protein, collagen, which is main fibrous protein widely found as the major constituent of skin, bones and connective tissue.

In pharmaceuticals, gelatin is normally used in the shells of capsules to make the powdery content easier to transport by ingestion [43] and even engineered nanoparticles have successfully been encapsulated in gelatin nanoparticles or coated with gelatin, with efficient loading and drug release properties [44]. Because of these advantages, the technology of nano-encapsulation has been extended to natural products to protect them from chemical damage and product degradation, especially from air oxidation [45]. Biodegradable nanoparticles have been used frequently as drug delivery vehicles due to their better encapsulation properties, high bioavailability and relative lack of toxicity. Ethanolic extract of *Nilgiranthus ciliatus* was reported to have appreciable inhibitory activities against the key enzymes related to type II diabetes mellitus namely intestinal α-glucosidase and pancreatic α-amylase [46]. Because of these advantages, *Nilgiranthus ciliatus* Nees encapsulated gelatin nanoparticles has been prepared by solvent evaporation technique using tween 80 as surfactant, tested its toxicity profile and confirming their antidiabetic activity using cell line studies. Nanoparticles achieved by this method were found to be simple, rapid, and cost effective and thus suggested because of its suitability for large scale production.

Characterization

Particle size and Zeta potential: Physicochemical properties such as size, morphology and charge are the critical factors that influence the functional performance of any nanoparticles based delivery systems [47]. We therefore measured the particle size distribution pattern of nanoparticle (Figure 2). From the results, the calculated average particle size of nanoformulation NC1, NC2, NC3, NC4 and NC5 were 116.4, 129.5, 117.7, 133.7 and 161.4 respectively with the narrow distribution of Polydispersity Index (PDI). Its values were less than 0.3, indicates a high degree of homogeneity in particle. The higher particle size is probably due to the high viscosity of the polymer solution that slow down the appropriate diffusion of the solvent toward the nonsolvent. The formation of large aggregates due to higher concentration of polymer was also previously reported by other authors. The zeta potential values lies between -16.4 to -17.9 mV (Figure 3), suggesting the stability of nanoparticles. In general, zeta potential values from +30 mV to -30 mV are considered as a standard value in providing enough repulsion forces to avoid particle aggregation [48]. These results agree with the published reports by others. Average particle size of PLGA encapsulated ethanolic extract of plant sample (*Gelsemium sempervirens*) was 122 nm and zeta potential was 14.8 mV [49].

Encapsulation efficiency: Amount of *Nilgiranthus ciliatus* Nees extract entrapped in the polymeric nanoparticles determined using UV-VIS spectrophotometer. The results showed that nanoparticles formulation had higher entrapment efficiency in the range of 70% to

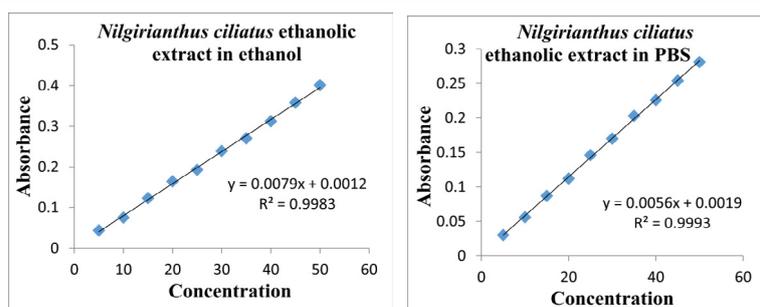


Figure 1: Calibration curve of *Nilgiranthus ciliatus* nees in ethanol at 402 nm and in PBS at 408 nm. Phenolic content was measured in the ethanolic extract which contributes to antidiabetic effect.

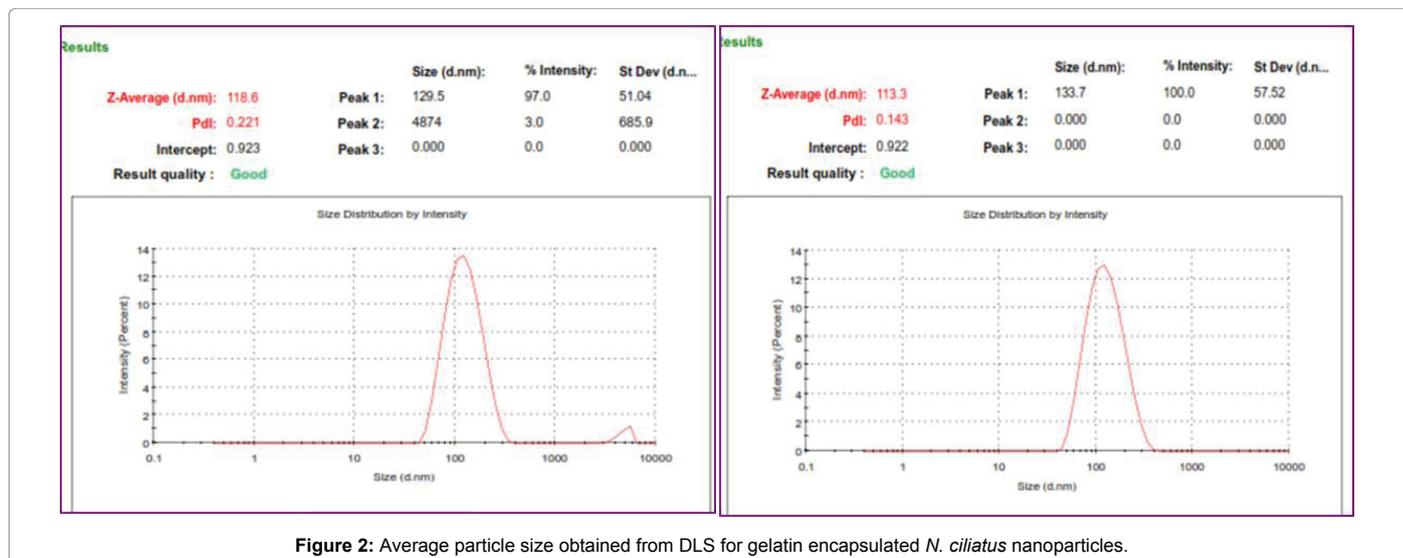


Figure 2: Average particle size obtained from DLS for gelatin encapsulated *N. ciliatus* nanoparticles.

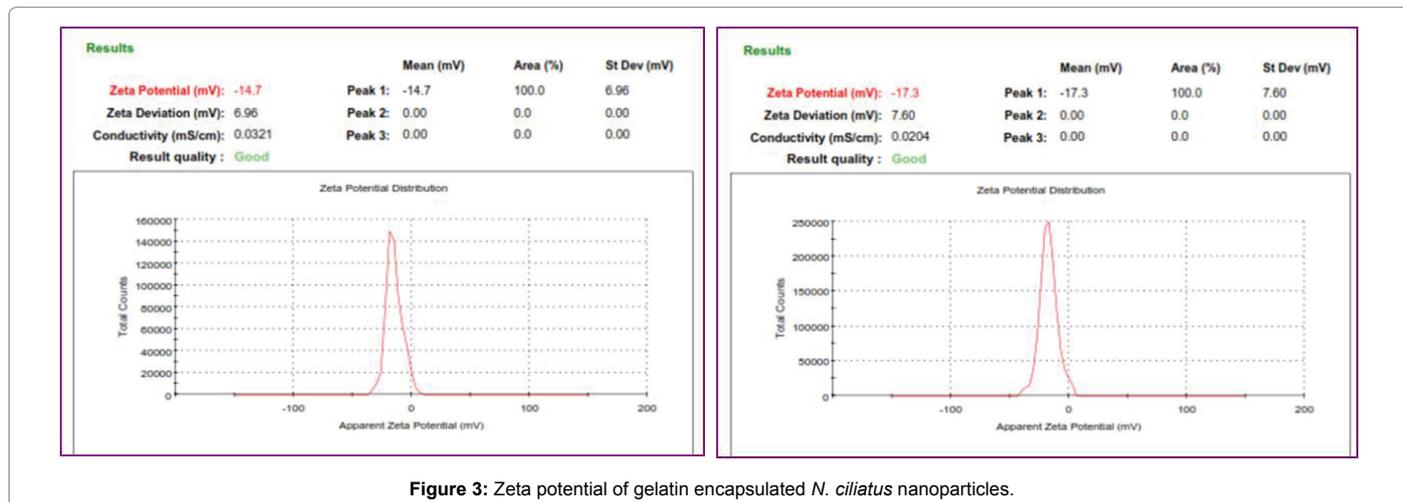


Figure 3: Zeta potential of gelatin encapsulated *N. ciliatus* nanoparticles.

82%. There was no significant difference observed in the formulations prepared with different concentrations of the polymer, since the surfactant level, amount of extract used and all other process variables were kept constant during the nanoparticles development. NCF3 has the greatest encapsulation efficiency because of smaller particle size and greater surface area. The overall encapsulation efficiency of the *N. ciliatus* extract in gelatin nanoparticles is 76% and this was significantly higher than the entrapment ratio of conventional gelatin nanoparticles (<45%) as reported by Saxena [50] and Vandervoort [51] and their respective co-workers. Based on these results, it was concluded that gelatin is a suitable carrier for the encapsulation of phytochemical extracts.

Surface morphology analysis: The structure of the nanoparticles plays an important role in determining their adhesion to and interaction with cells. The features of morphology of gelatin-encapsulated drug under scanning electron microscopy (Figure 4) and transmission electron microscopy image (Figure 5) displays a spherical shape of nanoparticles with a smooth surface with the size range of majority of the particles at below 100 nm. The gelatin encapsulated *N. ciliatus* nanoparticles lying in the optimal size range (below 200 nm) is suitable for drug delivery applications.

FTIR: The FTIR spectrum of gelatin, *N. ciliatus* and gelatin encapsulated plant extract nanoparticles showed in Figure 6. FTIR studies showed that significant peaks of gelatin appear at 3438 cm^{-1} (OH stretching), 2919 cm^{-1} (C-H stretching), 2856 cm^{-1} (C-H stretching), 2318 cm^{-1} (C-H stretching), 1640 cm^{-1} (C=O stretching), 1453 cm^{-1} (C-H stretching), and 1231 cm^{-1} (C-O stretching). *N. ciliatus* showed peaks at 3397 cm^{-1} (OH stretching), 2930 cm^{-1} (C-H stretching), 2127 cm^{-1} (C=C stretching), 1712 cm^{-1} (C=O stretching), 1632 cm^{-1} (C=O stretching), 1443 cm^{-1} (C-O stretching), 1261 cm^{-1} (C-O stretching), 1164 cm^{-1} (C-O stretching), 929 cm^{-1} (aromatic C-H bending) and 768 cm^{-1} (aromatic C-H bending). The common peaks that appear both in *N. ciliatus* and gelatin encapsulated nanoparticles were 3409, 2927, 2128, 1646, 1453, 1373, 1248, 1079, 876 and 810. From the FTIR profile, it is confirmed that all functional groups in the extracts were present after interaction with gelatin polymer also. It implies that FTIR spectrum of plant extracts and its nanoparticles does not show any significant changes. This result indicated that nanoparticles and polymers had all the characteristic peak and band values of extracts confirming the functional groups of extracts are well preserved.

In vitro drug release: The *in vitro* release study of *Nilgiranthus ciliatus* loaded gelatin nanoparticles was studied in phosphate buffer

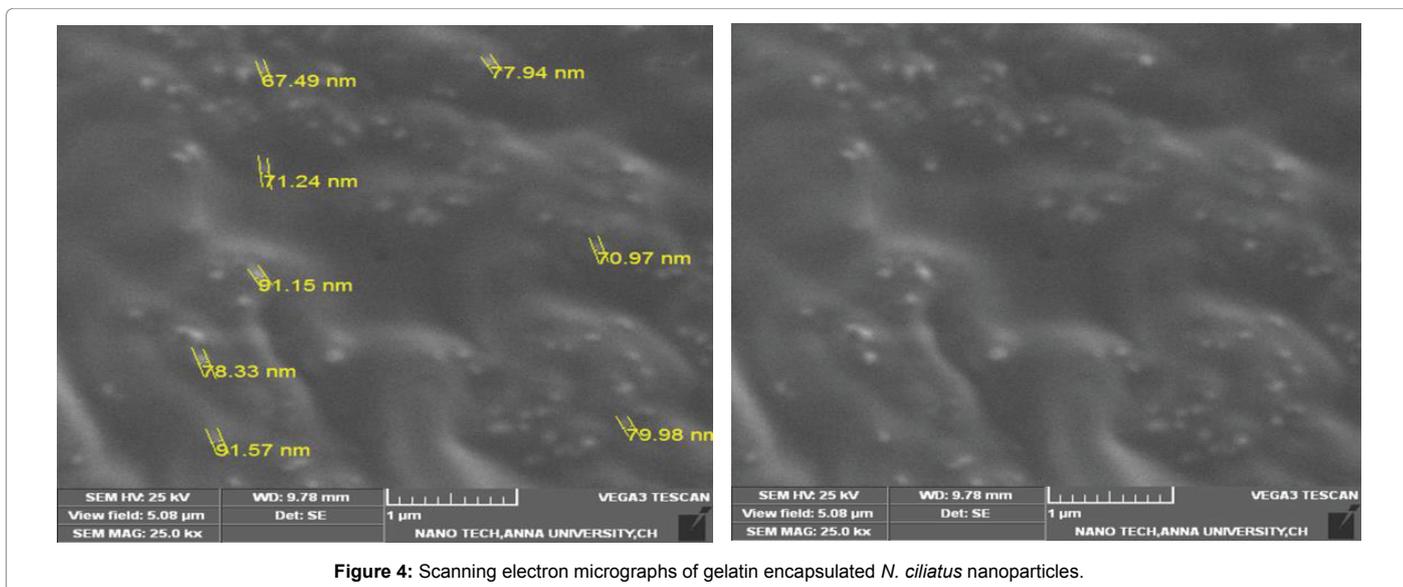


Figure 4: Scanning electron micrographs of gelatin encapsulated *N. ciliatus* nanoparticles.

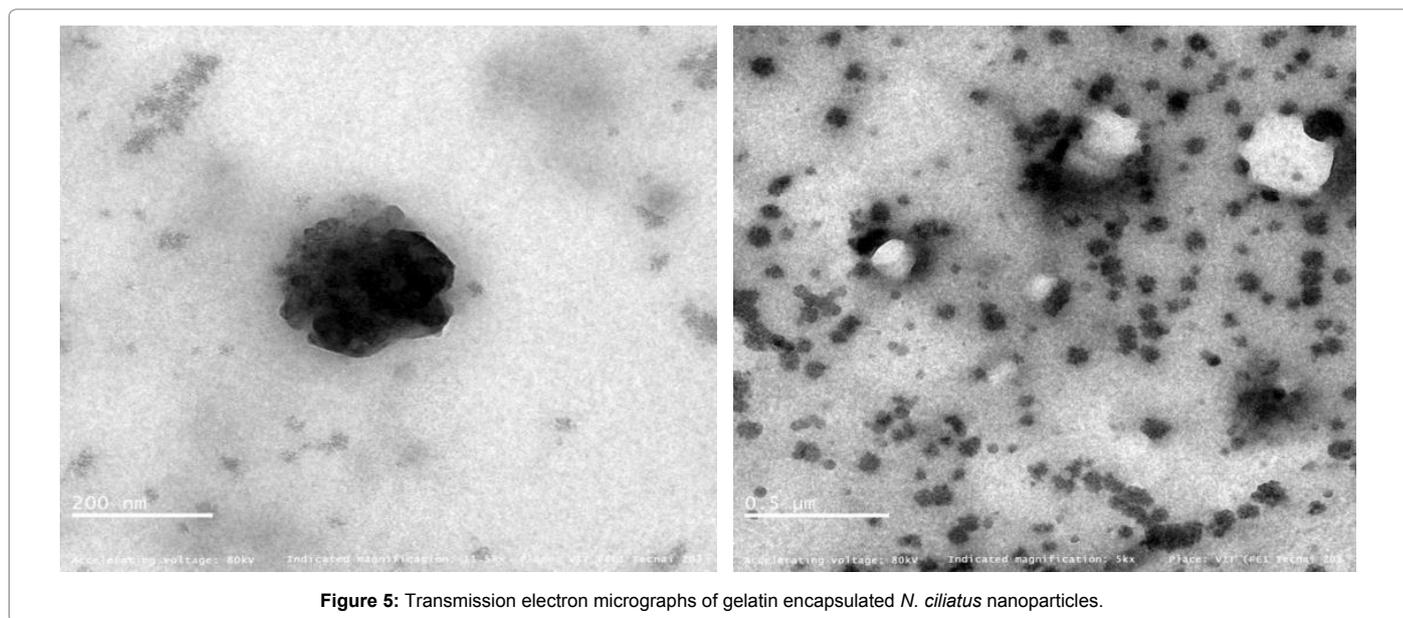


Figure 5: Transmission electron micrographs of gelatin encapsulated *N. ciliatus* nanoparticles.

saline to mimic the physiological conditions in the living organisms. Amount of drug released was calculated by using standard calibration curve generated by UV (Figure 1). *In vitro* release studies confirm that the dissolved drug molecules readily pass through the dialysis membrane. The drug release was found to be excellent and sustainable till 8 h. Drug release depends on surface properties, polymers used, nature of the drug, size of nanoparticles, swelling of particles etc. [52]. Kumar et al. have studied drug release of gelatin nanoparticles prepared by nanoprecipitation method [53], the drug loading efficiency was found to be 85% and drug release was 75% at 8 h, which is comparable with our results.

The drug release data of NCF1 to NCF5 showed good fit into zero order (Figure 7a) with the highest correlation coefficient ($r > 0.9846$), then Higuchi (Figure 7b) and followed by first order (Figure 7c). The results obtained were also put in Korsmeyer Peppas model (Figure 7d) in order to find out n value, which describes the mechanism of drug

release. The nanoformulation NCF1 to NCF5 showed good linearity ($r = 0.9831$ to 0.9976) with slope n values ranging between 0.5-1, the mechanism of drug release was found to follow anomalous non-Fickian diffusion i.e. the increased diffusivity of drug from the matrix by solvent-induced relaxation of the polymers.

Phytochemical screening of *N. ciliatus* Nees nanoparticles:

The phytochemical constituents of the plant extract were present in the gelatin encapsulated *N. ciliatus* nanoparticles also (Table 3). The result showed that none of the constituents were degraded during the preparation of nanoparticles.

Cytotoxicity

Cytotoxicity studies are useful in determining the potential toxicity of a plant extract and nanoparticles. Minimal to no toxicity is essential for the successful development of a pharmaceutical preparation and in this regard, cytotoxicity studies play a crucial role. The cytotoxicity

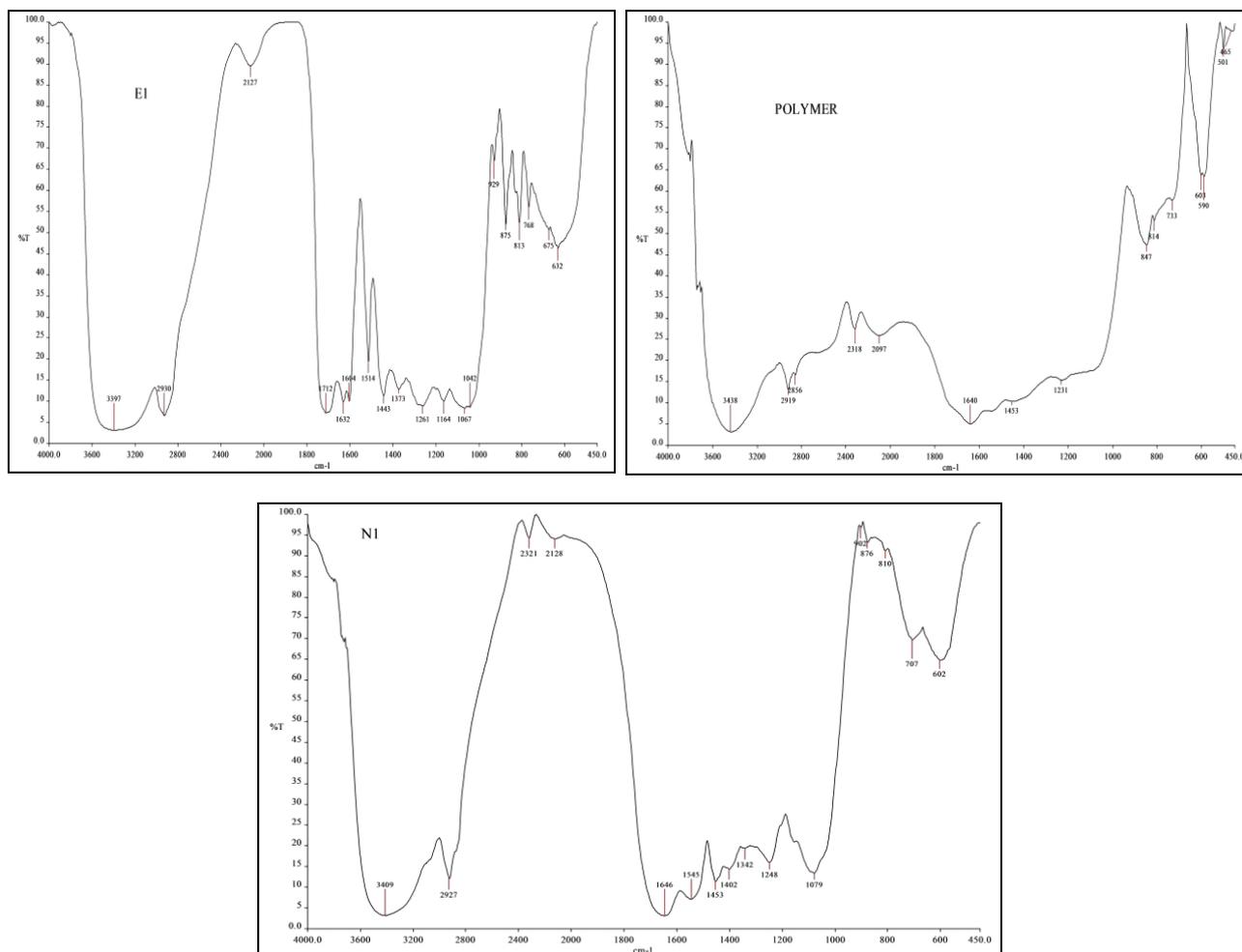


Figure 6: FTIR spectrum of *N.ciliatus* ethanolic extract (E1), gelatin (polymer) and gelatin encapsulated *N.ciliatus* nanoparticles (N1).

assays depends on both the number of viable cells and the mitochondrial activity of cells. MTT assay is based on the assumption that dead cells or their products do not reduce tetrathiazolium. Tetrathiazolium salts are reduced to blue colored formazan crystals by mitochondrial enzyme succinate dehydrogenase. The amount of formazan crystals produced is directly proportional to the number of active cells. In the present study, the ethanolic extract of the *Nilgiranthus ciliatus* Nees and its nanoparticles was screened for its cytotoxicity against L6 myoblasts and 3T3 L1 adipocytes at different concentration from 7.8 to 1000 $\mu\text{g/ml}$ to determine the IC 50 value. When the doses were increased, the cells viability reduced and there was a least viability at a dose of 1000 $\mu\text{g/ml}$. It did not confer any significant lethality to 3T3 L1 and L6 cells (Figure 8) with an IC 50 value greater than 1000 $\mu\text{g/ml}$, which means they do not influence the cell viability of both cells confirming the safe nature of the extract. Altogether, the present study demonstrated that the ethanolic extract do not cause any adverse effect and hence could be considered non-toxic and safe. The effective concentration of extracts and nanoparticles was 500 $\mu\text{g/ml}$, it indicates that the myoblasts and adipocytes tolerated the plant extracts and nanoparticles very well at a concentration of 500 $\mu\text{g/ml}$. Therefore, the concentration below 500 $\mu\text{g/ml}$ was used in the subsequent assays since myoblasts and adipocytes revealed optimal tolerance for it.

Antidiabetic activity

In an endeavor to recognize a small molecule that could stimulate glucose uptake like insulin, a cell based assay using L6 myoblasts was performed. This is an excellent experimental model to rapidly screen the effects of crude drugs on glucose uptake [54]. The influence of plant extract and its nanoparticles on basal and insulin stimulated glucose uptake in L6 cell lines was investigated by nonradioactive method. In the present study, we have established for the first time that plant extract and its nanoparticles have insulin like effects in L6 cells which is a typical target tissue of insulin and plays significant roles in the maintenance of glucose homeostasis. The differentiated cells were treated with increasing concentration of plant extract; gelatin encapsulated nanoparticles and incubated for 24 h. After the incubation period, the cells were evaluated for basal and insulin stimulated glucose uptake. It was found that the extract and its nanoparticles significantly enhanced the glucose uptake in L6 myoblasts in a concentration dependent manner comparable to the effects of standard insulin (injectable antidiabetic) and Rosiglitazone (RSG) (oral antidiabetic) and the best dose exhibiting maximum activity was found to be 125 $\mu\text{g/ml}$.

The extract and nanoparticles were also examined with insulin to confirm any synergistic effects but the results showed that the extract

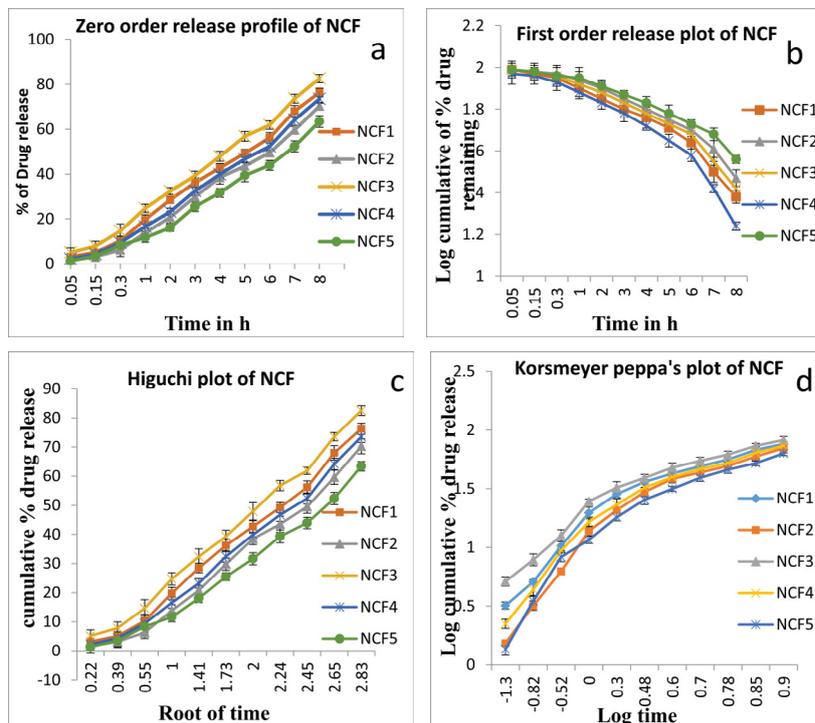


Figure 7: *In vitro* drug release kinetics of gelatin encapsulated nanoparticles. (a) Zero order release (b) First order (c) Higuchi plot and (d) Korsmeyer peppa's plot.

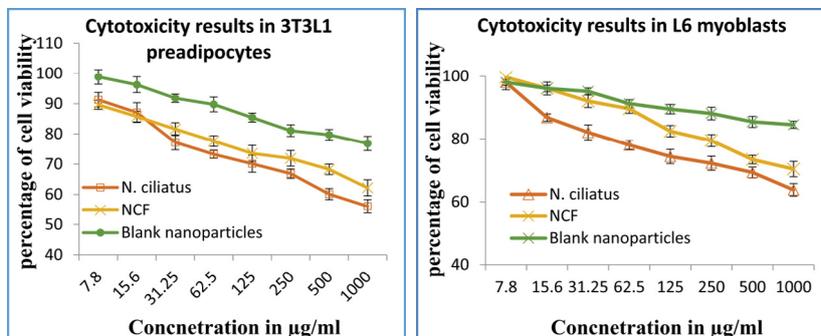


Figure 8: MTT assay results of *N. ciliatus* extract and gelatin encapsulated nanoparticles in L6 myoblasts and 3T3 L1 preadipocytes.

S.No	Phytochemical constituents	<i>Nilgiranthus ciliatus</i>	Polymer encapsulated <i>N. ciliatus</i>
1	Alkaloids	-	-
2	Carbohydrates	-	-
3	Flavonoids	+	+
4	Phenols	+	+
5	Proteins and amino acids	-	-

Table 3: Phytochemical screening of *Nilgiranthus ciliatus* extract and gelatin encapsulated *N. ciliatus* nees nanoparticles.

and nanoparticles does not have any synergistic effects with insulin. The extract and nanoparticles of *N. ciliatus* stimulate the glucose uptake in L6 cells by 212.60% and 254.74% respectively over control when used with insulin (Figure 9). This may be either by elevating the synthesis of the insulin independent (basal) glucose transporter GLUT - 1 or by increasing expression or translocation of the insulin

dependent glucose transporter GLUT 4. GLUT-4 is the major glucose transporter expressed in both insulin responsive tissues like skeletal muscle and adipose tissues. In acute insufficiency of insulin GLUT 4 is quickly translocated from an intracellular membrane storage site to the plasma membrane [55]. The results of the present study clearly indicated that both extract and its nanoformulation stimulates glucose uptake under *in vitro* conditions. This may be due to its effects on the various receptors located in the skeletal muscle L6 cells.

The extract and its nanoparticles can also be tested for their effect of adipogenesis in 3T3 L1 cells. Spindle shaped pre adipocytes were transformed into round shaped cells at the end of adipogenesis, that accumulated lipids and acquired the metabolic mechanisms to facilitate glucose uptake in response to insulin, synthesis fatty acids, accumulate triglycerides and secrete a wide variety of hormones and cytokines [56]. So the extent of adipogenesis in 3T3 L1 cells was demonstrated by monitoring the intracellular lipid accumulation as a general marker. We examined the anti-adipogenic effect of ethanol extracts of *N. ciliatus*

and its nanoparticles in 3T3 L1 adipocytes, nanoparticles lowered the lipid levels in 3T3 L1 adipocytes. The amount of lipid accumulated in the 3T3 L1 adipocytes subsequent treatment with extract and nanoparticles was calculated in terms of the absorbance of the oil red O dye extracted from stained cells. The present study results showed that the *N. ciliatus* plant extract and its nanoparticles having the ability to reduce lipid accumulation in 3T3 L1 adipocytes, expressing antiobesity effect which is an attractive property for an antidiabetic drug. The inhibitory effects of plant extracts and its nanoparticles on lipid accumulation during adipogenesis was dose dependent and nanoparticles significantly reduced the lipid accumulation by 42.7% at 250 µg/ml (Figures 10 and 11).

Numerous studies have revealed that natural phytochemical compounds like resveratrol, berberine, genistein inhibited adipogenesis by inhibiting preadipocyte proliferation and suppressing lipid accumulation [57]. Epicatechins, catechins and tannins are the most important active anti-oxidant constituents found in the medicinal plants to enhance the glucose uptake and reducing adipogenesis in differentiated adipocytes [58]. In both *in vitro* antidiabetic method, gelatin encapsulated *N. ciliatus* nanoparticles showed good anti-

diabetic effect when compared to its extract this may be due to the sustained release of active constituents. Further studies needed to confirm the molecular mechanisms by which these plants and their active compounds are responsible for the anti-diabetic effect.

Conclusion

This study has demonstrated in herbal drugs to realize the glucose-lowering drugs with minimal anti-adipogenic activity since the ability of existing therapies to target different features of the insulin resistance syndrome induces other metabolic abnormalities, mainly those involved in lipid metabolism. The results of the present study illustrated that *N. ciliatus* plant extract and gelatin encapsulated nanoparticles exerts its anti-diabetic properties by stimulating glucose uptake in myoblasts and inhibiting adipogenesis. *N. ciliatus* extract and its Nanoparticles have the ability to augment glucose uptake in L6 myoblasts in addition to its anti-adipogenic effects, this suggests that this extract may possibly be useful in the treatment of type 2 diabetes. To our knowledge, this is the first study of the potential use of *N. ciliatus* extract derived nanoparticles in the management of diabetes and associated complications. Further studies may be needed on the *in vivo*

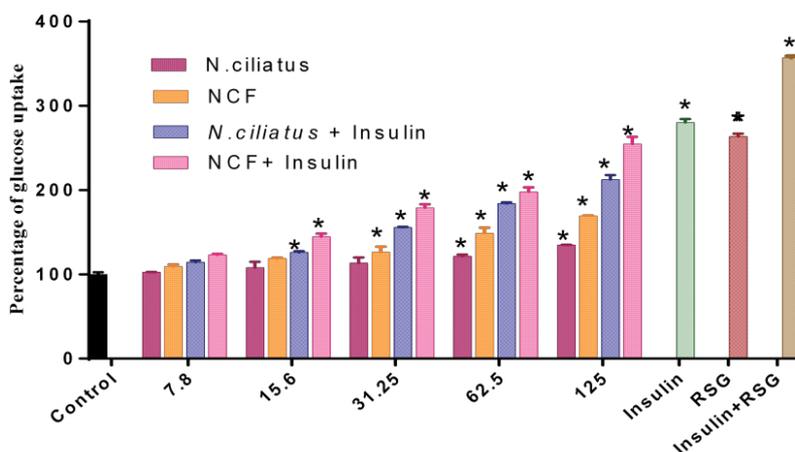


Figure 9: Glucose uptake assay of *Nilgiranthus ciliatus* Nees, its nanoformulation and Rosiglitazone (RSG) in L6 myoblasts. The plant extract and its nanoparticles were tested with insulin and without insulin at various concentrations. All the data were expressed as means ± SEM of the three independent experiments. *P<0.001 as compared with control group.

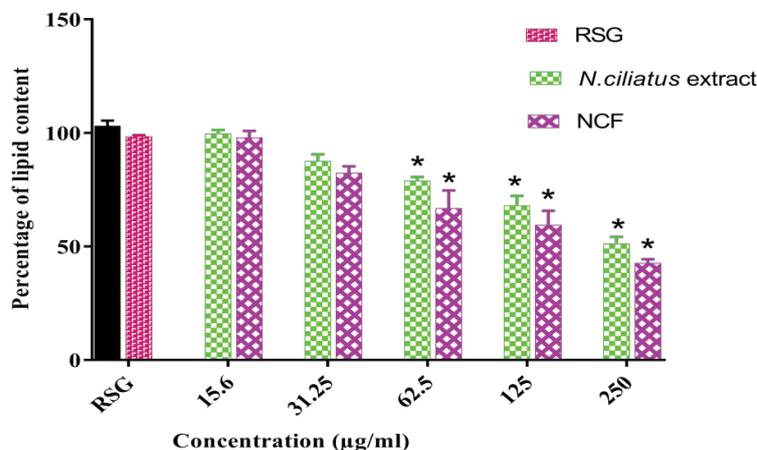


Figure 10: Anti adipogenesis assay of different concentrations of plant extract, its nanoparticles and Rosiglitazone (RSG) in 3T3 L1 preadipocyte. All the data were expressed as means ± SEM of three independent experiments. *P<0.001 as compared with control group.

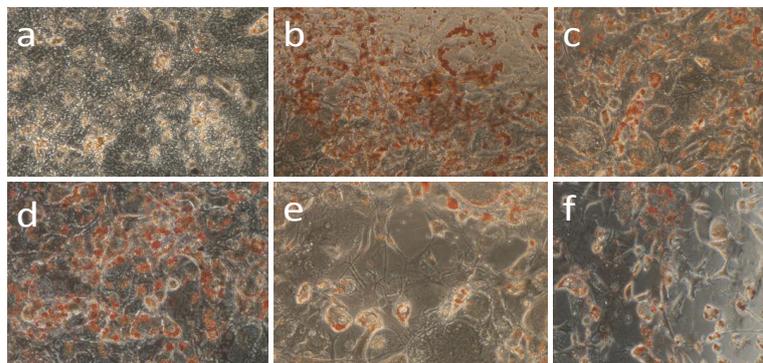


Figure 11: (a) Pre adipocytes (40x) (b) Control untreated (40x) (c) Differentiated induced+low dose of plant extract (40x) (d) Differentiated induced+low dose of nanoformulation (40x) (e) Differentiated induced + high dose of plant extract (40x) (f) Differentiated induced +high dose of nanoformulation (40x).

models and molecular mechanisms to substantiate the *in vitro* results by which these plants and their active compounds are responsible for the desired effect.

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