

Effect of Chi-alg Encapsulated 6% Curcumin Isolated from Mother Rhizome on Diabetes Mellitus in L6 Cell Lines

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Abstract

Introduction: In this study, antidiabetic activity of 6% curcumin and chi-alg-encapsulated 6% curcumin isolated from the *Curcuma longa* (commonly called as turmeric) mother rhizome were evaluated and compared. **Materials and Methods:** The 6% curcumin-chi-alg encapsulated nanoparticles were prepared and characterized using scanning electron microscope and Fourier transform infrared. The antidiabetic activity of 6% curcumin was estimated in L6 cell lines. Cell viability was assessed using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and 3T3L1 antiadipogenesis assay was performed and *in-vitro* α -amylase activity was done. **Results and Discussion:** The chi-alg encapsulated curcumin found to possess the maximum antidiabetic activity. Encapsulated curcumin demonstrated better α -amylase inhibition activity than curcumin alone with inhibitory concentration 50% value of 36.81 μ g/ml. The encapsulated curcumin reduced the lipid accumulation in differentiated adipocytes. Increase in the 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose uptake by the cells cumulatively reflects the uptake of the glucose by encapsulated curcumin treatment. **Conclusion:** The current results suggest that the chi-alg encapsulated curcumin exhibit its antidiabetic activity by various types of mechanisms, including enhancing the glucose uptake by adipose tissue, suppresses the adipogenesis.

Key words: Adipose cells, antidiabetic, curcumin, glucose uptake

INTRODUCTION

Type 2 diabetes is a disorder related to glucose metabolism and associated with insulin deficiency and lowered glucose uptake by the skeletal muscles. This may lead a condition like obesity, hypertension, glucose intolerance, and dyslipidemia, in addition to these disorders, nontraditional risk factors such as abnormalities in blood coagulation and inflammatory processes. Obesity is the main problem affects over a billion of people currently worldwide. It is the fast spreading problem in rich nations and should be considered as a chronic disease in epidemic proportions of the disease.^[1] Drugs used for the treatment of diabetes mellitus reduce the blood glucose levels by inducing the adipogenesis and obesity as side effects. Plant-derived compounds can serve as ideal candidates in treating the diabetes mellitus by acting on adipocytes and can act as an improved alternative in controlling the metabolic disorders.

The nanoparticle of chitosan alginate origin acts as novel drug delivery systems with the potential power to improve the drug stability, increase the duration of the therapeutic effect and allows the administration through parenteral and enteral, this helps to minimize (or) prevents the drug degradation, metabolism, and cellular efflux.^[2-4] Nanoparticles made up of synthetic biodegradable polymers, natural biopolymers, lipids, and polysaccharides are the proven carrier molecules from decades. Out of this chitosan and alginate are very capable and have been widely exploited in the pharmaceutical industry for controlling drug release.^[5,6] Alginate is chemically composed of α -L-guluronic and β -D-mannuronic acid residues with 1-4 glycosidic bonds, which is water soluble. Alginate promises a good drug delivery property

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due to its biodegradable, biocompatible, and mucoadhesive. Hence, alginate attained great importance in pharmaceutical and biomedical industry as encapsulating material and in drug delivery system.

Curcumin possesses a proved results associated with many medicinal properties including hypolipidemic, protective role in cardioprotective diseases, antibacterial, anti-HIV, anticarcinogenic, antitumor, and anti-arthritis activities.^[7] Hence, the current study is concentrated to evaluate the better targeted drug delivery for curcumin encapsulated with chi-alg in L6 and 3T3L1 cells.

MATERIALS AND METHODS

A collection of sample: 6% curcumin was obtained as gift sample from Rajkeerth Aromatics and Biotech. Pvt. Ltd., Kodambakkam, Chennai, isolated from the mother rhizome.

Synthesis of curcumin loaded chitosan-alg nanoparticles. The encapsulated particle synthesized by ionotropic gelation method based on electrostatic interaction among negatively- and positively-charged molecules like polyanionic and cationic polymers. The amino groups present on the chitosan interact with anionic groups of alginate. The concentration of stock solution for chitosan was made at 1 mg/mL in acidified double distilled water (DW) and alginate was prepared at 1 mg/mL in DW. The chitosan stock solution (1 ml) was vortexed for 10 min and to adjust to 1.5 ml with double DW. Followed by adding 5 µl of tween 80 to curcumin stock (1 mg/mL) prepared from 6% of curcumin dissolved in ethanol and transferred to chitosan solution. At the end alginate was added to initiate cross-linking in drop wise (100 ml), later this solution was stirred for 30 min and centrifuged at 4000 g for 5 min. Finally, the supernatant was transferred into a new tube and subjected to characterization of nanoparticles. A similar procedure was followed for preparation of blank nanoparticles.

Characterization of curcumin-loaded chitosan-alginate nanoparticles

The size of the particle, morphology (topography) and surface characterization of the nanoparticles was measured by scanning electron microscope (SEM) SEM-Carl Zeiss Supra-55. Curcumin nanoparticles separated from the suspension were made into powder using vacuum freeze dryer, and studied using Fourier transform infrared (FTIR) with KBr pellets with the help of FW-4A pelleter on an FTIR spectrophotometer (Thermo Nicolet, NEXUS, TM, USA).

Quantitative analysis of curcumin

Standard solution preparation: Standard solution of curcumin was prepared from the stock solution (10 µg/ml of

curcumin in methanol) by diluting with methanol to get the various working standards. The standards were prepared at concentrations of 1, 2, 3, 4, 5, 6, 7 µg/ml.

Sample preparation

The curcumin loaded in nanoparticles was assessed using different weights of curcumin with chitosan. Different concentrations of curcumin were dissolved in chitosan-alginate solution followed by centrifugation at 2000 rpm for 25 min. The supernatant was collected from the centrifuged curcumin-loaded chitosan-alginate nanoparticles, and absorbance was noted using ultraviolet-visible (UV-Vis) spectrophotometer. The encapsulation efficacy was calculated with the following equation: Loading efficiency (%) = [(Total amount of curcumin–Nanoencapsulated curcumin/Total amount of curcumin) × 100.

Antidiabetic activity

In-vitro α-amylase inhibition

In vitro amylase inhibition was studied according to the method of Benfield, 1992^[8]. Test samples in a concentration of 100 µl (1-1000 µg/ml) were allowed to react with 200 µl of α-amylase enzyme (Hi Media RM 638) in 100 µl of 2 mM of phosphate buffer (pH-6.9). 20-min later, 100 µl of 1% starch solution was added. The same was performed for the controls by replacing 200 µl of the enzyme with buffer. Then incubated for 5 min, and 500 µl of dinitrosalicylic acid reagent was added and boiled for 5 min. The absorbance was recorded at 540 nm using spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the formula % inhibition = [(control – test)/control]*100. Suitable reagent blank and inhibitor controls were simultaneously carried out.

Maintenance of cell lines and L6 myotubes

L6 myotubes culturing

L6 is a differentiating monolayer myoblast cell line (obtained from NCCS, PUNE – passage no. 15) was cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and supplemented with penicillin (120 units/ml), streptomycin (75 µg/ml), gentamycin (160 µg/ml), and amphotericin B (3 µg/ml) in a 5% CO₂ environment. L6 cells were transferred to DMEM for differentiation, with 2% FBS for 4 days, post-confluence. Multinucleate cell recognition helps to identify the degree of the differentiation. Pre-adipocytes grown in 48 well plates until 2 days post-confluence and the cells were induced by the differentiation medium (combination of 0.5 mm/L of 3-isobutyl-methylxanthine [IBMX], 0.25 µm/L of dexamethasone (DEX) and 1 mg/L of insulin in DMEM medium with 10% FBS) to differentiate into adipocytes. Once the cells differentiated, the medium was replaced with glucose medium (DMEM medium with an additional

supplement of 25 mM/L glucose) for the cells to reach the insulin resistant condition.

Cell viability

The viability of the L6 cell layers was assessed using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in 96-well plate at a concentration of 5×10^4 cells/well. Free curcumin chi-alg-curcumin cytotoxicities were standardized depending on the concentration and incubation period. After 24 h of incubation, the cells were washed with $\times 1$ phosphate buffered saline (pH 7.4) and MTT (0.5 g/L) was added. Cells were then incubated for 4 h at 37°C in CO₂ incubator. The purple formosan crystals formed were then dissolved by adding 150 μ l of dimethyl sulfoxide and mixed effectively by pipetting up and down. The spectrophotometrical absorbance of the purple blue formazan dye was measured using multimode reader (perkinelmer) at 570 nm. The optical density of each sample was compared with control optical density and graphs were plotted.

$$\text{Percentage of toxicity} = \frac{\left[\begin{array}{l} \text{Absorbance of control} \\ - \text{Absorbance of sample} \end{array} \right]}{\text{Absorbance of control}} \times 100$$

In vitro glucose uptake activity by 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) assay

Differentiated L6 myoblasts cell lines were used to perform the glucose uptake test.^[9] The L6 cells were pretreated with different concentrations (1, 3, 10, 30 μ g/ml) of control, free curcumin and chi-alg-curcumin were added to the wells along with the respective standard metformin for 24 h. Culture media was removed, and cells were washed with Kreb's ringer buffer. Fluorescent tagged analogue of glucose, 2-NBDG 20 μ g/200 ml was added and incubated for 30 min at dark. The reaction was stopped by removing the 2-NBDG and incubation medium from cells and washing with a precold Kreb's ringer washing buffer. The L6 cells in all the wells were trypsinized and followed by resuspending in 1 ml buffer. Glucose uptake (in %) was measured using fluorescence activated cell sorting with an excitation/emission filter 466/540 nm.

3T3L1 antiadipogenesis assay

3T3L1 preadipocytes were incubated with the combination of IBMX, DEX, and insulin to differentiate into adipocytes on day 0. 72 h later to induction; the differentiation medium was replaced with 10% FBS-DMEM which contains 1 mg/L insulin for 2 days (day 5). The differentiation medium was replaced with fresh culture medium and incubated for 2 days

(day 7). The differentiation percentage of the cells was investigated by adding different concentrations 1, 3, 10, and 30 μ g/ml of free curcumin and chi-alg-curcumin from day 0, a period of time which covered the entire induction and post-induction stages. Fenofibrate (30 μ g/ml) was used as a standard. Alternatively, pre-adipocytes were maintained with fresh FBS-DMEM every other day for the whole spectrum of the induction period. Adipo red lipid staining assay was performed using Oil Red O at the end of the induction period for 1 h to observe the degree of differentiation as described previously. Photomicroscopic evaluation was also carried out for the comparison of triglyceride accumulation using invert-microscope (nikon) was used for imaging at room temperature. For quantitative analysis, Oil Red O was extracted with isopropanol for 2 min, and optical density of each sample was determined at 540 nm.

Statistical analysis

Analysis of statistical significance of differences in measurements between samples was done by one-way ANOVA with Dunnett's *post-hoc* test (graph pad prism version 4) $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

6% curcumin loaded chitosan-alg nanoparticles

Curcumin-loaded chitosan-alg nanoparticles were prepared and optimized in shape, size, and charge. The average diameter of the nanoparticles was 10 μ m [Figure 1a].

Figure 1 shows the SEM images of the dried curcumin encapsulated beads at different magnifications. The diameter was noted for curcumin alone as 2 μ m and for the encapsulated curcumin it was noted as 10 μ m [Figure 1a and b] and energy dispersive X-ray analysis was shown in Figure 3. Curcumin-loaded chitosan-Alg nanoparticles showed the characteristic fluffy surface and spherical shape. FTIR spectra of curcumin alone and curcumin loaded chitosan-alg nanoparticles are showed in Figure 2a and b. From Figure 2a and b, two typical peaks (1420/cm of ν [OH] and 1087/cm of ν [C-O-C]), existed in the spectrum of curcumin and three characterization peaks (1077/cm of ν [C-O-C], 1632/cm ν [NH₂]) observed in the FTIR spectrum of chitosan. The three characteristic peaks at 3449/cm, 1632/cm corresponds to the C-O-C bonds for chitosan. In curcumin loaded spectrum different peaks were observed and new sharp peaks at 2318 and 2051 were appeared. Furthermore, the 1632 peak of amine was shifted to 1630 and 1420 is shifted to 1417. There may be a chance of that the ammonium groups of chitosan were reacted with hydroxide groups of curcumin during nanoparticle preparation. Similar studies were reported about the curcumin-loaded chitosan-alginate nanoparticles.^[10,11]

The encapsulation efficacy of curcumin loaded chitosan-alg nanoparticles. After the preparation of curcumin-loaded chitosan-alg nanoparticles, the nanoparticles were centrifuged and collected. The curcumin harvested from the supernatant of the solution was then measured by a UV-Vis spectrophotometer. The encapsulation efficacy was determined as 76%.

In-vitro α -amylase inhibitory assay

Carbohydrates are hydrolyzed into monosaccharides by various enzymes like α -amylase or α -glucosidase. Among these enzymes α -amylase, is an important enzyme in carbohydrate hydrolysis and digestion. Hence the inhibition of the α -amylase is one of the several therapeutic approaches to reduce postprandial hyperglycemia. The *in-vitro* α -amylase inhibitory activity of control, curcumin, and chi-alg-curcumin were investigated. In the current research on diabetes Type 2, management is becoming more prevalent through dietary practice. α -amylase is the primary enzyme in the process of digestion of the carbohydrates in the intestine and hence α -amylase inhibitors can retard the liberation of glucose from polysaccharides in the dietary carbohydrates and thus causing a reduction in postprandial hyperglycemia. Curcumin reported to be effective in controlling the blood sugar levels. In the present study, the results indicated that 6% curcumin encapsulated with chi-alg nanoparticle exhibited a promising α -amylase inhibitory activity. Among encapsulated curcumin and curcumin, encapsulated curcumin showed promising α -amylase inhibition potential with inhibitory concentration 50% (IC_{50}) value 36.81, 100 μ g/ml respectively. Acarbose was used as a standard positive control and IC_{50} value was found to be 30.83 μ g/ml [Table 1]. Currently, available diabetes drugs are acarbose, militol and metformin, these drugs cause various side effects. Hence, there is a need for alternative source of α -amylase inhibitors with the potential protective role and comparatively lesser side effects for the dietary management of Type 2 diabetic patient.

Cytotoxicity assay

There are previous studies about curcumin on the protection against diabetes. Therefore to enhance its activity further studies on cell lines were now carried

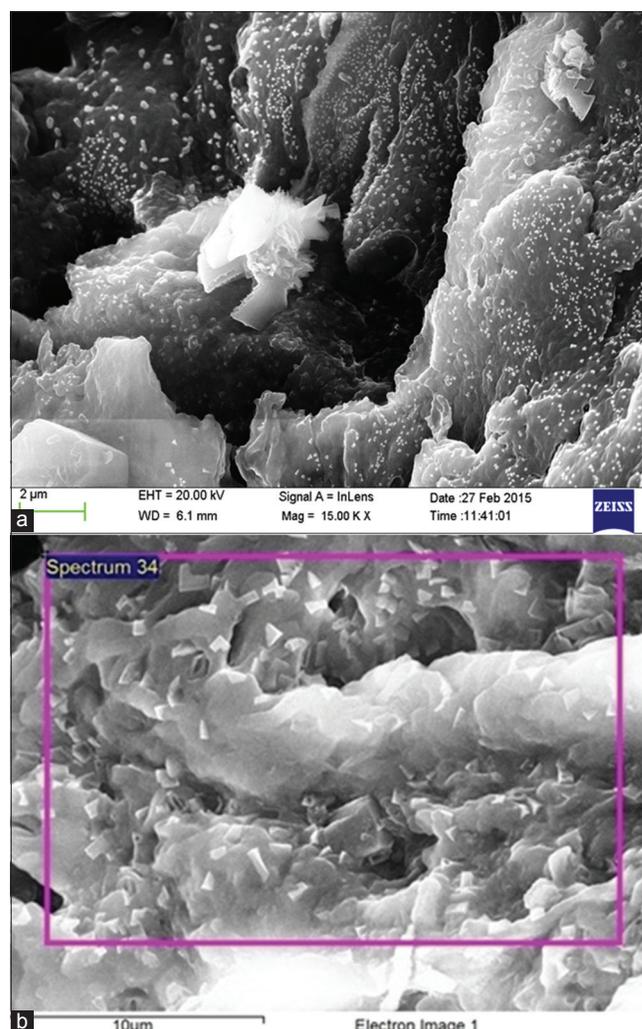


Figure 1: (a) Scanning electron microscope analysis of free curcumin, (b) scanning electron microscope analysis of encapsulated curcumin

Table 1: *In-vitro* α -amylase inhibitory activity

Concentration (μ g/ml)	% inhibition of α -amylase inhibitory activity			
	Control	Curcumin	Chi-alg-curcumin	Standard acarbose
1	-3.12 \pm 0.07	9.48 \pm 3.45	16.59 \pm 0.22	13.73 \pm 0.63
3	-1.26 \pm 0.05	18.10 \pm 6.47	23.71 \pm 1.29	23.84 \pm 0.09
10	-0.45 \pm 0.21	27.59 \pm 6.05	33.84 \pm 2.80	39.03 \pm 0.19
30	-8.19 \pm 0.92	36.85 \pm 3.54	46.77 \pm 1.08	50.26 \pm 0.09
100	-4.08 \pm 0.19	50.22 \pm 4.27	70.04 \pm 1.08	69.25 \pm 0.06
300	-7.68 \pm 0.86	63.79 \pm 4.48	85.13 \pm 1.08	76.51 \pm 1.51
1000	-8.93 \pm 2.80	81.47 \pm 4.74	89.87 \pm 0.22	93.32 \pm 1.08
IC_{50}	-	100 μ g/ml	36.81 μ g/ml	30.83 μ g/ml

All values are expressed as mean \pm SEM. IC_{50} : Inhibitory concentration 50%, SEM: Standard error of mean

Table 2: Cytotoxicity in L6 myotubes

Concentration ($\mu\text{g/ml}$)	% of cell death			
	Control	Curcumin	Chi-alg-curcumin	Standard metformin
Control	0.03 \pm 0.76	0.02 \pm 0.41	0.03 \pm 0.76	0.03 \pm 0.76
0.1% DMSO	10.17 \pm 3.02	13.58 \pm 1.49	10.17 \pm 3.02	10.17 \pm 3.02
1	2.83 \pm 0.19	1.27 \pm 0.09	1.17 \pm 0.09	1.46 \pm 0.49
3	5.58 \pm 2.03	4.38 \pm 2.10	1.16 \pm 0.64	2.05 \pm 1.25
10	0.30 \pm 0.08	6.67 \pm 0.40	4.13 \pm 0.61	1.50 \pm 0.69
30	2.88 \pm 1.86	9.80 \pm 0.77	7.42 \pm 0.95	3.71 \pm 0.67
100	8.07 \pm 1.25	11.40 \pm 0.32	9.08 \pm 1.27	10.37 \pm 0.54
300	5.55 \pm 1.06	15.33 \pm 0.88	12.26 \pm 0.84	15.26 \pm 0.85
1000	5.86 \pm 0.31	26.27 \pm 2.45	17.15 \pm 4.52	18.08 \pm 0.68
IC ₅₀	-	>1000 $\mu\text{g/ml}$	>1000 $\mu\text{g/ml}$	>1000 $\mu\text{g/ml}$

All values are expressed as mean \pm SEM. SEM: Standard error of mean, DMSO: Dimethyl sulfoxide, IC₅₀: Inhibitory concentration 50%

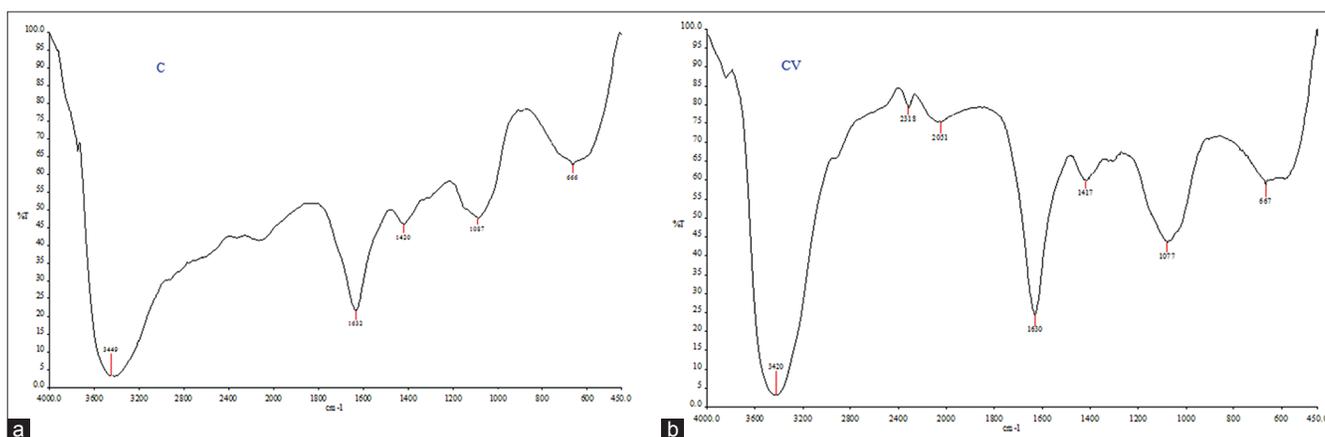


Figure 2: (a) Characterization of blank chi-alg nanoparticles by - Fourier transform infrared, (b) characterization of the Chi-alg-curcumin

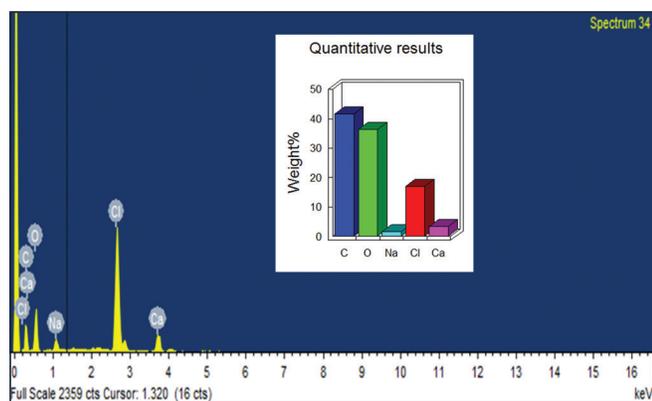


Figure 3: Energy dispersive X-ray analysis of encapsulated curcumin

out for the encapsulated curcumin. The effect of chi-alg-curcumin on viability of L6 cell lines was assessed by MTT assay. The results [Table 2] indicated that the chi-alg-curcumin exhibited in a dose-dependent manner. The IC₅₀ value of cytotoxicity of control, curcumin and chi-alg-curcumin was tested at concentrations of 1-1000 μg and was found to be protective up to 1000 $\mu\text{g/ml}$ concentration.

The cytotoxicity of the individual extracts was compared to standard metformin.

2-NBDG uptake assay in L6 myotubes

The non-metabolizable fluorescent glucose analogue (2-NBDG) is increasingly used to study cellular transport of glucose. Intracellular accumulation of exogenously applied 2-NBDG is assumed to reflect concurrent gradient-driven glucose uptake by glucose transporters (GLUT 4) which is mainly expressed in skeletal muscle and adipose cells.^[12] The enhanced glucose uptake potential by the cells would help in decreasing of blood glucose levels in an individual. One of the major hallmarks of the diabetes Type 2 is lowered sensitivity of muscle and adipose cells to insulin. Curcumin may increase the sensitivity of adipose tissue to insulin activates a signal transduction pathway leading to increased glucose uptake by GLUT 4 in adipocytes or myotubes.^[13] Skeletal muscles account for more than 80% of insulin-stimulated glucose uptake and an impaired glucose uptake in skeletal muscle plays an important role or the development of Type 2 diabetes mellitus. According to the previous studies, the

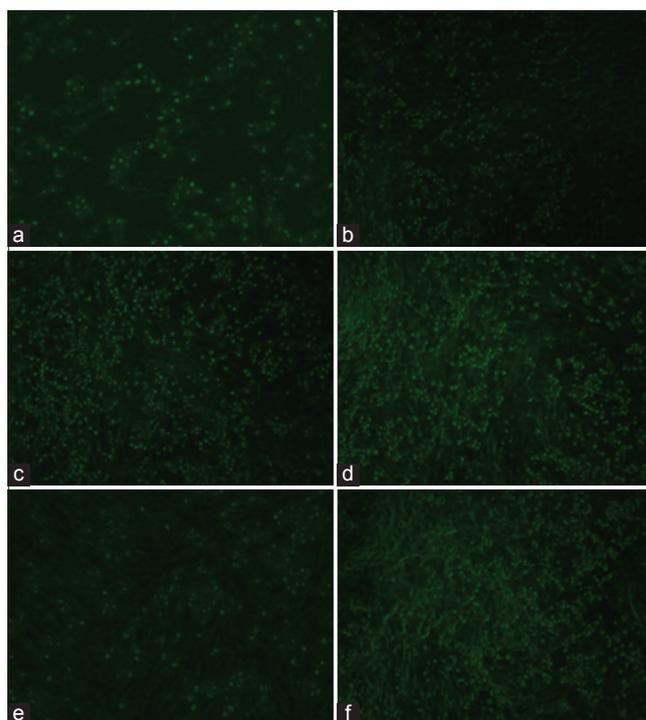


Figure 4: 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose glucose uptake potential of free curcumin and chi-alg-curcumin in L6 myotubes, (a) Control, (b) insulin (10 μ M/ml), (c) curcumin (10 μ g/ml), (d) curcumin (30 μ g/ml), (e) chi-alg-curcumin 10 μ g/ml, (f) chi-alg-curcumin (30 μ g/ml), (g) metformin (10 μ M/ml), (h) metformin (30 μ M/ml)

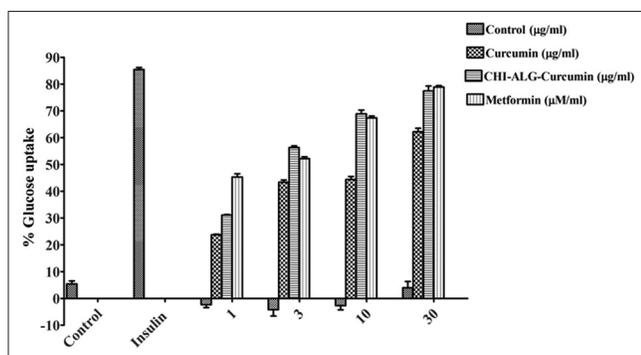


Figure 5: Effect of free curcumin and chi-alg-curcumin on 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose glucose uptake potential

curcumin and encapsulated curcumin evaluated for their glucose uptake potential based on 2-NBDG assay in L6 cells using flow cytometry. From the current results, it was found L6-myotubes incubated with encapsulated 6% curcumin induced glucose uptake [Figure 4] whereas free curcumin. Free curcumin and chi-alg-curcumin showed maximum 2-NBDG uptake of 62.21% ($P < 0.05$) and 77.43% ($P < 0.05$) respectively [Figure 5] at even very lower concentration of 30 μ g/ml. Chi-alg-curcumin nanoparticles showed significantly equal effect of glucose uptake potential with the metformin standard.

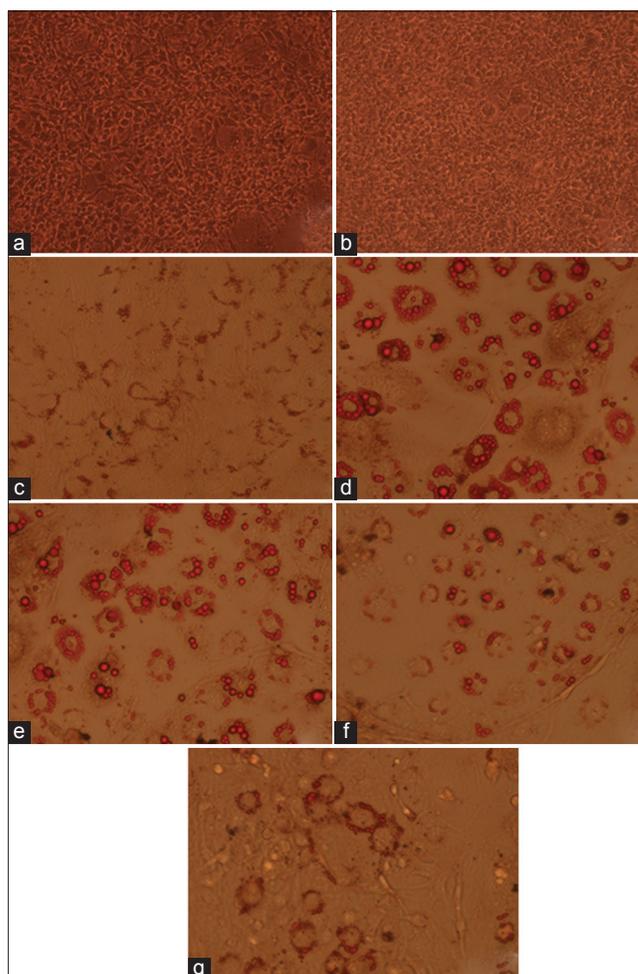


Figure 6: Impact of Free curcumin and CHI-ALG-Curcumin on adipogenesis study using 2NBDG glucose uptake by adipocytes and Oil Red O staining. Cells stained with Oil Red O were digitalized uniformly at 20X. (a) Unstained Preadipocytes, showing absence of lipid; (b) unstained differentiated adipocytes showing lipid droplets around the nucleus; (c) Stained Preadipocytes, showing no or very mild red stain indicating absence of lipid; (d) Stained differentiated adipocytes showing lipid droplets in red colour due to stain (e) Free curcumin(30 μ g/ml) treated stained adipocytes showing some of lipid droplets in red colour compared to untreated cells (f) CHI-ALG-Curcumin (30 μ g/ml) treated stained adipocytes showing some of lipid droplets in red colour compared to untreated cells and (g) Standard Fenofibrate (30 μ M/ml) treated stained adipocytes showing some lipid droplets.

Anti-adipogenesis assay

Adipogenesis a cellular process in which metabolically less active preadipocytes differentiate to metabolically active adipocytes this is highly controlled process by a mixture of hormones like insulin, DEX and IBMX.^[14] The intracellular lipid accumulation is a general marker for to indicate the adipogenesis in 3T3-L1 cell.^[15] Once the cells get differentiated to adipocytes, it becomes a depot for lipid

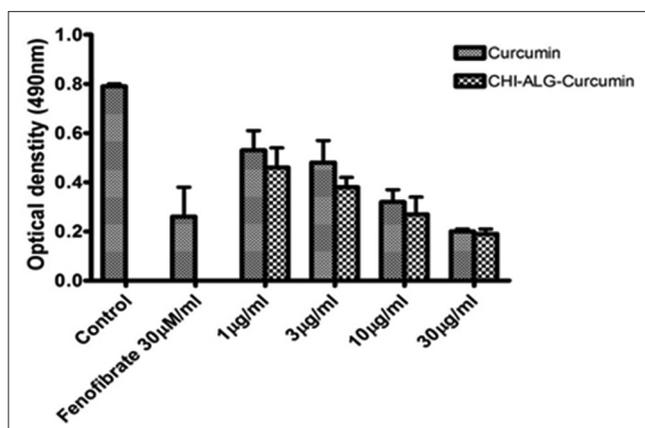


Figure 7: Quantification of Oil Red O stains. Oil Red O stain extracted and quantified spectrophotometrically and graph was plotted with mean \pm SEM

storage. Hence, studying the impact of the antidiabetic drug on this crucial process helps in elucidating the mechanism of action and also categorization of the drug [Figure 6]. With increasing concentrations of curcumin and chi-alg-curcumin, a significant inhibition of differentiation of preadipocytes to mature adipocytes was observed and it was evident from less accumulation of fat in the cells which was done using Oil Red O staining [Figure 7].

DISCUSSION

The murine pre-adipose cells 3T3-L1 was used to identify the mechanisms of action by which curcumin and encapsulated curcumin exerts their antidiabetic effects. However, some of the anti-diabetic drugs induce the obesity as a side effect therefore the effect of curcumin and encapsulated curcumin on adipogenesis was also evaluated. Inhibitors of adipocyte differentiation may be effective in preventing obesity, diabetes and atherosclerosis, diabetes and other associated complications. Under appropriate conditions, 3T3-L1 cells can differentiate into adipocytes and can be a major place for lipid and glucose metabolism. Excessive adipogenesis leads to overweight and obesity and therefore the obesity can be prevented by inhibition of 3T3-L1 cells differentiation this process is complicated by diabetes and atherosclerosis. Curcumin and its derivatives were found to be potential in inhibition of altering the gene expression involved in the adipocyte differentiation process.

The effect of curcumin and encapsulated curcumin on basal and insulin-stimulated glucose uptake into L6 myoblasts was examined, using the fluorescent method of measuring 2-NBDG uptake. Among the various concentrations of the free curcumin and encapsulated curcumin 30 μ g/ml of encapsulated curcumin enhanced the glucose uptake by L6 myoblasts cells. It is well known fact that curcuminoids have beneficial effects on hyperglycemia in Type 2 diabetes, but the molecular mechanism is still not known. This encapsulated

curcumin may enhance the insulin-independent GLUT-1 to stimulate glucose uptake or may increase expression or translocation of insulin dependent/sensitive GLUT-4.^[16]

This study concludes that encapsulated curcumin exhibits a significant glucose uptake and inhibits adipocyte differentiation and serves as the best candidate for the controlling obesity and its associated metabolic syndromes.

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