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Original Article

In Vitro Cytotoxicity and Glucose Uptake Activity of Gymnemic Acid Fraction of Gymnema Sylvestre Leaves

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ABSTRACT

Gymnema sylvestre is a native plant of India belonging to the family Asclepedaceae. The aim of the present study is to evaluate the cytotoxicity and glucose uptake activity of gymnemic acid fraction of gymnema sylvestre using L-6 cell lines. The results showed that the gymnemic acid fraction of gymnema sylvestre leaves did not confer any cytotoxicity and showed better glucose uptake potential. The results were compared with metformin, which were used as the standard type 2 antidiabetic drug. Metformin (100 µg/ml) enhance the glucose uptake over control.

Key words : Cytotoxicity, Gymnemic acid, L6 cell lines, metformin, Glucose transporter 4

1. INTRODUCTION

Diabetes is metabolic disease usually caused by a combination of hereditary and environmental factors, which result in hyperglycemia and other classical symptoms, especially polyuria, polydipsia, and polyphagia. Eventually, hyperglycemia leads to serious damage in blood vessels and the nerves as well as blurred vision and irritability. According to the World Health Organization (WHO), the number of people with diabetes will be doubled with in less than 30 years. Around 30 million people were characterized as

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diabetic in 1985 worldwide compared to around 171 million cases in 2010 and almost 377 million estimated in 2030 [1]. The prevalence of diabetes is the highest in the Middle East, where the number of diabetic subjects reached 15.2 million in 2000 and estimated to be triple by 2030.This remarkable increase is mainly due to population growth, lack of physical activity, obesity, aging and urbanization. Indeed, diabetes can be initially managed by exercise and dietary modifications. Drugs as well as medicinal food additives are necessary in advanced cases [2]. More than 1200 plant species are reported worldwide as antidiabetic .Over 400 plants as well as 700 recipes and compounds have been scientifically evaluated for DT2 treatment [3]. A healthy pancreas releases a regular supply of insulin into the blood supply. When blood glucose level rises pancreas will release insulin in response to that. The glucose in the blood reaches the pancreas and released to the beta cells from the capillaries, the beta cells will produce insulin in response to that. This insulin enters the blood stream and travels to three main destinations- the muscle, fat and liver cells. The surface of these cells contains insulin receptors. When insulin binds to these receptors it 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.

Gymnema Sylvestre is a woody, climbing plant of tropical forests of central and southern India and in parts of Africa. Gymnema has been referred to in some texts as *Asclepias geminata, Gymnema melicida,* and *Periploca sylvestris.* Gymnema has played an important role in Ayurvedic medicine for centuries. The major class of phytochemical belongs to *G. sylvestre* leaves contains triterpene saponins belonging to oleanane and dammarene classes. Oleanane saponins are gymnemic acids and gymnemasaponins, while dammarene saponins are gymnemasides. The other chemical constituents are flavones, anthraquinones, hentri-acontane, pentatriacontane, á and â-chlorophylls, phytin, resins, d-quercitol, tartaric acid, formic acid, butyric acid, lupeol, â-amyrin related glycosides and stigmasterol, some alkaloids and anthroquinones. The folklore claim of gymnema sylvestre leves are Antiflu, Antihistaminic, Antiinflammatory, Antiobesity, Antidiabetic Antipyretic, Antiseptic, Antiviral, Cyclooxygenase-Inhibitor, Fungicide, Gastrostimulant, Hypotensive, Hypothermic, Immunostimulant, Molluscicide, Mutagenic, Nematicide, Progesteronigenic, Sedative, Serotoninergic, Thyrotropic [4] . Hence, the objective of the present study was designed to investigate the free radical scavenging capacity of gymnema sylvestre leaves in various in-vitro models.

2. MATERIALS AND METHODS

Monolayer culture in log phase,Drug extracts (different concentration),DMEM without FCS,0.4µ filter,5ml sterile storage vial,Tissue paper, spirit, cotton, marker pen and gloves, Micropipette and tips,Discarding jar.

2.1 Collection and authentication

Gymnema sylvestre leaves were collected from Anna Herbal Garden, Chennai, Tamil Nadu. It was identified by Botanist, Plant Anatomy Research Centre (PARC), Tambaram, Chennai, Tamil Nadu and specimen voucher (**PARC/2012/1279**) are kept at Department of Pharmacognoscy, Vels College of Pharmacy, Chennai. The leaves of *Gymnema sylvestre* were shade dried grounded and stored dry until extraction.

2.2 Extraction of Gymnemic acid by Hoopers method

Step 1: extraction with petroleum ether (Defatting process)

I kg of dried *gymnema sylvestre* dry leaf powder was packed into a clean soxhlet extraction unit. Seven litres of petroleum ether (60-80^{o}C) was added and extracted for 24-36 hrs till all components are soluble in petroleum. petroleum ether extract is collected and distilled . Then a net of 240gm of petroleum extracts was obtained. petroleum ether extracts was obtained.

Step 2: Extraction with 90% methanol

The plant material is then extracted with 90% methanol. 90% methanol is added and the extraction was carried out for 24-36hrs till total methanol soluble extracts was obtained. Then methanol soluble extract was distilled and finally 185gm of thick paste were obtained.

Step 3: Isolation of pure gymnemic acid from methanol extract

175gm thick paste of methanol soluble extract was dissolved in 1% aq.KOH solution on continuously stirring for 45 min to 1hr.The solution is then filtered through filter paper to separate the undissolved particles. Diluted HCL was added slowly under constant stirring, during which the gymnemic acids were precipitated. Precipitated solution was filtered under suction and precipitate was dried. The pure gymnemic acid was obtained. The yield of crude gymnemic acid fraction was found to be 29.6%. The isolated gymnemic acid fraction was subjected to qualitative chemical test and thin layer studies and positive tests for steroids, terpenoids and glycosides. The gymnemic acid fraction was dissolved in ethanol used for further studies. [5]

2.3 Cell Culture

L6 Rat skeletal muscle Cells lines were obtained from the National centre for cell science Pune. All cells were grown under an atmosphere of 95% air and 5% CO2 in D-MEM (L6 cells) supplemented with 10% fetal calf serum (FCS), 1mM L-glutamine, 100U/mL penicillin, and 0.1mg/mL streptomycin.*2.4. MTT Assay*. The MTT assay is based on the protocol described for the first time by Mosmann (6). The assay was optimized for the cell lines used in the experiments. 3-(4, 5- Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium 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. Gymnemic acid fraction was added at increasing concentrations (0–2mg/mL) for 24 h. The cells medium was replaced with 100 μL fresh medium/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. The effect of the plants extracts on cell viability was expressed using the following formula:

Percent viability = (A570 nm of plant extract treated sample A570nm of nontreated sample) 100.

2.4 Glucose uptake assay

Cells were cultured on 6 well plates and incubated for 48 h at 37°C in a CO_2 incubator. When semi confluent monolayer was formed, the culture was renewed with serum free DMEM containing 0.2% BSA and incubated for 18 h at 37°C in the CO_2 incubator. After 18 h, the media was discarded and cells were washed with KRP buffer once. The cells were treated with Insulin, standard drug and plant extract and added glucose (1M) and incubated for half an hour. The supernatant was collected for glucose estimation and glucose uptake was terminated by washing the cells thrice with 1 ml ice-cold KRP buffer. Cells were subsequently lysed by freezing and thawing thrice. 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 GOD-POD method as follows:

Mix 10 µl of sample and 1 ml of reagent, incubate for 25 min at 15-25°C or 10 min at 37°C. Measure the absorbance of the standard ($A_{standard}$) and the sample (A_{sample}) against the reagent blank within 60 min, the time interval from sample addition to read time must be exactly the same for standard/control and sample.

$$\text{Glucose concentration} \quad \text{mmol} / l = \frac{A_{sample}}{A_{standard}} \times 55.5$$

$$\text{mg/dl} = \frac{A_{sample}}{A_{standard}} \times 100$$

3. RESULTS AND DISCUSSION

Medicinal plants offer a rich source of potentially useful antidiabetic drugs [7]. Gymnema sylvestre is recommended by asian practitioners for the treatment of diabetes . This plant has been recently reported as a potential anti-diabetic herb .Cytotoxicity and anti-diabetic properties of gymnemic acid fraction of gymnema sylvestre leaves were evaluated in the present in vitro study using L6-GLUT4myc muscle cells stably expressing myc epitope at the exofacial loop of glucose transporter-4 (GLUT4).

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 [8]. Gymnemic acid fraction of gymnema sylvestre toxicity was tested in vitro in L6-GLUT4myc cell following MTT as described in the materials and methods. Extract concentrations that kept at least 90% cell viability were considered as safe. Gymnemic acid fraction (Fig. 1, table 1 and plate 1-3) was found to be safe up to 0.25 mg/ml. Accordingly, all the efficacy studies (gymnic acid fraction) were performed at safe concentrations.

Medicinal plants enhance the glucose uptake by GLUT4 translocation and were proven by *in vitro* glucose model. The L6 and 3T3 cell lines are the best characterized cellular model origin to study glucose uptake and GLUT4 translocation. Glucose is the main source of energy in the brain. Uptaking of glucose is required by neurons during learning and memory. Alternatively, reduction of brain glucose metabolism caused cognitive deficits. Therefore, normal glucose metabolism is crucial in improving and maintaining learning and memory activities. Glucose metabolism is regulated by a comprehensive molecular network. Among these molecules, insulin is an essential factor in this processing and control.

Hence, in this study L6 cell lines are used to determine the glucose uptake activity of gymnemic acid fraction of gymnema sylvestre leaves and the results are presented in Table 1.

The glucose utilization in L6 cell lines showed that the gymnemic acid fractions of Gymnema sylvestre were found to be prominent over control. The L6 cell lines enhance the glucose uptake by 70.19**±1.72**at 500 µg/ml concentration (Fig. 2 & table 2). These results were compared with insulin and metformin, which were used as the standard antidiabetic drugs. Insulin at a concentration of 1IU/ml and metformin at a concentration of 100 µg/ml were found to enhance the glucose uptake over control. Similar results were reported by Mathews *et al*. [9], that the ethanolic extracts of root, fruit and aerial parts of *Solanum xanthocarpum* were found to have potent activity in enhancing the glucose uptake in L6 myotubes and it was compared with the insulin and metformin the standard

antidiabetic drugs. The study findings of Gupta *et al.* [10] have also clearly demonstrated that the fruits of *Helicteres isora* enhances glucose uptake under *in vitro* conditions by using L6 cell lines. Further the extract was tested with insulin for the conformation of the synergistic effects and the results indicated that the extract does not have any synergistic effect with insulin.

Hence, it can be concluded that the gymnemic acid fraction of gymnema sylvestre is found to be nontoxic and safe and also may be effective in glucose uptake. The major glucose transporter expressed in skeletal muscle and adipose tissue is GLUT-4 is translocated from an intracellular membrane storage site to the plasma membrane. The results of the present study demonstrated that the gymnemic acid fraction of gymnema sylvestre enhances the glucose uptake under *in vitro* conditions. This may due to the presence of phytoconstitutents in the gymnemic acid fraction of gymnema sylvestre or due to its effect on the receptors on the cell membrane.

Treated 500µg/Ml **Treated 125 µg/mL** **Treated 31.25 µg/mL**

Plate 1: Invitro Cytotoxicity Effect of gymnemic acid fraction on Skeletal Muscle Cells (L6 Myotubes).

Table 1 : Cytotoxicity effect of gymnemic acid fraction on skeletal muscle cell lines L-6

S.no	Concentration	Chloroform
1	3.906	96.33±1.27
2	7.8125	84.30±1.50
3	15.625	79.36±1.31
4	31.25	67.71±0.75
5	62.5	55.51±0.71
6	125	46.74±1.08
7	250	34.63±1.66
8	500	24.55±1.11
9	Cell control	100

Fig 1 : Cytotoxicity effect of gymnemic acid fraction on skeletal muscle cell lines L-6

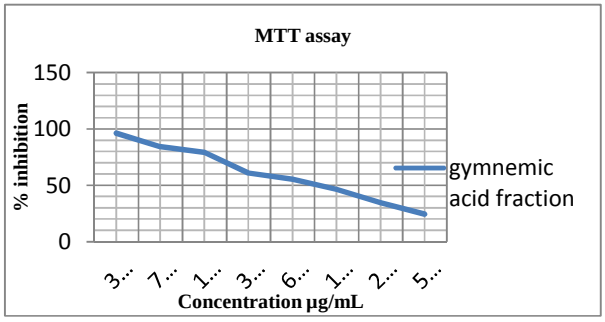
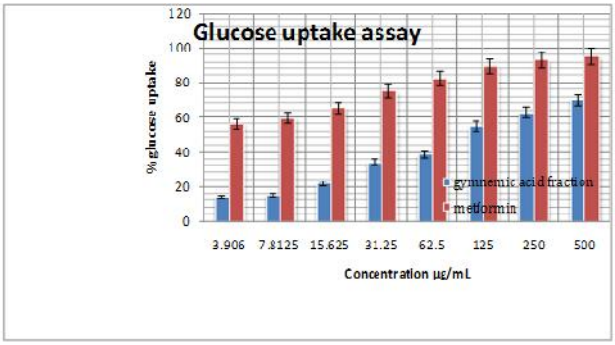


Table 2 : Invitro Glucose Uptake assay in L6 cell lines for gymnemic acid fraction

Sno	Concentration (µg/ml)	Gymnemic acid fraction	Metformin
1	3.906	14.12±0.97	56.47±0.41
2	7.8125	15.37±0.36	59.70±0.42
3	15.625	21.86±0.26	65.51±0.71
4	31.25	34.23±1.31	75.59±0.74
5	62.5	38.67±0.40	82.58±0.94
6	125	54.92±1.03	89.70±0.44
7	250	62.81±0.77	93.29±0.57
8	500	70.19±1.72	95.63±0.74

Fig 2: Invitro Glucose Uptake assay in L6 cell lines for gymnemic acid fraction



4. CONCLUSION

Gymnema sylvestre has traditionally been used to treat a number of diseases. Here experimental studies of gymnemic acid fraction of gymnema sylvestre leaves exhibited considerable antidiabetic activity and low cytotoxicity on L6 cell lines.

Here, L6 skeletal muscle cell lines expressing myc epitope at the exofacial loop of the glucose transporter-4 (GLUT4), were used as a model to follow GLUT4 translocation to the plasma membrane. We applied chemical and biophysical approaches to identify the active compounds in OB that might be involved in the

GLUT4 translocation to the skeletal muscle PM. The gymnemic acid fraction increased GLUT4 translocation to the PM up to 7 times, demonstrating safety, and the involvement GLUT4 translocation in the observed antidiabetic properties of gymnemic acid fraction of gymnema sylvestre leaves.

However, *in vivo* studies have to be carried out to substantiate the *in vitro* results by employing different *in vivo* models and clinical trials for their effective utilization as therapeutic agents.

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