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Research Article

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Glucose uptake assay of ELEGU and ELEAP on L6 cell line – *In vitro* study

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Abstract

Grewia umbellifera is an Indian traditional medicinal plant of the *Tiliaceae* family. *Grewia umbellifera* has potential for the treatment to control diabetes mellitus and hyperlipidemia. Ethanolic extract of *Grewia umbellifera* had an enhancing effect on glucose uptake in L6 cell lines. At the concentration 0.1mg/mL of ethanol fraction showed increase in glucose uptake activity compare to untreated. Glucose uptake activity was increased when compare to the insulin. These results suggest that the antidiabetic action of *Grewia umbellifera* may be mediated through the stimulation of glucose uptake and the potentiating of insulin action.

Keywords: Glucose uptake, *Grewia umbellifera*, L6 cell lines, antidiabetic

1.1 Introduction

According to the World Health Organization (WHO), the number of people with diabetes will be doubled with in less than 30 years. Indeed, diabetes can be initially managed by exercise and dietary modifications. Drugs as well as medicinal food additives are necessary in advanced cases (Kadan, S., Saad). More than 1200 plant species are reported worldwide as anti-diabetic .Over 400 plants as well as 700 recipes and compounds have been scientifically evaluated for DT2 treatment (Singh, Jand Cumming). When insulin binds to these receptor of liver and muscle cells, sit acts as a key for opening the door for glucose by sending signals to the cell which allows the special glucose transport channels on to the cell surface. These channels enable glucose to enter into the cell and thus decrease the blood glucose level. Diabetes will finally lead to heart attack, stroke, blindness, kidney failure and nerve damage.

The bioactive compounds of medicinal plants are used as anti diabetic, chemotherapeutic, anti inflammatory, anti arthritic agents where no satisfactory cure is present in modern medicines (Y. Tanko, A. Mohammed). Diabetes mellitus is a chronic endocrine disorder that affects the metabolism of carbohydrates, proteins, fat, electrolytes and water and includes a group of metabolic diseases characterized by hyperglycemia. Currently, there is growing interest in herbal remedies due to the side effects associated with the oral hypoglycemic agents for the treatment of diabetes mellitus (Bhalodi, M., S. Shukla). Biological actions of the plant products used as alternative medicines to treat diabetes are in relevance to their chemical composition. Herbal products or plant products are rich in flavonoids, phenolic compounds, coumarins, terpenoids and other constituents which help to reduce blood glucose levels (Jung M, Park M,).The present study, is aimed to determine the effect of ELEGU and ELEAP in vitro model of glucose uptake byL6 cell lines.

1.2 Material and methods for chemical and Cells

Fetal bovine serum was purchased from Hi-Media. Dulbecco's modified Eagle's medium (DMEM) and other culture products were purchased from Hi-Media. Trypsin, versene, glucose in PBS solution (TPVG) solution, bovine serum albumin (BSA), insulin, metformin and glucose kit were obtained from Randox Laboratory Ltd. ; dibasic sodium hydrogen phosphate, sodium bicarbonate, magnesium chloride, calcium chloride, potassium chloride and sodium chloride were from Ranbaxy Laboratories Ltd, SD Fine Chem. (Mumbai, India). All chemicals and solvents used were of analytical grade. The L6 cell line (RAT) was obtained from National Centre for Cell Sciences (Pune, India).

1.3 Experimental Design and Methodology

1.3.1 Materials and methods

1.3.1 Plant materials

Grewia umbellifera and *Aphanamixis polystchya* plants were collected from kanyakumari district andauthenticated by Dr.V.Chelladurai (Research Officer) Botany (C.C.R.A.S) Government of India. Voucher specimen (SIVET C-453/2012-2013) has been retained in the Dept of Biochemistry, S.I.V.E.T College of Arts & Science, Chennai. Materials were cleaned with water and dried in the shade until a constant weight was obtained.

1.3.2 Procedure for Extraction

The dried plant of *Grewia umbellifera* and *Aphanamixis polystachya* was coarsely powdered 50 gm was allowed to macerate for 48 hrs with 200ml of ethanol (50%), with occasional shaking. After 48 hrs, the ethanolic extract was

filtered through Whatman filter paper. The plant material was then macerated again with fresh 50% ethanol and the combined filtrate obtained from the first and the second maceration was then distilled under vacuum, the temperature of distillation being in the range of 55- 60ºC. After 3-5 hrs the three cycles were completed the extract weretransferred forthe distillation of alcohol, the extract was then evaporated to dryness and the total yield was noted. Further all the extracts were air dried till solid to semi solid mass was obtained and stored in air tight container in refrigerator below 10º C. Here after Ethanoic Leaf Extract of *Grewia umbellifera* and *Aphanamixis polystachya* mentioned as ELEGU and ELEAP respectively.

Percentage yield of *Grewia umbellifera*
Ethanol extract : 6.1 %
Percentage yield of *Aphanamixis polystachya*
Ethanol extract : 4.5 %

The ELEGU and ELEAP are thoroughly analyzed for the presence of different chemical group using standard methods (Brain and Tumer, 1975).

Table 1: Photochemical of screening ELEGU and ELEAP

S. No	Photochemical constituents	Name of the Test	Ethanolic Extract of GU	Ethanolic Extract of AP
1	Alkaloids	Mayer's test Dragondraff test Wagner Test	+ + +	+ + +
2	Tannins	Lead Acetate	+	+
3	Terpenoids	Noller Test	+	-
4	Steroidal Glycosides	Salkowaski	+	-
5	Protein	Biuret Test	+	+
6	Saponins glycosides	H_2So_4	+	-
7	Flavonoids	Shinoda's Test	+	-
8	Phenols	Ferric chloride	+	+

1.4 Preparation of cell culture

Monolayers of L6 cells were maintained at subconfluent conditions in growth media (DMEM with 4.5 g/l glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum). Cells were maintained in a humidified 37°C incubator with ambient oxygen and 5% CO2. Cells were maintained in continuous passage by trypsinization of subconfluent cultures using TPVG solution. (Figure 1A)

1.5 Cytotoxicity assay

The extract was separately dissolved in 1 ml of dimethyl sulfoxide (DMSO) and the volume was made up to 10 ml with maintenance medium to obtain a stock solution of 1 mg/ml concentration, sterilized by filtration and further dilutions were made from the stock. The cytotoxicity assays were carried out using 0.1 ml of cell suspension containing 10,000 cells seeded in each well of a 96-well microtitre plate (Tarsons India Pvt. Ltd., Kolkata, India).

Fresh medium containing different concentrations of the test sample was added after 24 hours of seeding. Control wells were incubated without test sample and with maintenance medium (2% serum). The microtitre plates were incubated at 37°C in a humidified incubator with 5% CO2 for a period of 72 hours. The morphology of the cells was inspected under the microscope for detectable alterations, i.e., loss of monolayer, granulation and vacuolization in the cytoplasm. The cytopathogenic effect (CPE) was scored. The 50% cytotoxic concentration (CTC50) was determined by the standard Microculture Tetrazolium MTT.(Francis and Rita, 1986) Table 2.

Table 2: Cell viability assay on L-6 cell lines for ELEGU and ELEAP

Sample Concentration (µl)	% Viability of ELEGU	% Viability of ELEAP
3.96	95.22 ± 1.25	60.45 ± 1.27
7.81	88.30 ± 1.45	55.54 ± 2.36
15.62	77.63 ± 1.26	40.25 ± 1.65
31.50	67.45 ± 0.74	31.58 ± 2.10
62.50	55.41 ± 0.76	21.45 ± 1.75
125.00	46.59 ± 1.07	15.28 ± 1.91
250.00	31.63 ± 1.12	10. 25 ± 1.74

1.5.1 MTT assay

The MTT assay is based on the protocol described for the first time by Mosmann (Mosmann, T. (1983). The assay was optimized for the cell lines used in the experiments. 3-(4, 5- Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) was applied to assess cell viability as described in. Cells (2 × 104/well) were plated in 100 µL of medium/well in 96- well plates and were allowed to attach to the plate for 24 hrs. ELEGU fraction was added at increasing concentrations (0–0.2mg/mL) for 24 h. The cells medium was replaced with 100 µL freshmedium/well containing 0.5mg/mL MTT and cultivated for another 4 h darkened in the cells incubator. The supernatant was removed and 100 µL isopropanol/HCl (1mM HCl in 100% isopropanol) were added per well. The absorbance at 570 nm was measured with microplate reader (Anthos). Two wells per plate without cells served as blank. All experiments were repeated three times in triplicates (Table 2). The effect of the plants extracts on cell viability was expressed using the following formula:

$$\%\ CellViability = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} X100$$

1.6 Glucose uptake by L6 cell line

Glucose uptake activity was estimated by the methods described by Pareek *et al.* Cells were cultured on 6-well plates and incubated for 48 hours at 37°C in a CO2 incubator. When semi-confluent monolayer was formed, the culture was renewed with serum free DMEM containing 0.2% BSA and incubated for 18 hours at 37°C in the CO2 incubator. After 18 hours, the medium was discarded and cells were washed with krebsringer phosphate (KRP) buffer once. The cells are treated with insulin (1IU/ml), standard drug and ELEGU and ELEAP. Glucosesolution (1 M) was added and incubated for half an hour. The supernatant was collected for glucose estimation and glucose uptake was terminated by washing the cells three times with 1 ml ice-cold KRP buffer. Cells were subsequently lysed by freezing and thawing three times. Cell lysate was collected for glucose estimation. Glucose uptake was calculated as the difference between the initial and final glucose content in the incubated medium by Glucose oxidase-Peroxidase (GOD-POD) method. All experiments were repeated three times in triplicates (Table 1).

Statistical analysis

Data were presented as mean ± standard error and analyzed using one way analysis of variance (ANOVA) with Dunnett test.

1.7 Results and Discussion

Many phytochemicals have been found to possess glucose uptake activity. Hypoglycemic herbs increase insulin secretion, enhance glucose uptake by adipose or muscle tissues, and inhibit glucose absorption from intestine and glucose production from the liver (Jesada P, Sutawadee C *et al* 2007).

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. It depends both on the number of viable cells and on the mitochondrial activity of cells. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is based on the assumption that dead cells or their products do not reduce tetrazolium. Tetrazolium salts are reduced only by metabolically active cells. Thus MTT can be reduced to a blue coloured formazan by mitochondrial enzyme succinate dehydrogenase. The amount of formazan produced is directly proportional to the number of active cells (Asokan and Thangavel, 2014). Skeletal muscle is the primary site responsible for postprandial glucose use. Furthermore, it is the most abundant tissue in the whole body, and thus, proper function of skeletal tissue is important to maintain normal blood glucose level (Defronzo RA, Jacot E, 1981).

Along with its pathological alterations to lipid metabolism and liver glycogen storage, insulin resistance disrupts proper clearance of glucose from the blood stream by both skeletal muscle and adipose tissue. As the pancreas begins to fail, insulin secretion decreases to a point where the plasma insulin concentrations are not sufficient to overcome the insulin resistance and type 2 diabetes is the result (DeFronzo, 1997).

Defects in insulin stimulated skeletal muscle glucose uptake are common pathological states in non-insulin-dependent diabetes mellitus (Baron AD, Laakso M*et al*, 1991). GLUT4 is the major glucose transporter expressed in insulin responsive tissue such as skeletal muscle and adipose tissue, where they respond to an acute insulin challenge by translocating GLUT4 rapidly from an intracellular membrane storage site to the plasma membrane (Suzuki K, Kono T. 1980).

Regulation of GLUT4 activity by insulin will enhance the muscle cell glucose uptake. Medicinal plants enhance the glucose uptake by GLUT4 translocation and were proven by *in vitro* glucose uptake model (Sudharshan Reddy D, *et al* : Yarasu N, *et al* : Anitha Mary M, *et al*: Premkumar N *et al* :). Insulin stimulates glucose uptake in skeletal muscle cells and fat cells by promoting the rapid translocation of GLUT4 glucose transporters to the plasma membrane. It regulates the release of GLUT4 from sequestered intracellular storage pools, and also has effects on docking and fusion of GLUT4 vesicles with plasma membrane (Sudharshan Reddy D, *et al* 2012). The insulin responsiveness of the membrane transport system for glucose (2-deoxy-D-glucose) in diaphragm was measured during postnatal development of the rat. The results indicate that the extent of insulin stimulation of glucose (2-deoxy-D-glucose) transport in the diaphragm during the first 20 days of life is not directly or simply related to the number of insulin receptors or the number of intracellular glucose transporters. (Suman Samaddar, *et.al*., 2015).

The extent of the insulin response depends on some other factors like phyto chemical compound present in the ELEGU that activates or is part of the machinery for translocation of the transporter. This mechanism has also been found that the sulfonylureas glibenclamide and glimepiride stimulates glucose utilization by rat diaphragm, and this stimulation is correlated with modulations of the cAMP regulatory cascade (Muller G, *et al*). The L-6 cell line is the best characterized cellular model origin to study glucose uptake and GLUT4 translocation (Patel MB., *et al*: Gupta RN, *et al*).

Hence in this study we have used L-6 muscle cells to determine the glucose uptake activity of ELEGU and ELEAP.The glucose uptake is shown by ELEGU and ELEAP comparable to Metformin and insulin treated group. ELEGU shows more effect when compared with ELEAP treated group. *In vitro* models comprising of skeletal muscle cells and adipocytes are widely used to study glucose uptake activity of drugs. The L-6 cell line is the best characterized cellular model origin to study glucose uptake and GLUT4 translocation (Patel MB, *et al* 2008, Yasungari K *et al* 1987). A 3T3-L1 adipocyte model has been used to study the effect of two sulfonylureas glibenclamide and glimepiride on glucose uptake. It has been found that they stimulate glucose uptake, and this stimulation is correlated with

modulations of the cAMP regulatory pathway (Muller G, Wied S.*et al*1994). ELEGU and ELEAP were evaluated for its cytotoxicity on L6 cells by MTT assay. The viability was found to be about 61% at a concentration of 50µg/ml for ELEGU and 24 % at concentration of ELEAP (Table 2). This indicates that ELEGU and ELEAP do not have significant cytotoxicity on L6 cells.

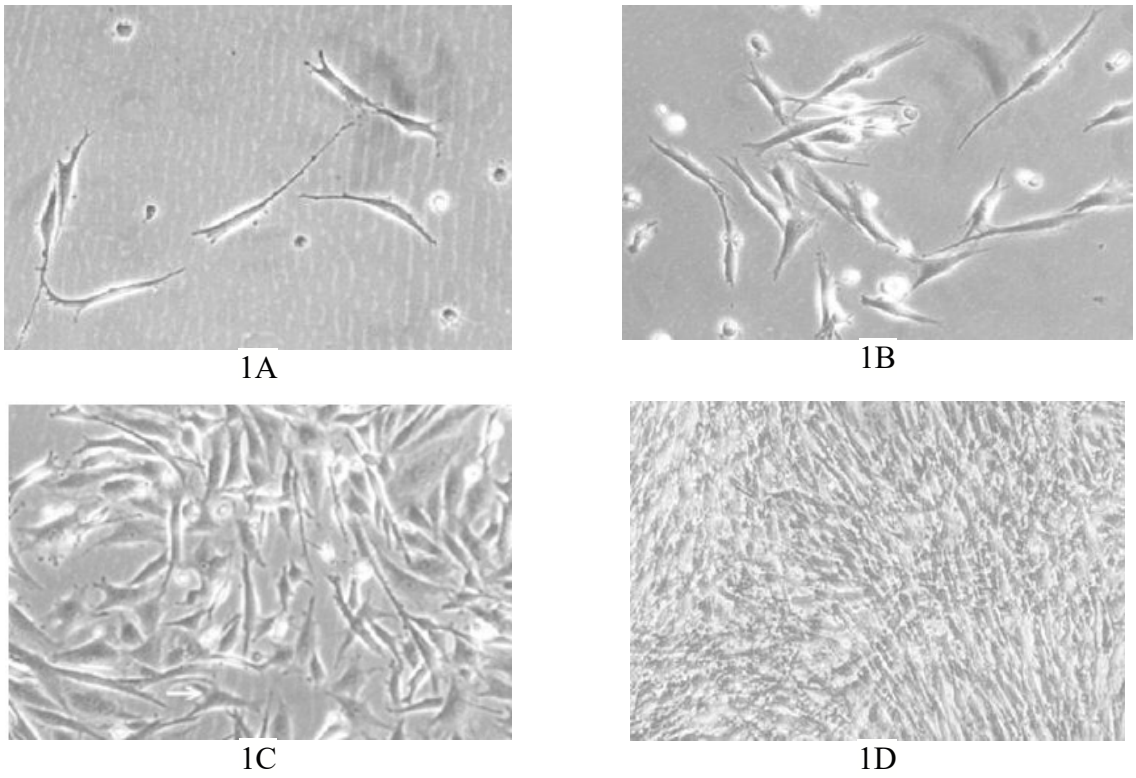
Glucose uptake by L6 in the presence of ELEGU, insulin and metformin occurred in a dose-dependent manner. The results show that uptake in the presence of ELEGU is higher as compared to ELEAP. The uptake peaked to 24.05 ± 5.45% for ELEGU and 12.56 ± 7.35% ELEAP as compared to control (Table 2).

Table 3: Effect of ELEGU and ELEAP on Glucose uptake by L6 cell line

Incubation medium used for glucose uptake assay –protocol.

Groups	Incubation medium (1000 µl)	% Glucose Uptake over control
Group 1 (control group)	900 µl of KRP Buffer 100 µl of glucose solution (1M)	2.0
Group 2	800 µl of KRP Buffer 100 µl of glucose solution (1M) 100 µl of Insulin (10 IU/ml)	131.79 ± 17.62
Group 3	800 µl of KRP Buffer 100 µl of glucose solution (1M) 100 µl of Metformin (1mg/ml)	69.45 ± 10.35^{a*}
Group 4	700 µl of KRP Buffer 100 µl of glucose solution (1M) 100 µl of Insulin (10 IU/ml) 100 µl of Metformin (1mg/ml)	135.85 ± 16.42*
Group 5	850 µl of KRP Buffer 100 µl of glucose solution (1M) 50 µl of ELEGU (2mg/ml)	24.05 ± 5.45^{ab*}
Group 6	850 µl of KRP Buffer 100 µl of glucose solution (1M) 50 µl of ELEAP (2mg/ml)	10.56 ± 7.35^{ab*}
Group 7	750 µl of KRP Buffer 100 µl of glucose solution (1M) 100 µl of Insulin (10 IU/ml) 50 µl of ELEGU (2mg/ml)	130.78 ± 14.51*
Group 8	750 µl of KRP Buffer 100 µl of glucose solution (1M) 100 µl of Insulin (10 IU/ml) 50 µl of ELEAP (2mg/ml)	132.79 ± 13.54*

Figure1A: Morphology and growth of L6 cell lines



1A 1B

1C 1D

1A: L6 cell lines shows that spindle-like shape before proliferation.
1B: L6 cell lines shows after proliferation day 5.
1C: L6 cell line shows after proliferation day 8. Large colonies cells were more densely distributed and spindle shape.
1D: The L6 cell line shows after proliferation day 12. Center of the colonies had several overcrowded layers and ceased proliferation.

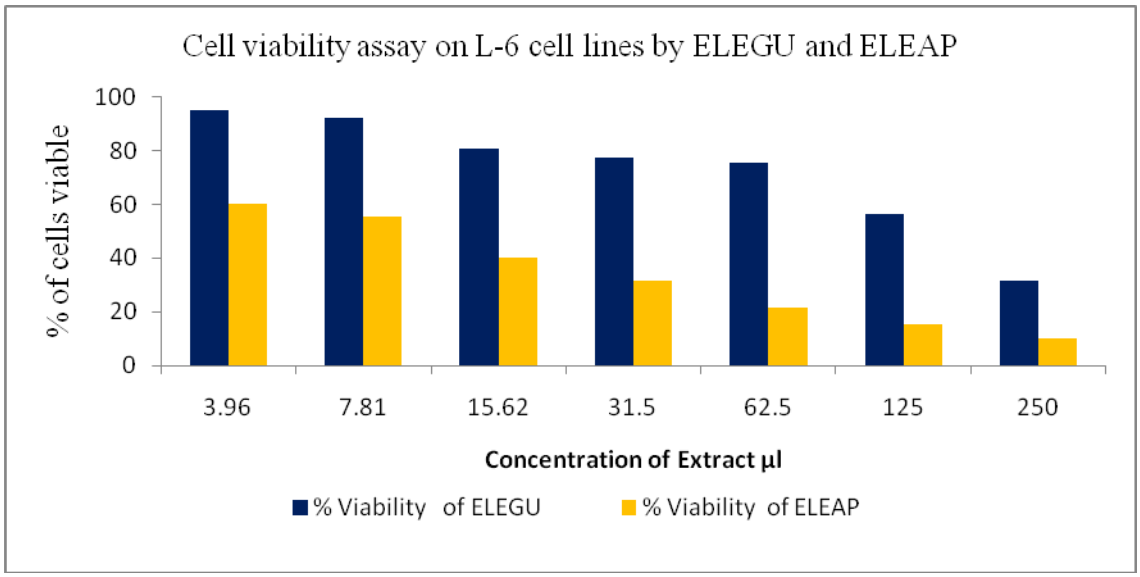


Figure 1B: Cell viability assay on L-6 cell lines for ELEGU and ELEAP

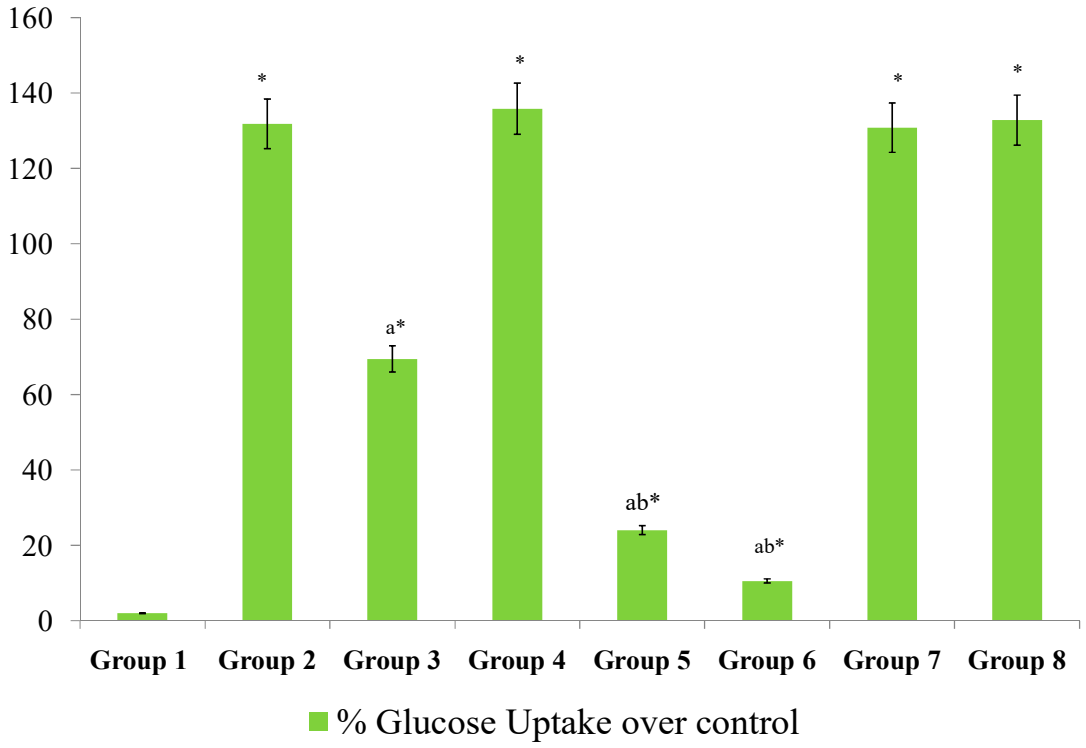


Figure 2: Effect of ELEGU and ELEAP on Glucose uptake by various groups of L6 cell line. Group 1 – control, Group 5 – ELEGU alone, Group 6 – ELEAP alone. Statistical significance was compared within the groups as follows: [a]control: [b]Insulin.
Values are statistically significant at * $p < 0.05$.

1.8 Conclusion

This study have established that plant extracts and its compounds have the ability to stimulate glucose uptake far better in the presence of insulin than in the absence of insulin i.e only ELEGU and ELEAP. The findings of this study indicate that ELEGU possesses significant type II antidiabetic activity than ELEAP (Figure 2). It enhanced glucose uptake in L6 cells considerably as compared to insulin and metformin. This may be due to its effect on the number of phytocompounds present in the extract that tend to enhance glucose uptake in the hypoglycemic state. Elucidation of its exact mechanism of action calls for further research. The phytoconstituent is a promising drug candidate against insulin-resistant hyperglycemia.

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