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ORIGINAL ARTICLE

Anti-diabetic Activities of *Dactylorhiza hatagirea* Leaf Extract in 3T3-L1 Cell Line Model

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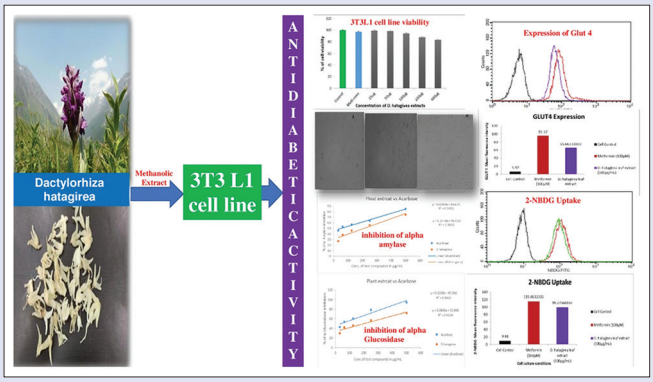
ABSTRACT

Background: Diabetes is an endocrine disorder that results in altered carbohydrate, protein, and lipid metabolism. Several synthetic drugs used to treat diabetes have adverse effects on prolonged usage. This has given the impetus to the search for alternative medicines with no or less side effects. The plants of *Orchidaceae* family have displayed antimicrobial, anti-inflammatory, antioxidant, anticancer, and antidiabetic activity. However, their antidiabetic properties are yet to be explored. **Materials and Methods:** The *in vitro* antidiabetic properties of *Dactylorhiza hatagirea* leaf extract were studied by biochemical assays such as α-amylase and α-glucosidase inhibition assays and *in vitro* cellular assays such as glucose uptake assay and glucose transporter type 4 (GLUT4) expression studies in 3T3-L1 cell line. **Results:** The methanolic extract of *D. hatagirea*, at varying concentrations (25 μg-400 μg/mL), did not exhibit cytotoxicity against 3T3-L1 cell line after 24 h of incubation. Methanolic extract of *D. hatagirea* leaves showed significant inhibition of α-Amylase and α-Glucosidase enzymes. After 24 h of exposure of 3T3-L1 cells to 100 μg/mL of *D. hatagirea* leaf extract and 100 μM of metformin, the relative expression rates of GLUT4 receptor were elevated when compared with untreated cells. The results also revealed that the amount of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose taken up by 3T3-L1 cells treated with *D. hatagirea* leaf extract and metformin is higher than that of untreated cells. **Conclusion:** Our results suggest that methanolic extract of *D. hatagirea* leaves has potential antidiabetic activity and could be a plausible resource for antidiabetic agents.

Key words: 3T3-L1 Cell line, *Dactylorhiza hatagirea*, flow cytometry, glucose uptake, glucose transporter type 4

SUMMARY

- Diabetes is an endocrine and metabolic disorder that has affected millions worldwide. Several medicinal plants have been used in traditional medicine to treat diabetes
- The *in vitro* anti-diabetic properties of *Dactylorhiza hatagirea* leaf extract were studied by biochemical assays like alpha-amylase and glucosidase inhibition assays and *in vitro* cellular assays like glucose uptake assay and glucose virtue of transporter type 4 (GLUT4) expression studies in 3T3-L1 cell line
- The leaf extract of *D. hatagirea* at different concentrations (25 μg–400 μg/mL) did not exhibit cytotoxic effect on 3T3-L1 cell line after 24 h of incubation
- Methanolic extract of *D. hatagirea* leaf showed significant inhibition of alpha-amylase and significant inhibition of α-glucosidase
- After 24 h of exposure of 3T3-L1 cells to 100 μg/mL of *D. hatagirea* leaf extract and 100 μM of Metformin, the relative expression rates of Glut4 receptor increased compared to untreated control and the mean fluorescence intensities
- The amount of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose taken up by 3T3-L1 cells treated with *D. hatagirea* leaf extract, and metformin is higher than untreated cells
- The methanolic extract of *D. hatagirea* leaves has shown promising anti-diabetic properties and could be used as a potential source to identify bioactive compounds that can be used as anti-diabetic agents.



Abbreviations used: *D. hatagirea: Dactylorhiza hatagirea;* GLUT4: Glucose transporter member 4; 2-NBDG: 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose; WHO: World Health Organization; DMEM: Dulbecco's Modified Eagle Medium; D-PBS: Dulbecco's phosphate-buffered saline; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; DMSO: Dimethyl sulfoxide; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC_{50}: Half maximal inhibitory concentration.

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder with multifarious etiologies that include chronic hyperglycemia and perturbance in the metabolism of carbohydrates, fats, and protein due to defective secretion and/or function of insulin.[1] DM has an estimated 382 million adult patients and 5.1 million deaths in 2013.[2] Long-term consequences of DM include dysfunction and damage to multiple organs, namely the kidney, nerves, heart, and gastrointestinal tract.[3,4] In India, diabetes is quickly becoming an epidemic with an additional 62 million recently diagnosed with the disease.[5,6]

Existent treatment regimens encompass a wide range of synthetic drugs which have adverse effects on prolonged use. For this reason, a remedy for DM with natural, accessible compounds that do not require arduous pharmaceutical processes is desired.[7,8] In this scenario, there has been a renewed interest in screening plants to validate their medicinal properties and to isolate their bioactive compounds. Systematic screening may pave way to the discovery of novel active compounds with antidiabetic properties.[9,10] Furthermore, the recommendations of the World Health Organization to evaluate the effectiveness of plants in conditions where we lack safe, modern drugs[11] has provided an added impetus for the same. These ideas have guided an increased interest in research on natural products, with antidiabetic properties, that have negligible or no adverse effects.[12,13]

Several studies guided by the interest to prepare oral antihyperglycemic agents from plants employed in traditional medicine have resulted in the identification of some plants with good antidiabetic properties.[14,15] One of the plant families that has been much appreciated for its general medicinal properties in traditional medicine is *Orchidaceae* (Orchids).[16] However, the scope to identify antidiabetic agents from plant species belonging to this family has not been tapped to its full potential. *Dactylorhiza hatagirea* (Salam panja) is an orchid that has been reported to have nutritive, astringent, aphrodisiac, demulcent, anti-diarrheal properties, and tonic properties. The mucilage jelly of the rhizome has also been used to treat diarrhea, dysentery, and chronic fever.[13] However, their antidiabatic properties are unknown.

In this study, we evaluated the antidiabetic properties of *D. hatagirea* leaf extract *in vitro* using the 3T3-L1 as a model system. The extract showed no cytotoxic effect on the cells and exhibited antidiabetic properties manifested by the inhibitory effect on α-amylase and α-glucosidase enzymes, enhancing the cellular uptake of glucose by inducing the expression of glucose transporter type 4 (GLUT4) on the cell surface.

MATERIALS AND METHODS

Chemicals and reagents

DMEM high glucose (#AL219A, Himedia), DMEM without glucose (#AL186, Himedia), Fetal Bovine Serum (#RM10432, Himedia), D-PBS (#TL1006, Himedia), 2-NBDG (Invitrogen: Cat no. 13195), Metformin (#PHR 1084, Sigma), Acarbose (#A8980, Sigma), Anti-Mouse Glut4–FITC Antibody (#NBPI-49533F, Novus Biologicals), MTT Reagent (# 4060 Himedia), DMSO (#PHR1309, Sigma),

Collection of plant material

Fresh leaves of *D. hatagirea* (D. Don) Soo. were collected from the western Himalayas in the month of May of the year 2018. The plant materials was examined and authenticated by Dr. Kotresha, Assistant Professor of Botany, East West College, Bengaluru. Figure 1 is a representative image of the plant.

Figure 1: *Dactylorhiza hatagirea*-Himalayan Marsh Orchid

Preparation of extract

The shade-dried leaves were ground to fine powder in a mixer and were extracted with 95% Methanol using a Soxhlet apparatus for 15 h. The filtrate was then concentrated in a rotary evaporator at 65°C. The concentrate was then freeze-dried to yield dried powder.

Cell culture

3T3-L1 cell line was procured from the National Centre for Cell Science, Pune. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (high glucose) with 10% FBS (Invitrogen, Canada), 10,000 U Penicillin G, 10,000 µg/mL streptomycin sulfate (Invitrogen), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Cultures were maintained at 37°C in 5% CO_2 in a humidified incubator.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay to determine the toxicity of a compound on the cells based on the conversion of MTT to formazan crystals by the lactate dehydrogenase present in live cells.[17,18] 3T3-L1 cells were seeded at an initial density of 20×10^4 cells per well/200 µL in 96 "well" plate and cultured overnight. The cells were then treated with desired concentrations of plant extract (25–400 µg/mL) for 24 h in same culture conditions. Post treatment, the medium was aspirated, 0.5 mg/mL of MTT reagent was added to cells and incubated at 37°C for 2 h. MTT reagent was then removed, and formazan crystals were dissolved with 20 µL of dimethyl sulfoxide (DMSO). Absorbance at 570 nm was measured by microplate reader. Percentage viability was determined using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD of test at 570 nm}}{\text{Mean OD of Untreated cells at 570 nm}} \times 100$$

Glucose uptake assay

3T3-L1 cells were cultured in 6-well plate at an initial density of $2*10^5$ cells/2 mL overnight. Cells were then washed with Dulbecco's phosphate-buffered saline (D-PBS) and treated with the extract (100 µg/mL) or the control - metformin (100 µM). The extract or control were prepared in glucose-free DMEM-containing 100 µM of 2-NBDG. After the treatment for 2 h, the cells were washed with D-PBS, trypsinized, and pellet was re-suspended in 0.5–1 mL of D-PBS. Cellular uptake of 2-NBDG was measured by flow cytometry

using BD FACS Calibur. The data were analyzed with Cell Quest Pro software (version 5.1, BD CellQuest™ Pro software, BD Biosciences, USA).

Glucose transporter type 4 expression studies

GLUT4 expression studies were conducted using flow cytometry. 3T3-L1 cells were cultured in 6-well plate at an initial density of 2×10^5 cells/2 mL. Cells were washed and treated with the extract (100 µg/mL) or control, metformin (100 µM) for 24 h. Post-treatment, the cells were trypsinized and incubated with Anti-Mouse Glut4-FITC antibody (#NBPI-49533F, Novus Biologicals) for 30 min in the dark and unbound antibodies were washed with D-PBS. GLUT4 expression was evaluated by flow cytometry in BD FACS Calibur. Data were analyzed with Cell Quest Pro software.

Inhibition of α-amylase activity

The α-amylase inhibitory activity was estimated using a previously described method.[19] Different concentrations of the extract or control, ranging from 31.25 µg/mL to 500 µg/mL, were prepared in DMSO. These solutions were then tested for the inhibitory effect on α-amylase activity. Equal volumes of the extract, control (100 µL) and α-amylase were incubated in microtubes for 10 min at 37°C. To this mixture, 100 µL of 1% soluble starch dissolved in buffer A was added and the mixtures were incubated for 30 min at 37°C. The reaction was arrested with 200 µL of dinitrosalicylic acid color reagent and microtubes were incubated at 100°C for 5 min. After cooling it to room temperature, 50 µL from each tube was transferred to the wells of 96-well microplate. This was further diluted with 200 µL of distilled water and the absorbance at 540 nm was measured by microplate reader (#EC800, Biotek). Acarbose was used as positive control. The half maximal inhibitory concentration (IC_{50}) values were also determined.

$$\% \text{ inhibition} = \frac{\text{Mean OD of control} - \text{mean OD of test}}{\text{Mean OD of control}} \times 100$$

Inhibition of α-glucosidase activity

The α-glucosidase enzyme inhibitory activity of the extract was assayed based on the procedure described by Shibano *et al.*[19] with a few modifications. Different concentrations of the extract ranging from 31.25 to 500 µg/mL were prepared. These were mixed with 600 µL of potassium phosphate buffer, and 25 µL of the α-glucosidase enzyme (1.2 EU/mL). The reaction mixture was incubated for 15 min at 37°C. Postincubation, 25 µL of PNPG was added to the tubes and was incubated at 37°C for 15 min. The reactions were stopped with 100 µL of 0.2M sodium carbonate. Absorbance at 410 nm was read in the microplate reader (EC800, Biotek). The results were compared with the positive control, Acarbose. Percentage inhibition of α-glucosidase was calculated using the below stated formula. IC_{50} values were also determined.

$$\% \text{ inhibition} = \frac{\text{Mean OD of control} - \text{mean OD of test}}{\text{Mean OD of control}} \times 100$$

Statistical analyses

All experiments were performed in triplicates and results were expressed as mean percentage inhibition ± standard deviation (SD) (n=3). IC_{50} values in enzyme inhibition assays were determined using linear regression graph (concentration versus percentage enzyme inhibition). Statistical significance was checked by single factor ANOVA and Student's t-test; P <0.05 was considered statistically significant. All statistical analyses and IC_{50} values determination were carried out in GraphPad Prism (version 3.1) software (San Diego, CA).

RESULTS

Cytotoxicity of *Dactylorhiza hatagirea* extract on 3T3 L1 cell line

3T3-L1 cells were treated with different concentrations (25 µg–400 µg/mL) of *D. hatagirea* extract and were assayed for their cytotoxic effect. The extract had no cytotoxic effect on the cells. The concentrations of the extract used and the respective percent cell viability were tabulated and plotted [Table 1 and Figures 2, 3]. The lowest concentration of *D. hatagirea* (25 µg/mL) showed 99.4% viability, and the highest concentration (400 µg/mL) showed 83.69% of viability after 24 h of exposure. These results indicated that methanolic extract of *D. hatagirea* leaves is not toxic to mammalian cells even at higher concentrations and could be used to analyze other parameters of *in vitro* antidiabetic studies. Metformin (100 µM) treatment – positive control – also had percent viability of 97.4% post 24-h exposure.

Inhibition of α-amylase activity

Percentage inhibition of α-amylase activity by *D. hatagirea* leaf extract was estimated with acarbose as the positive control. The extract showed 27.13±0.3% inhibition of α-amylase activity at 31.25 µg/mL and 74.53±0.5 % inhibition at 500 µg/mL concentration. The IC_{50} value of the extract was found to be 210.28 ± 5.4 µg/mL. The standard drug acarbose exhibited 46.18±0.1% inhibition of α-amylase activity at 31.25

Table 1: Cell viability of 3T3-L1 cells treated with different concentrations of *Dactylorhiza hatagirea* extract and 100 µM of control drug

Culture conditions	Percentage of viability
Control (untreated cells)	100
25 µg/mL	99.4
50 µg/mL	98.7
100 µg/mL	94.6
200 µg/mL	87.99
400 µg/mL	83.69
Metformin (100 µM) (standard drug)	97.4

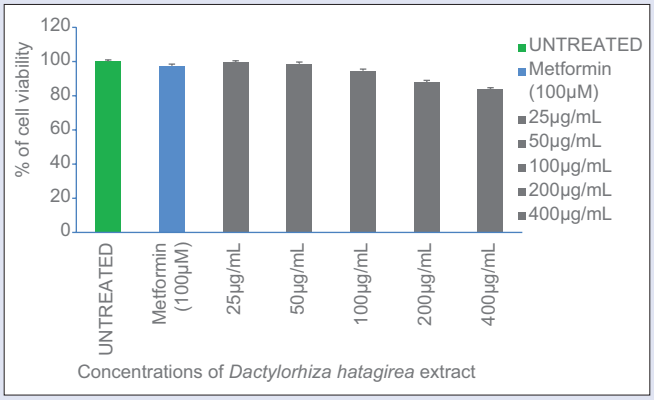

Figure 2: The effect of *Dactylorhiza hatagirea* leaf extract on 3T3-L1 cell line viability as determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay, after 24 h of incubation with various concentrations of the extract ranging from 25, 50, 100, 200, and 400 µg/mL. Each bar represents the data as the mean ± standard deviation of triplicate experiments and *indicates significant difference in comparison to positive control (*P < 0.0001)

μg/mL and 85.27±1.2% inhibition at 500 μg/mL concentration. The IC_{50} value for Acarbose was found to be 51.24 ± 4.8 μg/mL. The percentage inhibition of activity by the extract and drug at different concentrations are tabulated in Table 2 and represented in Figure 4.

α-glucosidase inhibition activity

Percentage inhibition of α-Glucosidase activity by *D. hatagirea* leaf extract was estimated with acarbose as the positive control. Methanolic extract of *D. hatagirea* leaves showed significant inhibition of α-Glucosidase activity. The lowest concentration of the extract showed 30.16±0.16% inhibition and the highest concentration showed 72.13±0.78% of inhibition in α-Glucosidase activity. Acarbose, the positive control drug, showed a percent inhibition, in α-Glucosidase activity, of 43.20±0.09% at 31.25 μg/mL and 94.41±0.49% at 500 μg/mL. The IC_{50} values of acarbose and *D. hatagirea* leaf extract were found to be 38.86 ± 4.1 μg/mL and 199.8 ± 4.7 μg/mL, respectively, as shown in Table 3 and Figure 5.

Glucose transporter type 4 expression study

GLUT4 expression was analyzed by flow cytometry using Anti-Mouse Glut4-FITC antibody (#NBPI-49533F, Novus Biologicals). Metformin was used as a positive control. Metformin-treated cells showed the highest expression of GLUT4 and the extract treated cells showed elevated levels of GLUT4 when compared with untreated cells. The relative expression levels of Glut4 and the mean fluorescence intensities are represented in Figures 6 and 7.

Glucose uptake assay

2-NBDG, a fluorescent deoxyglucose analog, was used to probe for the cellular uptake of glucose in 3T3-L1 cells. The results showed that the amount of cells that took up 2-NBDG were higher in the population of cells treated with *D. hatagirea* leaf extract when compared to the untreated cells.

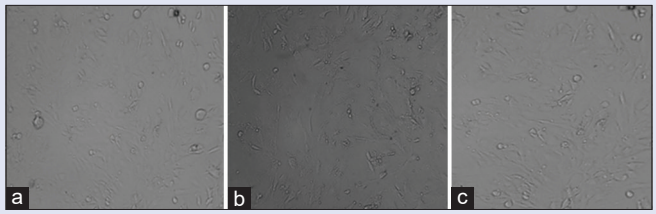

Figure 3: Images of 3T3-L1 cell line in inverted light microscopy after exposure to *Dactylorhiza hatagirea* leaf extract for 24 h. From "A" to "C" where (a) untreated control, (b) Metformin treated (100 μM), and (c) 100 μg/mL concentration of *Dactylorhiza hatagirea* leaf extract

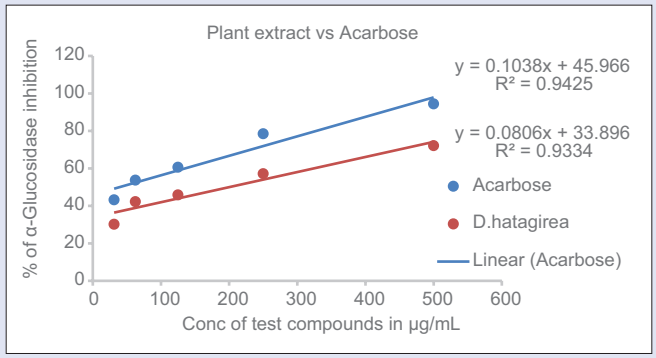

Figure 5: Percentage inhibition of α-glucosidase activity against increasing concentrations of *Dactylorhiza hatagirea* leaf extract (Orange) and the standard drug, Acarbose (Blue); Values are presented as mean ± standard deviation

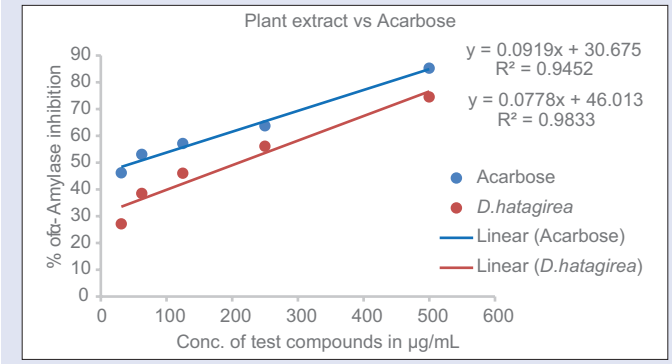

Figure 4: Percentage inhibition of α-Amylase against increasing concentrations of *Dactylorhiza hatagirea* leaf extract (Orange) and standard acarbose (Blue). Values are presented as mean ± standard deviation of triplicate experiments

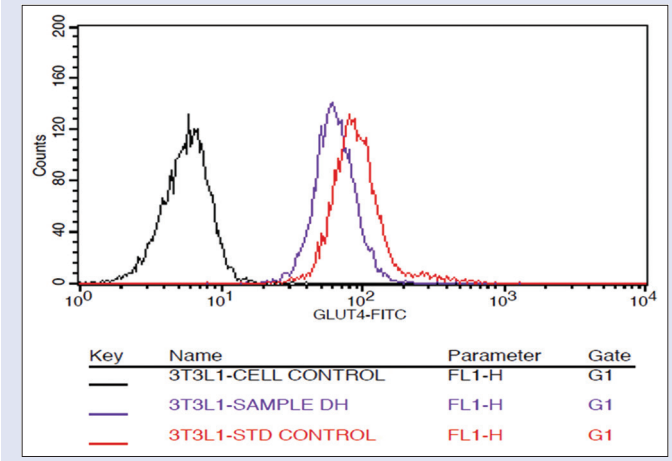

Figure 6: Overlaid expression of glucose transporter member 4 in untreated 3T3-L1 cells (Black colour line) and standard drug treated cells (Metformin 100 μM, Red color line) and 100 μg/mL of *Dactylorhiza hatagirea* leaf extract treated cells (Purple color line)

Table 2: *In vitro* antidiabetic activity of *Dactylorhiza hatagirea* leaf extract analyzed by α-amylase inhibition assay and comparison with standard drug acarbose

Concentration of test compounds (μg/mL)	Percentage of inhibition of α-amylase		IC_{50} values	
	Acarbose	*Dactylorhiza hatagirea* leaf extract	Acarbose (μg/mL)	*Dactylorhiza hatagirea* leaf extract (μg/mL)
31.25	46.18±0.1	27.13±0.3	51.24±4.8	210.28±5.4
62.50	53.06±0.8	38.52±0.6		
125.00	57.13±0.8	46.06±0.2		
250.00	63.74±1.8	56.10±0.1		
500.00	85.27±1.2	74.53±0.5		

Table 3: *In vitro* anti diabetic activity of *Dactylorhiza hatagirea* leaf extract, analyzed by α-glucosidase inhibition assay and comparison with standard drug acarbose

Concentration of test compounds (µg/mL)	Percentage of inhibition ofα-glucosidase		IC_{50} values	
	Acarbose	*Dactylorhiza hatagirea* leaf extract	Acarbose (µg/mL)	*Dactylorhiza hatagirea* leaf extract (µg/mL)
31.25	43.20±0.09	30.16±0.16	38.86±4.1	199.8±4.7
62.50	53.73±0.37	42.24±0.15		
125.00	60.59±0.57	45.83±0.44		
250.00	78.48±0.56	57.14±0.78		
500.00	94.41±0.49	72.13±0.78		

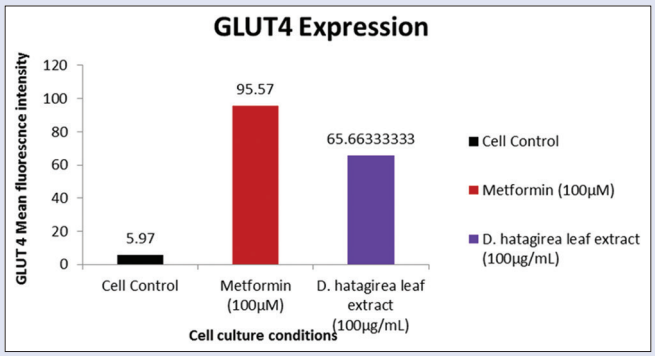

Figure 7: Expression of glucose transporter type 4 in Untreated 3T3-L1 Cells, Standard drug Metformin (100 µM) treated cells and 100 µg/mL of *Dactylorhiza hatagirea* leaf extract treated cells

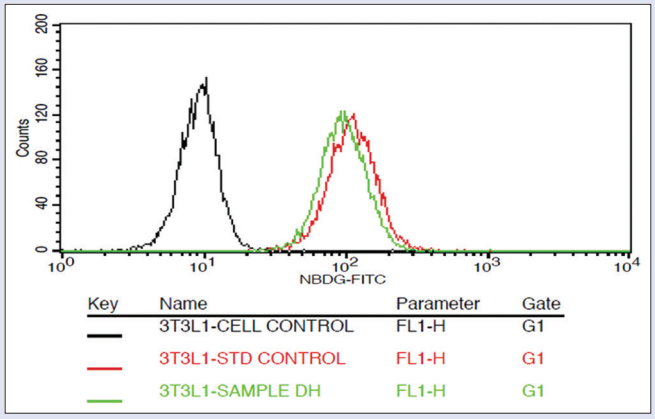

Figure 8: Overlaid fluorescence intensities of given untreated 3T3-L1 cells (Black colour line) and Standard drug treated cells (Metformin 100 µM, Red colour line) and 100 µg/mL of *Dactylorhiza hatagirea* leaf extract treated cells (Green Colour line)

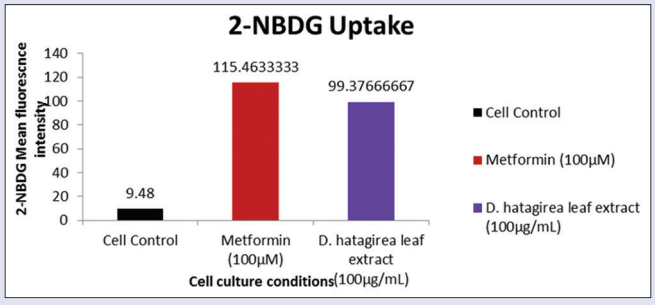

Figure 9: Relative mean fluorescence intensity values of intracellular 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose taken up by Untreated 3T3-L1 Cells, Standard drug Metformin (100 µM) and 100 µg/mL of *Dactylorhiza hatagirea* leaf extract treated cells

Metformin-treated cells showed the highest cellular uptake of 2-NBDG. The relative mean fluorescence intensity values are given in Figures 8 and 9.

DISCUSSION

One of the approaches in tackling DM has been the use of agents that inhibit the action of enzymes involved in the absorption and metabolism of carbohydrates. The major enzymes involved in the absorption of glucose from the gut include the pancreatic α-amylase and α-glucosidase enzymes. Inhibition of these enzymes regulate the postprandial blood sugar level in Type 2 DM patients by reducing the absorption of sugars from gut.[20,21] Existent antidiabetic drugs that specifically inhibit the activity of enzymes include acarbose, voglibose, and miglitol. However, usage of these drugs can have adverse effects such as flatulence and abdominal bloating.[22] Natural compounds from ethnomedicinal plants that do not have such effects are candidates of interest in the treatment of Type 2 diabetes.[23] Such medicinal plants play a vital role in herbal medicine, particularly in treating ailments like diabetes.[20,21,24,25]

In this study, we evaluated the antidiabetic properties of *D. hatagirea*. Our results indicate that the methanolic extract of *D. hatagirea* effectively inhibits α-amylase and α-glucosidase activities. These inhibitory effects were estimated with acarbose as the standard drug. Furthermore, *D. hatagirea* had no cytotoxic effect. The implications of this study corroborate previous studies on the inhibitory effect of other natural compounds on α-amylase and α-glucosidase activities.[26-28]

Cellular uptake of glucose from blood is critical in reducing DM. This is generally mediated by GLUT4 in the cell. GLUT4, also known as SLC2A4, is a 509 a multi-pass type membrane protein and shares 65% as identity with mouse GLUT4.[29] It is an insulin-regulated glucose transporter. GLUT4 is most highly expressed in adipose and striated muscle tissues but has also been reported in multiple other tissues including the nervous system and breast cancer. Insulin-stimulated transport of GLUT4 has been shown to be impaired in type 2 diabetes patients.[30] On stimulation with some antidiabetic drugs, GLUT4 is translocated from the sites in the cell to the cell surface. Insulin induces GLUT4 translocation through the phosphatidylinositol-3-kinase pathway.[31] Glucose transport by insulin-stimulated by the PKB/Akt pathway has been reported in rat adipocytes and L6 muscle cells.[32] Metformin is an antidiabetic drug that increases the uptake of glucose by inducing the translocation of GLUT4. This drug is known to act by AMP-activated protein kinase signaling pathway.[33]

In this study, metformin was used as the positive control in cellular glucose uptake assay and to evaluate the expression of GLUT4. Upon treatment with plant extract the Mean Fluorescence Intensity (MIF) values corresponding to GLUT4 expression and 2-NBDG uptake (65.66 MFI and 99.37MFI respectively) were increased compared to cell control as indicated in figures 7 and 9. These findings suggest that the leaf extract of *D. hatagirea* could enhance cellular glucose uptake by inducing GLUT4 translocation. However, further studies need to be carried out to understand the exact mechanism of action.

CONCLUSION

This novel study reports the antidiabetic properties of *D. hatagirea* as evidenced by biochemical assays and *in vitro* cellular assays. *D. hatagirea* extracts effectively inhibited the activity of the enzymes α-amylase and α-glucosidase, enhanced glucose uptake and GLUT4 translocation in 3T3-L1 cells. This suggests that *D. hatagirea* has potential antidiabetic properties and may serve as a source of lead molecules possessing potential uses in the treatment of DM. However, further studies to identify and characterize these bioactive constituents must be performed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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