

In vitro Cytotoxicity and Glucose Uptake Activity of Fruits of *Terminalia bellirica* in *Vero*, L-6 and 3T3 cell lines

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ABSTRACT

Terminalia bellirica is a native plant of India belonging to the family Combrataceae. The aim of the present study is to evaluate the cytotoxicity and glucose uptake activity of ethanolic extract of fruits of *Terminalia bellirica* using *Vero*, L-6 and 3T3 cell lines. The results showed that the extracts did not confer any cytotoxicity and the ethanolic extract showed better glucose uptake potential. The results were compared with insulin and metformin, which were used as the standard antidiabetic drugs. Insulin (1IU/ml) and metformin (100 µg/ml) enhance the glucose uptake over control.

INTRODUCTION

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 (Sangeetha *et al.*, 2014). 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 (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 (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. L-6 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 (Ammerman *et al.*, 2008). *Terminalia bellirica* belongs to the family Combretaceae, commonly known as dhandrika in Tamil, is a deciduous tree found throughout the Indian forests and plains. It is reported to promote digestive power, wound healing, curative of ulcers, local swelling, anemia and chronic recurrent fever. The fruits are purgative, laxative, gastroprotective and are used to alleviate asthma, piles and cough. It has been reported to exhibit a variety of biological activities, such as anticancer, antimutagenic and antiviral activity (Manohar *et al.*, 2012).

Hence the present study was aimed to screen the cytotoxicity of the ethanolic extract of the fruits of *Terminalia bellirica* by MTT assay and to evaluate their glucose uptake using different cell lines namely L-6 and 3T3 and compared with normal healthy *Vero* cell lines.

MATERIALS AND METHODS

Plant material and extract preparation

The fruits of *Terminalia bellirica* were collected from Pondi area, Coimbatore and duly authenticated by Botanical Survey of India, TNAU, Coimbatore. The authentication number is BSI/SRC/5/23/2014-15/Tech 510. The collected fruits were washed, dried and powdered. The powdered fruits were then subjected to ethanolic extraction by using Soxhlet apparatus.

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Chemicals

3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Modified Eagle's Medium (DMEM) and trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl sulfoxide (DMSO) and propanol from E.Merck Ltd., Mumbai, India.

Cell lines and Culture medium

L6 (Rat muscle), 3T3 and *Vero* cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells 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 a humidified atmosphere of 5% CO₂ 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 cm² 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 10⁵ 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 37° C for 3 days in 5% CO₂ 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 37° C in 5% CO₂ 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 microplate 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 (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 drugs were determined in differentiated L6, 3T3 and *Vero* 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 (Takigawa-Imamura *et al.*, 2003; Yap *et al.*, 2007).

RESULTS AND DISCUSSION

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).

In the present study, the ethanolic extract of the fruits of *Terminalia bellirica* was screened using MTT for its cytotoxicity against three cell lines namely *Vero*, 3T3 and L6 at different concentrations to determine the IC₅₀ value.

The cytotoxicity of the ethanolic extract fruits of *Terminalia bellirica* was found to be dose dependent. The ethanolic extract of fruits of *Terminalia bellirica* did not confer any significant lethality to the healthy *Vero* cell lines, L6 and 3T3 cell lines with an LC₅₀ value greater than 1000 µg/ml confirming the safe nature of the extract. Among the cell lines studied, less cytotoxicity was seen towards *Vero* cell line than L6 and 3T3 cell line.

Our results are similar to that of work done by Srividhya and coworkers (2012) that they had shown a moderate cytotoxicity of

Polygonum chinensis extracts (400 - 125 µg/ml) in *Vero* cell lines and L6 cell lines. Banu *et al.* (2014) have studied the anticancer activity of *Datura metel* on MCF-7 and *Vero* cell line. MCF-7 exhibited greater cytotoxicity in comparison with a normal *Vero* cell line. Wild plant extracts from Saudi Arabia were screened for cytotoxicity in breast cancer cell line and control cell line. The extracts possessed greater cytotoxicity to cancer cell lines but were less sensitive to the normal cell line (Ali *et al.*, 2014).

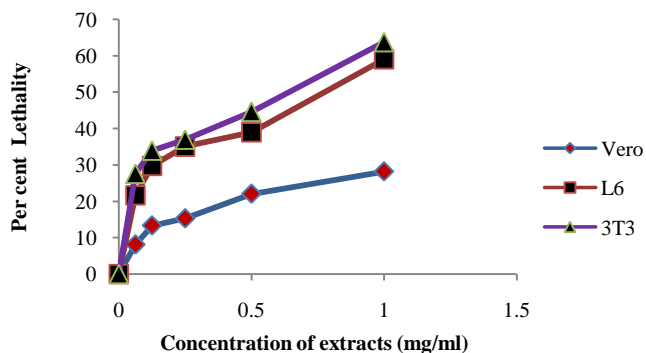


Fig. 1: *In vitro* cytotoxic effect of *Terminalia bellirica* using MTT Assay.

Altogether, the present study demonstrated that the ethanolic extract do not cause any adverse effect and hence could be considered nontoxic and safe. The results of the present study confirm that it can be safely consumed. The extracts were then subjected to analyze for its glucose uptake. On the basis of cytotoxicity assay the dose was decided for glucose uptake using cell lines.

Glucose uptake activity of ethanolic extract of fruits of *Terminalia bellirica* using *Vero*, L6 and 3T3 cell lines

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 non-insulin-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 (Gupta *et al.*, 2010).

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. Hence, in this study *Vero*, L6 and 3T3 cell lines are used to determine the glucose uptake activity of ethanolic extract of fruits of *Terminalia bellirica* and the results are presented in Table 1. The glucose utilization in *Vero*, L6 and 3T3 cell lines showed that the ethanolic extract of fruits of *Terminalia bellirica* were found to be prominent over control. The *Vero*, L6 and 3T3 cell lines enhance

the glucose uptake by 27.15 ± 1.19 , 18.73 ± 1.29 and 24.43 ± 0.88 at 500 µg/ml concentration. 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.

Table 1: *in vitro* glucose uptake studies in *Vero*, L6 and 3T3 cell lines.

Sample	Concentration (µg/mL)	% Glucose uptake over control		
		Vero	L6	3T3
	500	27.15 ± 1.19	18.73 ± 1.29	24.43 ± 0.88
EFTB	250	14.56 ± 0.94	9.60 ± 0.92	12.77 ± 0.84
Insulin	1 IU/ml	94.81 ± 1.76	88.67 ± 2.52	90.61 ± 2.25
Metformin	100	58.80 ± 1.40	55.93 ± 1.10	54.53 ± 1.10

Similar results were reported by Mathews *et al.* (2013), 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.* (2010) 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 fruits of *Terminalia bellirica* 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 ethanolic extract of fruits of *Terminalia bellirica* enhances the glucose uptake under *in vitro* conditions. This may due to the presence of phytoconstituents in the fruits of *Terminalia bellirica* 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.

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