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## ***In Vitro* antioxidant and glucose uptake potential of *Tinospora cordifolia* leaves extract**

G. Sriram Prasath<sup>a\*</sup>, S. Rahini<sup>a</sup>, P.T. Srinivasan<sup>a</sup> and S.Subramanian<sup>b</sup>

<sup>a</sup>PG and Research Department of Biochemistry, Dwaraka Doss Goverdhan Doss Vaishnav College, Arumbakkam Chennai, India

<sup>b</sup>Department of Biochemistry, University of Madras, Guindy Campus, Chennai, India

### **ABSTRACT**

*Tinospora cordifolia* (Willd.), belonging to the family Menispermaceae, is known to exhibit a wide array of pharmacological properties such as antiulcer, anti-inflammatory and antiarthritic properties. In the present study an attempt has been made to evaluate the antioxidant and glucose uptake potential of *T. cordifolia* leaves extract in vitro. Phytochemical analysis of the leaves extract revealed the presence of phenols, alkaloids, flavonoids, glycosides, saponins, tannins, phytosterols and triterpenoides. The free radical scavenging activity of the leaves extract was determined against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS, Nitric oxide(NO) and Superoxide Scavenging assays. At a concentration of 800µg/ml, the leaves extract significantly scavenged 87.1 % of DPPH radicals and 86 % ABTS radicals. The leaves extract exhibited a maximum of 88.1% superoxide scavenging activity and 80.1% nitric oxide scavenging activity. Further, *T. cordifolia* leaves extract increases the uptake of glucose in rat L6 myotubes. These findings suggest that *T. cordifolia* possess antioxidant as well as glucose uptake potential and has complimentary potency to develop as an antihyperglycemic agent for the treatment of diabetes mellitus.

**Key words:** *Tinospora cordifolia*; Antioxidant; Antidiabetic; L6 myotubes.

### **INTRODUCTION**

Diabetes mellitus is multisystemic, multifactorial endocrine disorder resulting from the deficiency and/or insulin inefficiency of insulin, which in turn leads to chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism. It is broadly classified into Type 1 diabetes mellitus and Type 2 diabetes mellitus. Type II diabetes mellitus is the most common endocrine disorder, covering about 90–95% of all diabetes cases [1]. The pathogenesis of T2DM diabetes involves abnormalities in insulin secretion from pancreatic β-cells and resistance to insulin activity. The rising rates of obesity in youth have concurrently led to an increase in the prevalent rates of type 2 diabetes mellitus. Reducing the incidence of T2DM by preventing pediatric obesity through the implementation of lifestyle changes in the community should be the primary objective of healthcare systems [2].

Currently available therapeutic measurements to treat type II diabetes mellitus often elicit undesirable adverse effects like causing hypoglycemia at higher doses, liver dysfunctions, lactic acidosis and diarrhea. Hence, the search for drugs, preferentially from plant origin continues.

*Tinospora cordifolia* (Willd.) Miers ex Hook. F. and Thoms which belongs to the family Menispermaceae, is a large, deciduous, climbing shrub found throughout India. It is known as “Moonseed plant” in English, “seenthil kodi” in tamil, “Guduchi” in Sanskrit and “Giloy” in Hindi. *Tinospora cordifolia* is a widely used medicinal plant for the treatment of various ailments which includes inflammation, arthritic, allergy, infection, liver dysfunction, urinary disorders, ulcer, infertility, leprosy and diabetes. Anti-diabetic properties of *T. cordifolia* is well recorded in

traditional literature and is also supported by the modern scientific community [3]. The pharmacological activity of *T. cordifolia* could be related to the presence of various phytoingredients, of which the alkaloid fraction is thought to have anti-diabetic activity.

A variety of biologically important compounds belonging to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides are present in *T. cordifolia* plant. Water soluble isoquinoline alkaloids viz., jatrorrhizine, palmatine, berberine, tembetarine, magnoflorine, choline, tinosporine, isocolumbine and hydrastine are present [4-11]. Even though, *T. cordifolia* contains numerous alkaloids, berberine and palmatine are found to be present in significant amounts [12]. Berberine is a well proven anti-diabetic alkaloid in both pre-clinical and clinical studies [13]. However, the biological activity of palmatine has not been systematically studied. Hence, the mechanism of action of the anti-diabetic *T. cordifolia* along with its phytoingredient, palmatine has to be studied, for its proper clinical use. Hence, in the present study an attempt has been made to evaluate the antioxidant as well as glucose uptake potential of *T. cordifolia* leaves extract.

## MATERIALS AND METHODS

### Plant Material

The plant of *Tinospora cordifolia* was collected from the local surroundings in Chengalpattu. The plant was identified and authenticated by a taxonomist at the Centre for Advanced studies in Botany, University of Madras.

### Preparation of Extract

The leaves of *T. cordifolia* were collected and washed thoroughly under running tap water and then was rinsed in distilled water; they were allowed to dry for some time. The leaves (2kg) were shadow dried without any contamination for about 3 to 4 weeks.

The leaves of *T. cordifolia* were dried at room temperature and powdered in an electrical grinder, which was then stored in an airtight container at 5° C until further use. The powdered leaves were delipidated with petroleum ether (60 - 80° C) for overnight. Almost all the chlorophyll and lipids are deposited which was selectively removed. The extraction was done with ethanol. The dried powdered was subjected to Soxhlation using ethanol. The ethanolic extract was filtered, dried and weighed. The extract obtained was evaporated in rotary evaporator to get a powdery mass. The extract was dried under reduced pressure using rotator evaporator to get the crude. The yield was 13.6g. It was stored below 4 °C until further use.

### Preliminary phytochemical screening

The ethanolic extract of *Tinospora cordifolia* leaves were subjected to preliminary phytochemical screening for the identification of various plant constituents [14, 15].

### In vitro antioxidant assays

#### DPPH radical scavenging assay

The free radical scavenging capacity of the ethanolic extract of *T.cordifolia* was determined using DPPH [16]. Briefly, DPPH (200µM) solution was prepared in 95% methanol. From the stock plant extract solution 100, 200, 400, 800 µg/ ml were taken in five test tubes. 0.5ml of freshly prepared DPPH solution was incubated with test drug and after 10 minutes, absorbance was taken as 517 nm using spectrophotometer. Standard ascorbic acid was used as reference.

#### ABTS assay

ABTS radical scavenging activity of ethanolic extract of *T.cordifolia* was determined [17]. Briefly, ABTS radical cation (ABTS<sup>•+</sup>) was produced by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Then, ABTS<sup>•+</sup> solution was diluted with ethanol to an absorbance of 0.7 at 734 nm. To 3.0 ml of diluted ABTS<sup>•+</sup> solution, different concentration (100-800 µg/ ml) of leaves extract in ethanol was added and after 1 min, the decrease in absorbance was measured at 734 nm spectrophotometrically.

#### Assay for nitric oxide scavenging activity

Sodium nitroprusside (5 mM) in phosphate buffer pH 7.7 was incubated with 100, 200, 400 and 800 µg/ml concentrations of drug dissolved in a suitable solvent (alcohol) and tubes were incubated at 25°C for 120 minutes. At intervals, 0.5ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent N-naphthyl ethylenediamine was measured at 546 nm in [18].

**Superoxide anion (SO) radical scavenging assay**

The Superoxide radical scavenging activity of *T.cordifolia* was measured [19]. In this method, the activity is measured by reduction of riboflavin/light/NBT (Nitro blue tetrazolium). The 1 ml of reaction mixture contained Phosphate buffer, NADH, NBT and various Concentrations of sample solution. The method is based on generation of superoxide radicals by autooxidation of riboflavin in presence of light. The Superoxide radical reduces NBT to a blue coloured formazon that can be measured at 560 nm.

**Cell culture of L6 myoblasts and myotubes**

L6 myoblast cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India. 2-Deoxy-D-glucose, bovine serum albumin (BSA),  $\alpha$ -MEM and other cell culture solutions were procured from Hi-media, India. The fetal bovine serum (FBS), penicillin, streptomycin, gentamycin, amphotericin B was purchased from Genetix, India. Wortmannin, and insulin were obtained from Sigma Aldrich, USA.

L6 myoblasts were maintained in  $\alpha$ -MEM with 10% FBS and supplemented with penicillin (120units ml<sup>-1</sup>), streptomycin (75 $\mu$ g ml<sup>-1</sup>), gentamycin (160 $\mu$ g ml<sup>-1</sup>) and amphotericin B (3 $\mu$ g ml<sup>-1</sup>) in 5% CO<sub>2</sub> environment. For differentiation of L6 cells, they were transferred to  $\alpha$ -MEM with 2% FBS, and the cells were seeded in a collagen-coated 96 well (4x10<sup>3</sup> cells/well) micro plate and cultured in  $\alpha$ -MEM for 3 days with 10% FBS till semi confluent. Next, the cells were cultured in  $\alpha$ -MEM with 2% FBS for 5 days to differentiate into myotubes which were then used for glucose uptake assay. The extent of differentiation to myotubes was established by observing multinucleation of cells with the aid of inverted tissue culture microscope (Euromex, Finland).

**Effect of *T. cordifolia* leaves extract on Glucose Uptake**

The determination of cell viability was performed by MTT assay (Denizot and Lang, 1986). Twenty four hour cell cultures with 70–80% confluency in 40 mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4–6 days. The differentiated cells were used to measure the cell-associated glucose using glucose assay kit (Biovision Inc., USA) (Yap et al., 2007). For PI3-K inhibition studies, L6 myotubes were treated with wortmannin (100nM), 30min prior to the incubation with the *Tinospora cordifolia* (500  $\mu$ g/mL) and standard Insulin (1IU/mL) followed by the glucose uptake assay[20].

**Statistical analysis**

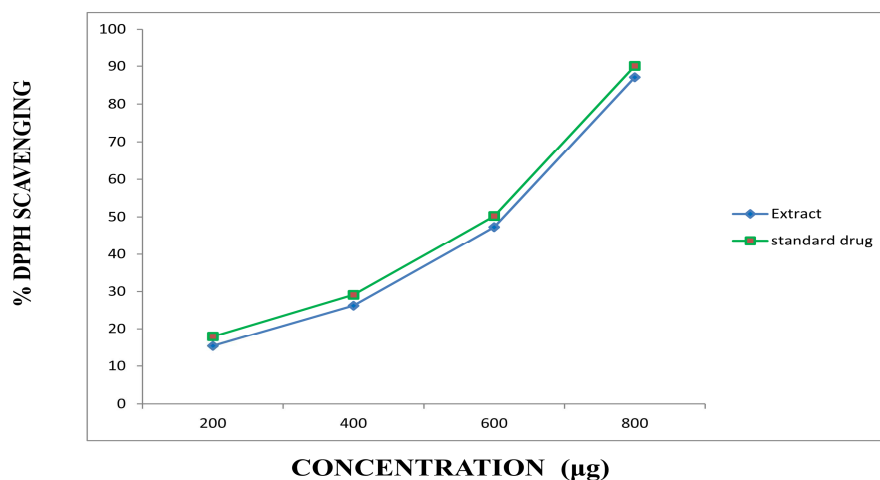
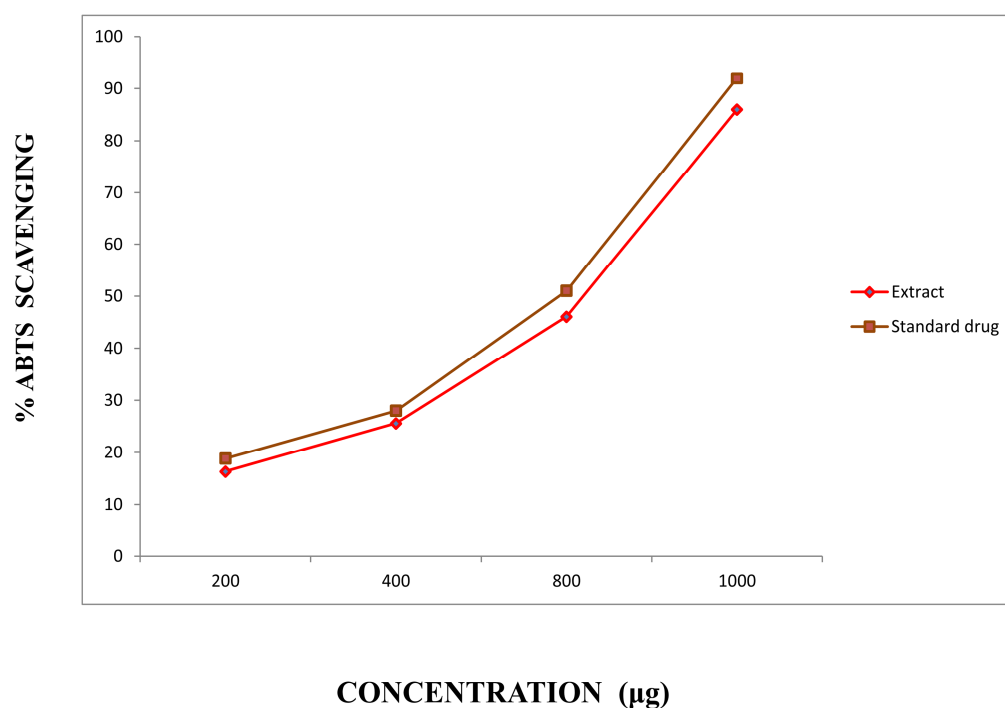
The results were expressed as mean  $\pm$  S.E.M and statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS (version 11.5) program followed by LSD. Values were considered statistically significant when  $p < 0.05$ .

**RESULTS**

Phytochemical screening of *T. cordifolia* leaves extract showed the presence of biologically active phytochemical constituents like alkaloids, flavonoids, glycosides, proteins, saponins, tannins, terpenoids and anthraquinones ethanolic extract (Table 1).

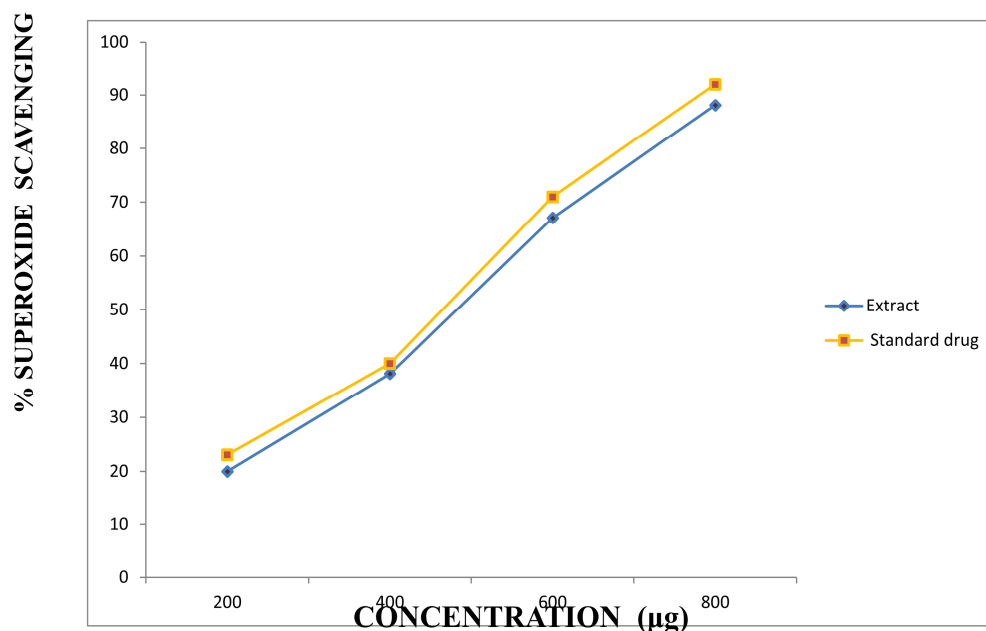
PHYTOCONSTITUENTS	INFERENCE
Alkaloids	+
Flavonoids	+
Saponins	+
Tannins	+
Phytosterol	+
Diterpenes	-
Triterpenoids	+
Glycosides	+
Anthraquinones	+
Phenols	+

Figure 1 and 2 shows the dose dependent effect of *T. cordifolia* leaves extract on the percentage inhibition of DPPH and ABTS radicals present in the reaction mixtures respectively. The ethanolic extract of *T.cordifolia* scavenges DPPH and ABTS radical in a concentration dependent manner. The leaves extract of *T. cordifolia* significantly and concentration dependently reduced the DPPH and ABTS radicals. However, at a concentration of 800 $\mu$ g/ml, the extract significantly scavenged 87.1 % of DPPH radicals and 86 % ABTS radicals.

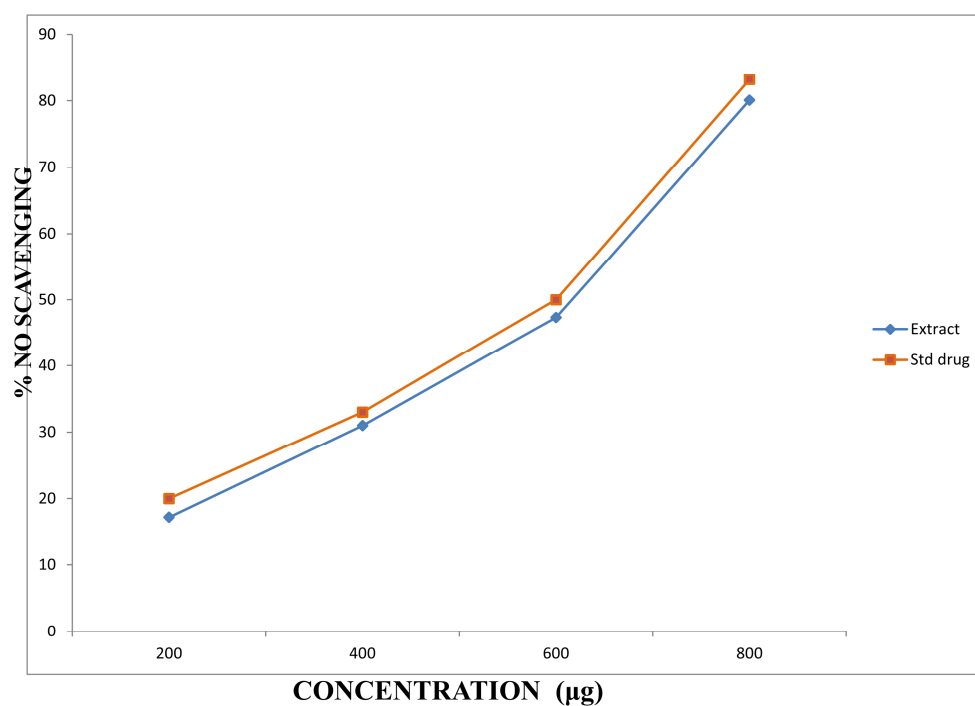
**Figure 1: DPPH radical scavenging potential of *Tinospora cordifolia* leaves extract****Figure 2: ABTS radical scavenging potential of *Tinospora cordifolia* leaves extract**

Superoxide radical and Nitric oxide radical scavenging potentials of the *T. cordifolia* leaves extract in a concentration dependent manner are depicted in Fig 5 and 6 respectively.. The test extract exhibited a maximum of 88.1% superoxide scavenging activity (Fig 3) with a significant extent at a concentration of 800 µg/ml. *T. cordifolia* leaves extract at a concentration of 800 µg/ml also quenched 80.1% NO released by a NO donor (Fig 4).

**Figure 3: Superoxide radical scavenging potential of *Tinospora cordifolia* leaves extract**



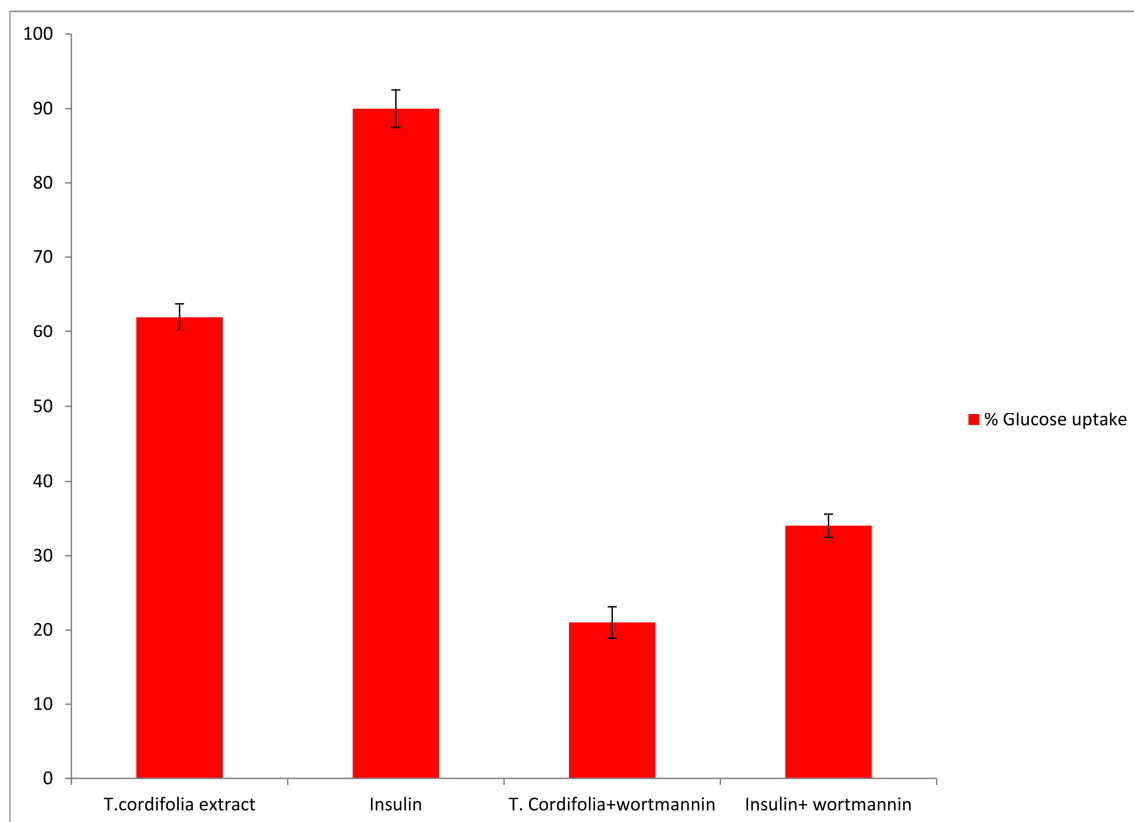
**Figure 4: NO scavenging potential of *T.cordifolia* leaves extract**



In Glucose uptake assay, *T. cordifolia* leaves extract showed  $62.0 \pm 1.79\%$  glucose uptake over control compared with the standard insulin (1 IU/mL) which showed  $90 \pm 2.5\%$  glucose uptake over control. In the presence of PI3K

inhibitor wortmannin, *T. cordifolia* leaves extract showed  $21 \pm 2.1\%$  glucose uptake over control and the standard insulin (1 IU/mL) showed  $34 \pm 1.55\%$  glucose uptake over control (Fig 5).

**Figure 5: Effect of *Tinospora cordifolia* and insulin on glucose uptake in L6 muscle cell lines in the absence and presence of wortmannin (100 nM).**



## DISCUSSION

Plants are the major source of medicine in the past centuries and today scientists recognize their value as a source of new or complimentary medicinal products. Over the past twenty years, interest in medicinal plants has increased enormously from the use of herbal products as natural remedies by the general public to the scientific investigations of plants for their biological effects in human beings. Beyond this pharmaceutical approach to plants, there is a wide tendency to utilize herbal product to supplement the diet, mainly with the intention of improving the quality of life and preventing the diseases of elderly people [21].

Phytochemical investigation of *T.cordifolia* has led to the exploration of a variety of constituents which are responsible for its wide range of pharmacological activities. *T.cordifolia* extract contain most of the biologically active phytochemicals. The present study reveals that *T. cordifolia* leaves extract showed the presence of phytochemical constituents like alkaloids, flavonoids, glycosides, proteins, saponins, tannins, terpenoids and anthraquinones which readily accounts for its pharmacological properties.

The free radical scavenging potential of natural products can be assessed by several assays. Among them, DPPH, ABTS, Nitric oxide and Superoxide radical scavenging assays are routinely practiced for the assessment of antioxidant properties of different natural compounds, as they are easy, affordable and reliable. The ability of natural compounds to scavenge the DPPH radical can be expressed as its magnitude of antioxidative ability. DPPH radical in alcoholic solution is deep purple in colour with an absorption peak at 515 nm. DPPH assay is based on the principle that DPPH radical on accepting a hydrogen atom from the scavenger molecule i.e. antioxidant, results in reduction of unpaired valence electron at one atom of nitrogen bridge in DPPH leading to the change of purple color to yellow with concomitant decrease in absorbance at 515 nm [16]. The change in colour from deep purple to yellow or the decrease in intensity signifies the antioxidant potential of the test compound.

ABTS assay is used for the screening of antioxidant activity of both water and lipid soluble compounds. The assay involves reduction of the color intensity of ethanolic solution containing pre-formed radical monocation of ABTS, generated by oxidation of ABTS with potassium persulfate due to the radical scavenging activity of antioxidants [17]. The change in color intensity is proportional to the antioxidant efficiency of compounds.

The ethanolic extract of *T. cordifolia* scavenges both DPPH and ABTS radical in a concentration dependent manner. The leaves extract of *T. cordifolia* significantly and concentration dependently reduced DPPH and ABTS radicals. However, at a concentration of 800 µg/ml, the extract significantly scavenged 87.1 % of DPPH radicals and 86 % ABTS radicals

Superoxide radical scavenging activity was shown by the extract in a concentration dependent manner. Superoxide anions play an important role in the formation of the ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA. The leaves extract exhibited a maximum of 88.1% superoxide scavenging activity with a significant extent at a concentration of 800 µg/ml. *T. cordifolia* leaves extract at a concentration of 800 µg/ml also quenched 80.1% NO radical.

Skeletal muscle contributes up to 50% of total body mass, making it the largest organ in the body. In the fasting and resting state, skeletal muscles utilizes relatively small amount of glucose whereas, in postprandial state, skeletal muscle accounts for 75% of glucose disposal. Hence, abnormalities in the skeletal muscle metabolism can affect the metabolic budget of the body [22]. During diabetes mellitus, the uptake of glucose by skeletal muscles is reduced either due to the deficiency of insulin secretion or its resistance, resulting in impaired glucose homeostasis [23].

In Glucose uptake assay, *T. cordifolia* leaves extract showed  $62.0 \pm 1.79\%$  glucose uptake over control compared with the standard insulin (1 IU/mL) which showed  $90 \pm 2.5\%$  glucose uptake over control. In the presence of Phosphoinositide 3-kinase inhibitor wortmannin, *T. cordifolia* showed  $21 \pm 2.1\%$  glucose uptake over control and the standard insulin (1 IU/mL) showed  $34 \pm 1.55\%$  glucose uptake over control (Fig 7). *T. cordifolia* may stimulate metabolic effects in skeletal muscle cells. *T. cordifolia* may enhance glucose uptake by increasing the translocation of Glucose transporter-4 (GLUT4) to the cell membrane.

Skeletal muscle, a primary tissue responsible for postprandial uptake of glucose from blood, accounts for nearly 50% of body mass and >30% energy expenditure. Glucose transport is the rate-limiting step in glucose utilization in insulin targeted skeletal muscle. Glucose transport is facilitated by the major glucose transporter proteins, GLUT4 and GLUT 1 in skeletal muscle. Thus, skeletal muscle is quantitatively the most important glucose-utilizing tissue and the acceleration of glucose uptake into skeletal muscle upon exposure to insulin is accompanied by a redistribution of the “insulin-responsive” facilitated glucose transporter isoform, GLUT4 [24]. The insulin signaling pathway leading to increased muscle glucose uptake involves the binding of insulin to its receptor, phosphorylation of downstream insulin receptor substrates (IRS) and activation of phosphatidylinositol 3 kinase (PI3-K) and Akt which promotes the membrane translocation of GLUT4 from the intracellular pools [25]. Defects in GLUT4 translocation contribute to insulin resistance, a characteristic of diabetes mellitus [26].

Cellular glucose consumption in the presence and absence of inhibitors of insulin pathway were studied individually for each test drug and the results are shown in Fig. 1A and B. Presence of inhibitors suppressed the glucose consumption efficiency of *T. cordifolia* which concludes the anti-diabetic activity is mediated through insulin dependent pathway through the activation of Phosphoinositide 3-kinase (PI-3 kinase). However, presence of these inhibitors did not completely arrest the glucose consumption efficiency. Berberine a close analogue of palmatine is shown to mediate its action by promoting Glut-4 translocation and by increased AMP-activated protein kinase (AMPK) activity in L6 myotubes, and modulated lipogenic genes thereby reducing lipid accumulation in 3T3-L1 cells [27, 28]. Hence, alkaloids like berberine and palmatine are considered to share similar activity in mediating anti-diabetic property.

Insulin mediates muscle glucose transport via a signaling pathway which involves the binding of insulin to the insulin receptor, tyrosine phosphorylation of insulin receptor substrate-1/2 (IRS-1/2), activation of phosphatidylinositol 3-kinase (PI3-kinase), phosphorylation of Akt, and phosphorylation of the Akt substrate of 160 kDa (AS160) resulting in the translocation of GLUT4 to the plasma membrane. From the present study, it is evident that *T. cordifolia* increases glucose uptake. In the Presence of Wortmannin, a PI3 kinase inhibitor, the glucose uptake is reduced which evidence the fact *T. cordifolia* leaves extract may facilitates the translocation of GLUT4 via PI3 kinase mediated pathway.



## CONCLUSION

*T. cordifolia* leaves extract shows the presence of phytochemical constituents like alkaloids, flavonoids, glycosides, proteins, saponins, tannins, terpenoids and anthraquinones. The medicinal properties of the *T.cordifolia* leaves extract may be due to the presence of pharmacologically active phytoingredients. The leaves extract exhibited free radical scavenging potential which is evident from DPPH, ABTS, Superoxide and NO radical scavenging assays. The results of the present study also showed that *T. cordifolia* increases glucose uptake by GLUT4 translocation through PI3kinase pathway. The observed effect of the leaves extract may be due to the existence of palmatine and berberine which are of antidiabetic in nature.

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