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IN VITRO ANTIOXIDANT AND GLUCOSE UPTAKE EFFECT OF *TRICHODESMA INDICUM* IN L-6 CELL LINES

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ABSTRACT

The goal of the present study was to provide *in vitro* evidences for antioxidant and anti diabetic potential of *Trichodesma indicum* to generate a stronger biochemical rationale for further *in vivo* studies. The hydroalchohol extract of *Trichodesma indicum* whole plant was screened for its *in vitro* antioxidant activity using 2, 2-Diphenly 1-picryl hydrazyl assay, Scavenging of Nitric oxide and Superoxide radical methods, evaluation of Total antioxidant capacity of the extract and Reducing power assay. Cytotoxic effect of the extract was evaluated by MTT assay and *in-vitro* antidiabetic effect was studied using the glucose uptake model in rodent skeletal muscle cells (L-6 cells) involved in glucose utilization. The extract of *Trichodesma indicum* showed good antioxidant properties against 2, 2-Diphenly 1-picryl hydrazyl radical with low IC_{50} values 135μg/ml and against nitric oxide and superoxide radical exhibited poor scavenging properties with IC_{50} value of > 1000μg/ml. The total antioxidant activity which expressed equivalent to Ascorbic acid has 225.28 mg per gram of dried extract. The increased absorbance at 700 nm indicates an increase in reducing power of samples. The drug extract showed percentage growth inhibition value of 500 μg/ml and showed average glucose uptake ($P<0.05$) with percentage of glucose uptake of 91.03±10.12 over the control. The extract of *Trichodesma indicum* exhibited significant antioxidant activity and moderate antidiabetic activity and merits further investigation in animal models and support traditional claim.

KEYWORDS: **Trichodesma indicum, In-vitro antioxidant, Antidiabetic, Glucose uptake and L6 cell lines**

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INTRODUCTION

Trichodesma indicum (Linn.) R.Br. (Boraginaceae) is an herb found as a weed throughout the greater part of India, on roadsides and stony dry wastelands. In Ayurveda, the plant is beneficial in diseases of the eye; it is also prescribed for expulsion of the dead foetus[1]. The whole plant and root are used to treat arthritis, dysentery, skin diseases, snake-bite poisoning and fever[2]. The root paste is applied to reduce swellings, particularly of the joints; extract is given to children in dysentery and fever[3]. It is useful in vitiated conditions of *Vata* and *Kapha*, arthralgia, inflammations, dyspepsia, diarrhoea, dysentery, leprosy, skin diseases and diabetes[4,5]. Chemical constituents isolated from the plants include nonsteroidal compounds; hexacosane, ethyl hexacosanoate and 21, 24-hexacosadienoic acid ethyl esters from leaves[5], and oleic, linoleic, palmatic, stearic, and linolenic acid from seed oil[6]. Based on these traditional and phytochemical reports the plant has been screened for various pharmacological activities. Srikanth *et al.,* 2002 evaluated the effect of *Trichodesma indicum* extract on cough reflex induced by sulphur dioxide in mice (anti-tussive activity) [7]. Perianayagam *et al.,* 2005, 2006 and 2011 reported anti-inflammatory, anti-diarrheal, analgesic and antipyretic potential[8-10]. Khan et al., 2009 reported the antispasmodic and lipoxygenase inhibitory effects on the isolated rabbit jejunum[11]. The exact mechanism of these aforesaid pharmacological activities is yet to be elucidated and might be due to the role of antioxidant chemical constituents present in the plant. There are extensive evidences to implicate free radicals for the development of degenerative diseases. Free radical has been implicated in causation of ailments such as diabetes, liver cirrhosis, nephrotoxicity, inflammatory conditions etc. Together with other derivatives of oxygen they are inevitable by products of biological redox reactions. Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing tissue injury through covalent binding, and thus have been shown to augment collagen synthesis and fibrosis. The increased production of toxic oxygen derivatives is considered to be a universal feature of stress conditions. An anti-oxidant principle from natural sources possesses wide range of mechanisms to provide enormous scope in correcting the imbalance[12].

Diabetes mellitus is associated with insulin deficiency and decreased glucose uptake in skeletal muscles[13]. The increased plasma free radicals observed in diabetes mellitus may impair insulin action, thus contributing to the generation of hyperglycemia[14]. Defects in GLUT- 4 and GLUT-1 may explain the insulin-resistant glucose transport. Skeletal muscle is a major tissue for blood glucose utilization and a primary target tissue for insulin action. Insulin increases 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[15]. Free radical impairs insulin-stimulated GLUT-4 translocation and exerts an inhibitory effect on muscle contractility that is major pathological feature of diabetes[16, 17]. 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 GLUT-4[18].

A detailed review of literature afforded no information on the anti-oxidant and antidiabetic potential of *Trichodesma indicum*, it was therefore thought worthwhile to investigate the *in-vitro* anti-oxidant and antidiabetic potential in standard models.

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MATERIALS AND METHODS

Plant material and Preparation of Extract

The plant material was collected from Deshmukhi village of Nalagonda district and authenticated from Head, Department of Botany, Osmania University, Hyderabad (S.No.162, Voucher No: 0475). The collected plant material was cleaned, air dried and coarsely powdered. The coarse powder obtained was extracted exhaustively with 70% ethanol in Soxhlet apparatus and filtered. The extract was concentrated under reduced temperature and pressure to get dry residue and stored in a desiccators.

Chemicals

2, 2-Diphenyl-2-picryl hydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. Ascorbic acid and rutin were obtained from Merck Ltd., Mumbai, India. Naphthyl ethylene diamine dihydrochloride, sulphanilic acid, sodium hydroxide, sodium phosphate, Ammonium molybdate, potassiumm ferricyanide, trichloroacetic acid, ferric chloride, dimethylsulfoxide (DMSO), Propanol and glacial acetic acid were purchased from Ranbaxy Laboratories Ltd., Mohali, India and S. D. Fine Chem., Mumbai, India. 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. All chemicals and solvents used were of analytical grade.

In-vitro Antioxidant Activity

The extract was tested for in-vitro antioxidant activity using several standard methods. The absorbance was measured specrtrophotometrically against corresponding blank solution. The percentage inhibition was calculated by the following formula.

$$\text{Radical scavenging activity (\%)} = \frac{\text{OD control- OD sample}}{\text{OD control}} \times 100$$

IC_{50} which is the concentration of the sample required to scavenge 50% of the free radicals was calculated.

DPPH (2, 2-Diphenly 1-picryl hydrazyl) Assay

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in color and upon reaction with hydrogen donor changes to yellow color. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490nm. The assay was carried out in a 96 well microtitre plate. To 200 μl of DPPH solution, 10 μl of each of the test sample or the standard solution was added separately in wells of the microtitre plate (Tarsons Products (P) Ltd, Kolkota, India). The final concentration of the test and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625, 7.812 μg/ml. The plates were incubated at 37° C for 30 min and the absorbance of each solution was measured at 490 nm, using an ELISA microplate reader (Bio Rad Laboratories Inc, California, USA, Model 550). [19, 20]

Scavenging of Nitric Oxide Radical

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by

the use of modified Griess Ilosvay reaction. In the present investigation, Griess Ilosvay reagent is modified by using Naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Nitrite ions react with Griess reagent, which forms a purple azo dye. In presence of test components, likely to be scavengers, the amount of nitrites will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The absorbance of the chromophore formed was measured at 540 nm. The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, pH 7.4, 1 ml) and extract in DMSO at various concentrations or standard was incubated at 25° C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min for completion of diazotization. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink coloured chromophore was formed. The absorbance of these solutions was measured at 540 nm[21].

Scavenging of Superoxide Radical by Alkaline DMSO Method

The scavenging activity towards the superoxide radical (O_2^-) was measured in terms of inhibition of generation of $O2^-$. The method was performed by using alkaline DMSO method. In alkaline DMSO method, superoxide radical is generated by the addition of sodium hydroxide to air saturated DMSO. The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium into formazan dye at room temperature and that can be measured at 560 nm. Superoxide scavenger capable of reacting inhibits the formation of a red dye formazan. To the reaction mixture containing 1 ml of alkaline DMSO (1 ml, 1% distilled water, 5 mM NaOH) and 0.3 ml of the extracts in DMSO at various concentrations or standards, 0.1 ml of NBT (1 mg/ml) was added to give a final volume of 1.4 ml. The absorbance was measured at 560 nm[20].

Evaluation of Total Antioxidant Capacity of the Extract

The total antioxidant capacity was determined by phosphomolybdenum method and is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex which has the maximal absorption at 695 nm. An aliquot of 0.1 ml of the sample solution containing a reducing species in DMSO was combined in an eppendorff tube with 1 ml of reagent solution (0.6 M Sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in water bath at 95 °C for 90 min. The samples were cooled to room temperature, and the absorbance of each solution was measured at 695 nm. The total antioxidant capacity was expressed as mM equivalent of ascorbic acid[22].

Reducing Power

The reducing power of extracts was determined according to the method of Oyaizu (1986). Extract solution in methanol and water at different amounts were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min. After 2.5 ml of TCA (10%) was added, the mixture was centrifuged at 3000 rpm for 10 min. Supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of ferric chloride (0.1%) and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power[23].

In vitro anti-diabetic studies

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_2 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^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For *in vitro* 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.

Cytotoxic assay

Determination of cell viability by MTT Assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used[24].The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 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% CO_2 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% CO_2 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% (CTC_{50}- cytotoxic concentration at which 50% of the cells are dead after drug exposure) values is generated from the dose-response curves for each cell line.

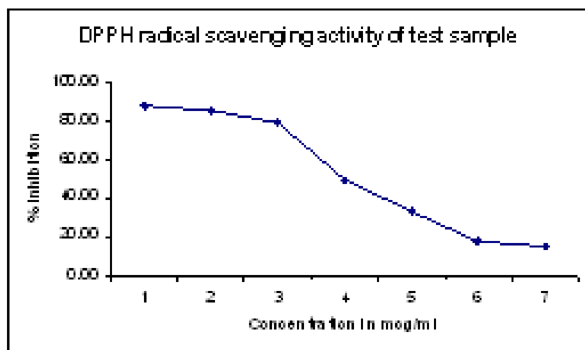
$$\%\ \text{Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of Control}} \times 100 \right)$$

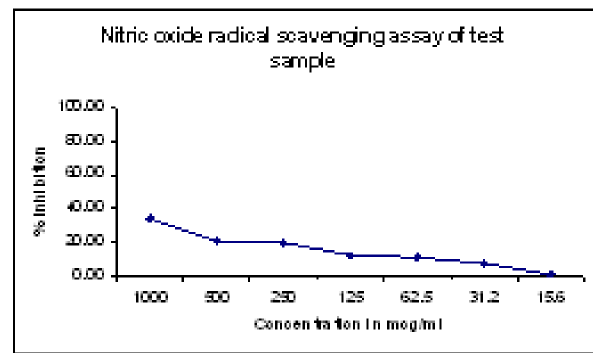
In vitro glucose uptake assay[25,]

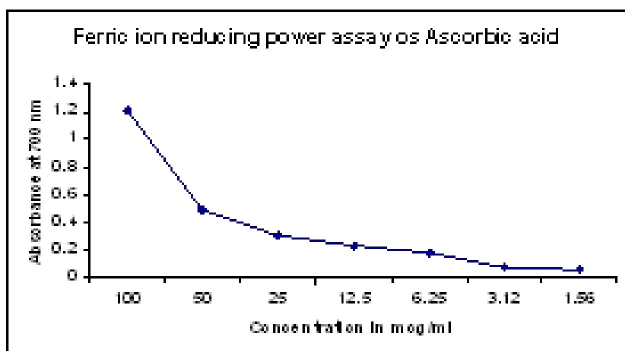
Glucose uptake activity of test drugs were determined in differentiated L6 cells. In brief, the 24 hr cell cultures with 70-80% confluency in 40mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4-6 days. The extent of differentiation was established by observing multinucleation of cells. The differentiated cells were serum starved over night and at the time of

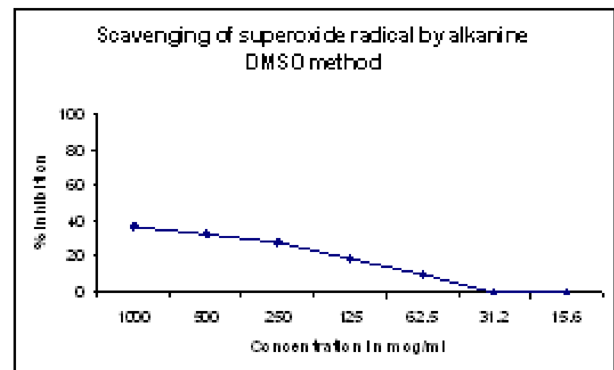
experiment cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) once and incubated with KRP buffer with 0.1% BSA for 30min at 37^0C. Cells were treated with different non-toxic concentrations of test and standard drugs for 30 min along with negative controls at 37^0C. 20µl of D-glucose solution was added simultaneously to each well and incubated at 37^0C 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 (Biovision Inc, USA). Three independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls.

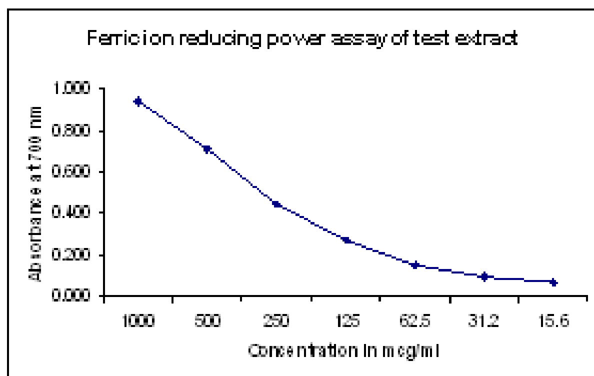
RESULTS AND DISCUSSION











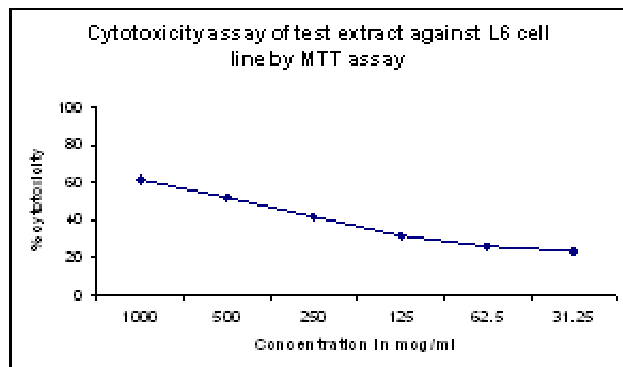


Figure 1

Antioxidant activity of different concentrations of hydroalchoholic extract in a) DPPH b) Nitric oxide c) Superoxide d) Ferric ion radical scavenging method e) Ferric ion reducing assay of Ascorbic acid and f) Cytotoxic assay against L6 cell line by MTT assay.

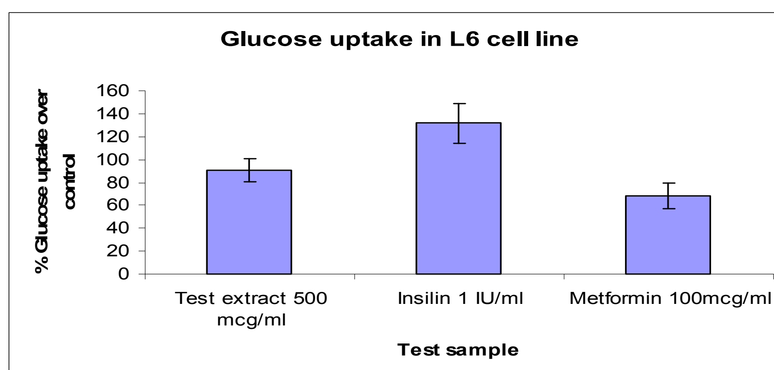


Figure 2

Glucose uptake of Trichodesma indicum, Insulin and Metformin in L6 cell line

Table 1

Absorbance of Trichodesma indicum Extract and Standard Ascorbic acid at various concentrations (µg/ml) in Ferric Reducing Power Determination Model.

Trichodesma indicum	**Absorbance**		**Ascorbic acid**
Concentration	700 nm		Concentration
1000	0.945	1.211	100
500	0.712	0.497	50
250	0.445	0.309	25
125	0.272	0.239	12.5
62.5	0.154	0.189	6.25
31.2	0.096	0.08	3.12
15.6	0.067	0.06	1.56

Table 2

Cytotoxic Properties of Test Drugs on L6 Cell Line.

Concentration (µg/ml)	**% Cytotoxicit**	**CTC_{50} (µg/ml)**
1000	61.43	500
500	51.75	
250	41.88	
125	31.41	
62.5	26.18	
31.25	23.56	

Table 3
In vitro glucose uptake studies in L-6 cell line

Sample	Concentration	% Glucose Uptake Over Control
Trichodesma indicum	500 µg/ml	91.03±10.12
Insulin	1 IU/ml	131.50 ± 17.62
Metformin	100 µg/ml	68.35 ± 11.45

The hydroalchohol extract of *Trichodesma indicum* was tested for its antioxidant activity in five different *in vitro* models. It was observed that free radicals were scavenged by the test compound in a concentration dependent manner up to the given concentration in all the models. *Trichodesma indicum* showed good antioxidant property against DPPH radical with IC_{50} value 135.00 µg/ml which was comparable to that of rutin (IC_{50} =3.91 ± 0.10 µg/ml). On the other hand against nitric oxide and superoxide radicals, test drug demonstrated poor scavenging properties with IC_{50} value >1000 µg/ml which was comparable to that of rutin with IC_{50} = 65.44 ± 1.56 and >1000 µg/ml respectively. The total antioxidant capacity of the extract which expressed equivalent to Ascorbic acid was found to be 225.28 mg per gram of dried extract (Fig 1). The reducing power of the hydroalchoholic extracts and standards increases with the increase in amount of sample and standard concentrations (Table 1). Increased absorbance of the reaction indicated increased reducing power suggesting strong reducing power potential of the extract. With these properties plant extract acts as reducing agent, hydrogen donator and singlet oxygen quencher. In addition, a good correlation between antioxidant activity and reducing power in some plant extracts has been observed[27]. Therefore the reducing capacity may be used as an indicator of the potential antioxidant activity. The extract of *Trichodesma indicum* was evaluated for its cytotoxic activity by MTT assay. It showed CTC_{50} value of 500 µg/ml in L6 cell lines and results are shown in Table 2. The extract showed average glucose uptake with percentage of glucose uptake of 91.03±10.12 over the control. Results were compared with insulin and Metformin, which were used as the standard anti diabetic drugs. Insulin (1IU/ml) and Metformin (100 µg/ml) enhance the glucose uptake over control (Fig 2 & Table 3). 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[28]. Defects in insulin stimulated skeletal muscle glucose uptake are common pathological states in non-insulin-dependent diabetes mellitus[29]. 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[30]. *Trichodesma indicum* showed antioxidant activities in *in-vitro* assays. These data show direct evidence that it has antidiabetic activity and suggest that the antidiabetic activity is due to improvement of antioxidant status. The results obtained in the present study clearly demonstrate that the *Trichodesma indicum* extract enhances glucose uptake under *in vitro* conditions. This may be due to its effect on the number of receptors located in the skeletal muscle cell line and further studies with estimation of insulin and insulin receptors may give more insight into the mechanism of the antidiabetic activity of *Trichodesma indicum.*

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