



**IN VITRO CYTOTOXICITY AND GLUCOSE UPTAKE ACTIVITY OF SIDDHA FORMULATION NAAVAL KOTTAI MATHIRAI IN L-6 CELL LINES**

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**ABSTRACT**

The Siddha system of medicine is a traditional Indian system which evolved with the development of mankind and is more of an evolution rather than invention. Siddha system describes the health of an individual as an ideal perfect state of the physical, physiological, social, and spiritual components of a human being. Diabetes is a chronic disorder linked with the metabolism of carbohydrate, protein and fat due to absolute or relative deficiency of insulin secretion with or without varying degree of insulin resistance. There is sufficient number of drugs in different systems of medicine for the management of diabetes but the incidence of type-II diabetes is too high. In Siddha system many more traditional formulations are available. Most of these medicines are clinically used by Siddha physicians but have not been evaluated scientifically. *Naaval kottai Mathira* is a anti-diabetic Siddha herbal formulation which is prepared from the seeds of *Syzygium cumini* and leaves of *Aristolochia bracteolata* and it has been used for the management of Diabetes mellitus (*Madhumegam*). The aim of the present study is to evaluate the cytotoxicity and glucose uptake activity of herbal preparation NKM using L-6 cell lines. The results showed that the test drug did not confer any cytotoxicity and the drug NKM showed better glucose uptake potential. The result of glucose uptake percentage for standard drug Rosiglitazone is  $113.26 \pm 7.72$  and  $32.67 \pm 4.25$  for *Naaval Kottai Mathirai*. The findings of this investigation concluded that the study drug NKM has anti-diabetic activity in glucose uptake assay.

**KEYWORDS:** Glucose Uptake - Cytotoxicity - *Naaval Kottai Mathirai* - Siddha medicine.

**INTRODUCTION**

Diabetes mellitus, one of the most common endocrine metabolic disorders caused significant morbidity and mortality due to microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (heart attack, stroke and peripheral vascular disease) complications<sup>(1)</sup>. According to the fifth edition of the World Diabetes Atlas released by the International Diabetes Federation (IDF), as of 2011, the total adult population in the age group of 20-79 years stands at 4.3 billion, out of which 366 million live with diabetes, which is set to increase to 552 million by 2030.<sup>(2)</sup>

According to World Health Organization the diabetic population is likely to increase up to 300 million or more by the year 2025.<sup>(3)</sup>

There is sufficient number of drugs in different systems of medicine for the management of diabetes but the incidence of type-II diabetes is too high. The mainstay of the treatment includes oral hypoglycaemic and insulin<sup>(4,5)</sup>. They have many side effects with their long term usage<sup>(6,7)</sup>. Herbal drugs may be preferred as alternatives or adjuncts' because of their relative safety, efficacy and affordability. Herbal drugs are widely prescribed and used, in many forms, in different systems of medicine. They are used though we act data substantiating their usefulness and efficacy.

Skeletal muscle is a major tissue for blood glucose utilization and a primary target tissue for insulin action.

Insulin decreases glucose uptake in skeletal muscle by increasing functional glucose transport molecules (GLUT-4) in the plasma membrane. Glucose transport in skeletal muscle can also be stimulated by contractile activity<sup>(8)</sup> (Dachani et al., 2012). Most drugs used for treating diabetes causes obesity as a side effect by reducing blood glucose level and inducing adipogenesis. Traditional medicinal plants can serve as an ideal candidate in treating obesity and type 2 diabetes by acting on adipocytes and can act as a better alternative for the treatment of metabolic disorders<sup>(9)</sup> (Prathapan et al., 2012). L6 cell lines are derived from the skeletal muscle and are used in antidiabetic research to study cytotoxicity / uptake of glucose. L6 cells represent a good model for glucose uptake because they have been used extensively to elucidate the mechanisms of of glucose uptake in muscle, have an intact insulin signaling pathway and express the insulin-sensitive GLUT-4<sup>(10)</sup> (Ammerman et al., 2008).

*Naaval Kottai Mathirai*<sup>(11)</sup> is a herbal Siddha preparation which has been used for diabetes (*Madhumegam*) and mentioned in classical Siddha literature *Kannusamiyam ennum Pathartha Guna Vilakkam Mooligai Vakuppu*. Hence the present study was aimed to screen the cytotoxicity of the *Naaval Kottai Mathirai* by MTT assay and to evaluate its glucose uptake using L-6 cell line.

**MATERIALS AND METHODS****Plant material and Naaval Kottai Mathirai preparation**

The seeds of *Syzygium cumini* (L.) skeels (Figure:1) and *Aristolochia bracteolata* Lam. (Figure:2) were collected from Seeganendal village, Pudukkottai district, Tamilnadu and duly authenticated by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, Chennai-45. The authentication number is PARC/2016/3255 and 3256. The collected seeds of *S.cumini* were powdered and grounded by adding the leaf juice of *A. bracteolata* until its waxy consistency and made into 500mgm pills (*Pattani size*) (Figure:3).

**Chemicals**

3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Bovine Serum Albumin (BSA), D- glucose, Dulbecco's Modified Eagle's Medium (DMEM), Metformin and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Antibiotics from Hi-Media Laboratories Ltd., Mumbai. Insulin (Torrent Pharmaceuticals, 40IU/ml) was purchased from a drug store. Dimethyl Sulfoxide (DMSO), NaOH and Propanol from E.Merck Ltd., Mumbai, India.

**Cell lines and Culture medium**

L-6 (Rat, Skeletal muscle) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of L-6 were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO<sub>2</sub> at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm<sup>2</sup> culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

**Preparation of test solutions**

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

**Determination of cell viability by MTT Assay**

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10<sup>5</sup> cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The

plates were then incubated at 37o C for 3 days in 5% CO<sub>2</sub> atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37o C in 5% CO<sub>2</sub> atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC50) values is generated from the dose-response curves for each cell line<sup>(12)</sup> (Francis and Rita, 1986).

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

**In vitro glucose uptake assay**

Glucose uptake activity of test drug was determined in differentiated L6 cells. In brief, the 24 hr cell cultures with 70-80% confluency in 40mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4-6 days. The extent of differentiation was established by observing multinucleation of cells. The differentiated cells were serum starved over night and at the time of experiment cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) once and incubated with KRP buffer with 0.1% BSA for 30min at 37 °C. Cells were treated with different non-toxic concentrations of test and standard drugs for 30 min along with negative controls at 37°C. D-glucose solution was added simultaneously to each well and incubated at 37°C for 30 min. After incubation, the uptake of the glucose was terminated by aspiration of solutions from wells and washing thrice with ice-cold KRP buffer solution. Cells were lysed with 0.1M NaOH solution and an aliquot of cell lysates were used to measure the cell associated glucose. The glucose levels in cell lysates were measured using glucose assay kit. Three independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls.<sup>(13,14)</sup> (Takigawa-Imamura *et al.*, 2003; Yap *et al.*, 2007).

**RESULTS**

The cytotoxicity of anti diabetic Siddha medicine Naaval Kottai Mathirai was evaluated by MTT assay. The CTC value of NKM is 166.45±5.4 in L 6 cell lines (Table 1. Fig.1). In this glucose uptake study, the drug NKM was found to have potent activity in enhancing the glucose uptake in L-6 myotubes with 18.73±6.36 and 32.67 ± 4.25 percent uptake over controls for the drug concentration of 50 and 100 mcg/ml respectively. The standard drug Rosiglitazone showed 113.26 ±7.72 percentage glucose uptake over control. (Table 2).

**Table 1: In - vitro cytotoxic effect of Naaval Kottai Mathirai using MTT Assay**

Sl.No	Name of Test drug	Test Conc.( µg/ml)	% Cytotoxicity	CTC <sub>50</sub> ( µg/ml)
1	<i>Naaval Kottai Mathirai</i>	1000	82.68±0.4	166.45±5.4
		500	81.15±1.0	
		250	80.36±1.2	

		125	33.64±3.8
		62.5	15.75±5.1

**Table 2: In -vitro glucose uptake activity of Naaval Kottai Mathirai in L6 cell lines**

Sl.No.	Name of the Test substances	Test Conc. In mcg/ml	Glucose uptake percentage (%)
1	Control	-	0.74±1.19
2	Rosiglitazone	100	113.26±7.72
3	Naaval Kottai Mathirai	100	32.67±4.25
		50	18.73±6.36

**DISCUSSION**

The drug NKM has been used for the management of Diabetes (*Madhumegam*) in Siddha system of medicine. This present study was aimed to prove its antidiabetic effect by *In vitro* Glucose uptake in L<sub>6</sub> cell lines method. The cytotoxicity of NKM was evaluated by MTT assay. The results showed that the test drug NKM did not show any cytotoxicity.

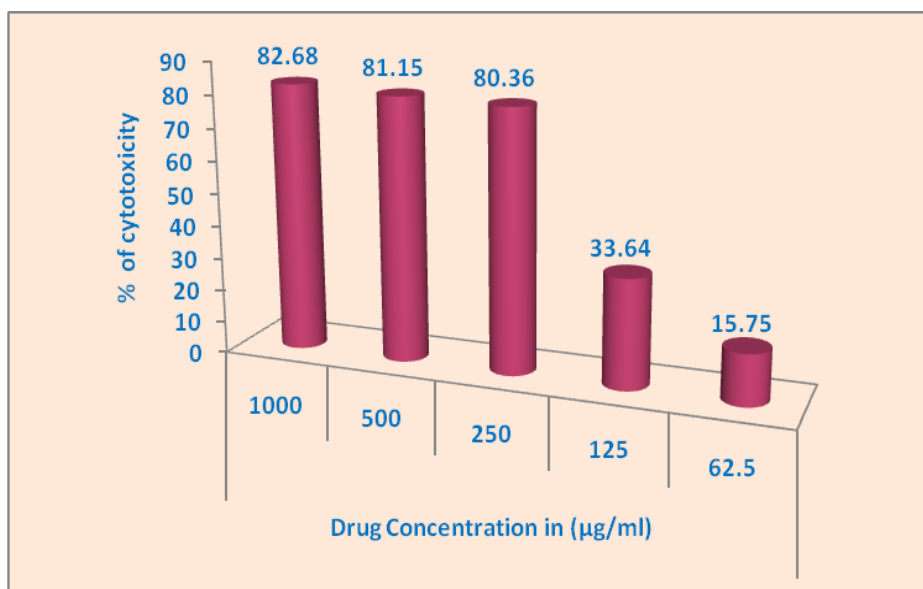
Skeletal muscle is a major tissue involved in insulin induced stimulation of glucose uptake. In the skeletal muscle, insulin increases glucose uptake by increasing functional glucose transport molecules in the plasma membrane. Glucose transport in skeletal muscle can also be stimulated by contractile activity (Atmakuri and Dathi, 2010). Defects in insulin stimulated skeletal muscle glucose uptake are common pathological states in noninsulin-dependent diabetes mellitus (Dachani et al., 2012). L6 cells represent a good model for glucose uptake because they have been used extensively to elucidate the mechanisms of glucose uptake in muscle, have an intact insulin signaling pathway and express the insulin-sensitive GLUT4<sup>(15)</sup> (Gupta et al., 2010).

The results of in-vitro anti diabetic effect of NKM by glucose uptake assay revealed the increase in percentage of glucose uptake in lymphocyte culture preparation. When compared to Rosiglitazone on increasing the dose of NKM, the % glucose uptake also increased proportionally. Rosiglitazone increases the % glucose uptake by skeletal muscle via glucose transporters

and the report of the NKM also showed the increase in percentage of glucose uptake. So the current observations confirms the role of % glucose uptake and are indeed may be due to the activation of PPAR $\gamma$  by PPAR $\gamma$  agonist (insulin sensitisers) which are currently being used in the treatment of insulin resistance associated with type-2 diabetes mellitus and thus influenced the peripheral glucose uptake. Drugs like thiazolidinediones and Insulin cause differentiation of pre-adipocytes into adipocytes. The adipocytes then stimulate glucose uptake and this aid in reducing the blood glucose levels. Therefore, drugs which exhibit glucose uptake activity would be desirable for patients with T2DM. The drug NKM exhibited increase in % glucose uptake and thus can be explored as glucose lowering agent to treat T2DM<sup>(16, 17)</sup>. The study supports this hypothesis and given a lead to explore the role of NKM in glucose uptake.

**CONCLUSION**

The results of the present study demonstrated that the drug NKM enhances the glucose uptake under in vitro conditions. This may due to the presence of phytoconstituents in the seeds of *Syzygium cumini* or due to its effect on the receptors on the cell membrane. 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.



**Fig. 1: In vitro cytotoxic effect of Naaval Kottai Mathirai using MTT Assay**





Fig: 1. *Syzygium cumini* seed



Fig: 2. *Aristolochia bracteolata*



Fig: 3. Naaval Kottai Mathirai

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