**In-vitro Study of Trachyspermum ammi extract against Diabetes Mellitus**

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Abstract

Diabetes resulting from the low secretion of insulin and insulin resistance, is a chronic metabolic disorder. Medication from plant materials for treatment of Diabetes Mellitus is advised as good source for novel drug or leading to preparation of novel drug. We investigated the antidiabetic property of fractions and subfractions obtained from the ethyl acetate extract of *Trachyspermum ammi* seeds. TLC analysis of crude extract was done. Then column chromatography was used to isolate the fractions of *T. ammi*. Primary screening of fractions was done by Starch-agar Gel Diffusion assay for identification of Alpha-amylase inhibitors. Then after chromatographic separation of subfractions, their secondary screening was carried out by Glucose-Uptake assay in Yeast cells. Finally the in vitro Anti-diabetic activity of crude and screened subfraction of ethyl acetate extract of *T. ammi* was evaluated in 3T3-L1 cell lines. The results presented the antidiabetic activity of *T. ammi* and their fractions along with subfractions. Further in vitro cell line assay also proved the antidiabetic activity of *T. ammi*. It is suggested that the *T. ammi* had antidiabetic activity by inhibiting the α-amylase enzyme, also by facilitating the glucose utilization in adipose tissues and skeletal muscles.

**Keywords:** Antidiabetic screening, Starch plate assay, Yeast assay, In vitro antidiabetic assays, Glucose uptake assay

Introduction

Diabetes Mellitus (DM) is an incurable illness triggered due to defective insulin secretion from pancreas and insulin resistance along with alterations of lipid and protein metabolism which leads to hyperglycemia [1]. Insulin deficiency causes increase in level of glucose in blood leading to damages in body systems, particularly, the blood vessels and nerves. Around 2 - 3 % of the population worldwide has been affected by Diabetes and becomes third ‘Killer’ of mankind health along with cancer, cardiovascular and cerebrovascular diseases [2]. Prevalence rate of Diabetes is expected to reach up to 4.4 % in 2030 [3]; and highly occurs in India, China and U.S.A [4].

Therapeutic method for treatment of Diabetes is to bring down the post-prandial glucose levels. The α-amylase (α-1, 4-glucan-4-glucanohydrolases; E.C. 3.2.1.1) majorly secreted by pancreas [5] and salivary glands catalyzes the initial breakdown of starch into smaller oligosaccharides through the cleavage of α-D-(1 - 4) glycosidic bonds [6-9]. Delaying glucose absorption through inhibition of carbohydrates-hydrolyzing enzymes, α-glucosidase and α-amylase, present in small intestinal brush border that are responsible for breakdown of oligosaccharides and disaccharides into monosaccharides suitable for absorption helps in reduction of glucose absorption rates and lowers the post-prandial glucose levels since carbohydrates not broken down into glucose molecules [10,11]. Few currently used synthetic suppressors of these enzymes acarbose and miglitol inhibits glycosidases (α-glucosidase and α-amylase) while voglibose inhibits α-glucosidase. In spite of their restrictions are non-specific, produce serious side effects and fail to elevate diabetic complications. Side effects of these inhibitors are gastrointestinal viz., bloating, abdominal discomfort, diarrhea and flatulence [12].

Therapies presently used for Diabetes include Insulin and numerous oral antidiabetic agents such as Sulfonylureas, Biguanides, Thiazolidinedione’s, Glucosidase inhibitors and Glinides. They cause a number of serious adverse effects such as hepatotoxicity, abdominal pain, flatulence, diarrhea and hypoglycemic etc. Resistance of drugs to these medications is often conveyed after persistent usage [13].
Thence, investigations to obtain hypoglycemic drugs from therapeutic plants have expanded acceptance in present. 45,000 plants have been recorded in India out of which 7,500 species are of medicinally important [14]. For this reason, search for highly effective and safer hypoglycemic agents is one of the crucial areas of investigation [15]. Traditional Medicine preparations from plant sources are possibly are a good source of peculiar antidiabetic compounds or phytomedicines/supplements [16].

*Trachyspermum ammi* (Ajwain seeds) comes under the family Apiaceae. *T. ammi* is identified in different regions of the world by different names such as Ajwain, Ajma, Omam, Yaviniki, Bishop’s weed, etc., Different herbalists also use the scientific name *Carum copticum* for *Trachyspermum ammi* [17]. Ajwain fruits are bitter, pungent which works as anti-inflammatory, anti-tussive, analgesic, antibacterial, antifungal and antiviral also works as bronchodilatory, anticancer and antioxidant [18,19]. It is also used for relieving flatulence, dyspepsia, spasmodic disorders, flatulence, common cold, acute pharyngitis, sore and congested throat [20]. Nonetheless the antidiabetic activity of *T. ammi* seeds are not yet scientifically established. In the present study we aimed to scientifically establish the glucose lowering potential of *T. ammi* by various assays.

**Materials and methods**

**Sample collection and extraction of phytochemicals**

The fresh seeds of *Trachyspermum ammi* were collected from Chennai, Tamilnadu, India. The dried plant seeds were physically cleaned and powdered using a blender and stored in a clean glass ware air tight container. Soxhlet extraction assembly was used for extraction purpose. 50 g dried and powdered seeds were mixed separately with 250 mL of Ethyl Acetate and continuously extraction was done for 5 - 6 h [21]. Then the mixture was filtered using Whatman No.1 filter paper. The crude extract thus obtained was concentrated by using rotary vacuum evaporator under reduced pressure and stored at 4 °C.

**Thin layer chromatography of ethyl acetate extract of *T. ammi***

TLC Plates using glass plates were prepared and activated at 110 °C for 30 min for activation [22]. TLC tank glass chamber with airtight lid was developed in saturated glass chambers using the following different solvent systems as mobile phases: Toluene:Methanol:Chloroform (5:1:4), Chloroform:Methanol:Toluene:Acetone (2:1:2:0.5), Chloroform:Ethyl acetate:n-Hexane (70:15:15) and Ethyl acetate:Toluene:Methanol (1:8:1). The 5 µl (10 mg/mL) of extract in ethyl acetate was applied as a small spot on the TLC plates using capillary and solution was dried. Plates were run in the solvent mixtures till the solution reached the line mark [23]. The plates were then air-dried. The plates were placed in the UV light of 312 and 365 nm, many fluorescent spots were observed of varying colors and photographed. TLC plates were then placed in the iodine chamber for the development of brown spots on the TLC plates. $R_f$ value of the bands were calculated by using the formula: $R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$.

**Column chromatography of ethyl acetate extract of *T. ammi***

Wet packing method was adopted for packing the column. Silica gel (Kieselgel 60, mesh size 60 - 120) was used for column packing. Silica gel slurry was added into a glass column [24]. Gradient elution technique was used which involves the use of a continuously changing eluting medium. The crude ethyl acetate extract (10 g) was dissolved in running solvent n-hexane (10 mL) and loaded onto packed column, followed by graded mixtures of n-hexane: Ethyl acetate, ethyl acetate 100 %, graded mixtures of ethyl acetate: Toluene, toluene 100 %, graded mixtures of toluene: Chloroform, chloroform 100 %, graded mixtures of chloroform: Methanol and finally with 100 % methanol [25]. Eluted fractions were collected at the rate of about 5 mL. Totally 80 fractions were obtained. Each fraction was subjected to TLC on silica gel G. The fractions with same $R_f$ were pooled together and concentrated to obtain major fractions. Totally 9 number of major fractions were obtained.

**Primary screening of fractions by starch-agar gel diffusion assay**

The 9 fractions obtained were subjected to starch-agar gel diffusion assay to screen the fraction having highest α-amylase inhibition activity. Starch-agar gel diffusion assay was done according to Fossum and Whitaker [26] (1974) with some modifications. 0.5 cm thick 1 % agar containing 0.5 % starch gel was prepared and poured in sterile petri plates. Wells were bored and labeled. The reaction mixtures were prepared by adding 50 µl of α-amylase solution (0.1 mg/mL in phosphate buffer), 50 µl of fraction and 25 µl of 0.02 M phosphate buffer pH 6.9. Blank reaction mixture was prepared without adding fraction; instead 50 µl phosphate buffer was added. Mixtures was prepared at room temperature.
and incubated for 10 min to allow enzyme-inhibitor interaction. Standard acarbose solution (1 mg/mL in phosphate buffer) alone served as positive control. 100 μl of each reaction mixture was loaded in the respective wells and allowed to diffuse overnight. After 24 h, the plate was flooded with iodine solution. Clear circular zone of starch hydrolysis was seen in blank well against deep blue or purple background indicating activity of alpha-amyloses. Diameters of the zones were measured and used to calculate percentage of inhibition. The fraction with high α-amylase inhibition activity was further sub-fractioned using chromatography techniques and TLC analysis of the subfractions were done to pool them accordingly.

**Column separation of fraction in to sub-fractions**

1 gm of the fraction-8 was run in column chromatography for sub-fractionation. The solvents used for running the column were hexane (100 %); n-hexane and toluene (2:1, 1:1, 1:2); toluene (100 %); toluene and ethyl acetate (2:1, 1:1, 1:2); ethyl acetate (100 %); ethyl acetate and chloroform (2:1, 1:1, 1:2); chloroform (100 %); chloroform and methanol (2:1, 1:1, 1:1) and methanol (100 %). Then the column sub-fractions were analyzed by TLC. Based on the R<sub>f</sub> values 4 major Subfractions were obtained namely subfraction-I, subfraction-II, subfraction-III and subfraction-IV. The subfractions were subjected to glucose uptake activity in yeast cells to further screen the subfraction based on percentage increase in glucose uptake by yeast cells.

**Secondary screening of sub-fractions by glucose-uptake assay in yeast cells**

Preparation of Yeast cells was done by the method of Cirillo [27], (1962). Baker’s yeast dissolved in distilled water was repeatedly centrifuged at 3,000×g, for 5 min until clear supernatant fluids were obtained. 10 % (v/v) of the suspension was prepared in distilled water. Following concentrations of plant extracts (250, 500, 750, 1,000 μg/mL) were added to 1 mL of glucose solution (20 mm) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 μL of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged at 2,500×g, for 5 min and amount of glucose was estimated in the supernatant. Metronidazole was used as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

\[
\text{Increase in glucose uptake (\%)} = \left( \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \right) \times 100
\]

where, Abs control was the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample was the absorbance of the test sample. All determinations were carried out in triplicate manner and values are expressed as mean ± S.D.

**In Vitro anti-diabetic activity evaluation in 3T3-L1 cell lines**

**3T3-L1 cell culture maintenance**

Mouse preadipocytes cell lines 3T3-L1 was procured from cell repository, National Centre for Cell Sciences, Pune. The 3T3-L1 (mouse fibroblast) cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10 % Fetal Calf Serum (FCS), 4 mm Glutamine and 1 % antibiotic/antimycotic solution, in a 5 % CO<sub>2</sub> incubator at 37 °C. The experiments were performed at 90 % confluence.

**Cell differentiation and processing**

The 3T3-L1 cells were seeded at cell density of 6×10<sup>4</sup> cells/well at a final volume of 1,000:1 in a 24 well plate with DMEM containing 10 % FBS and incubated for 48 h until the cells become confluent, then the old media was replaced by 10 % FBS and incubated again for 48 h. Preadipocytes cultured in DMEM containing 10 % FCS, 4 mm Glutamine, 2 % NaHCO<sub>3</sub> and antimycotic, in an atmosphere of 5 % CO<sub>2</sub> at 37 °C, which were undifferentiated fibroblasts, were induced to differentiate to mature adipocytes using differentiation Medium (DMEM with 10 % FBS, 1 % antibiotic-antimycotic, 1 μM Dexamethasone, 0.5 mm 3-isobutyl-1-methylxanthine (IBMX) and 1 μg/mL Insulin) for 9 days.

**In Vitro MTT Assay**

MTT assay was carried out based on the method described by Mosmann [28] (1983). The MTT reagent was prepared by dissolving 5 mg of 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl tetrazolium
bromide (MTT) in 1mL of phosphate buffer saline (PBS). Preadipocyte were seeded at density, 10,000 cells per well in 96-well were allowed to attach for 24 h prior to the cell viability assay. To study the toxicity of extract on 3T3-L1 cell viability, the monolayer cell culture was trypsinized and the cell count was made 1.0×10^5 cells/mL in DMEM with 10 % FBS. In each well, 0.1 mL of the diluted cell suspension 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 crude and Sub-fraction of Ethyl acetate extract at different concentrations (10, 50, 100, 250 and 500 μg/mL) were added. 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. Plates were gently shaken and incubated for 3 h at 37 °C in 5 % CO₂ atmosphere. 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 at a wavelength of 570 nm using a microplate reader. The percentage growth inhibition was calculated using the following formula, 

Cell viability (%) was calculated by using the formula:

\[
\left(\frac{AC - AT}{AC}\right) \times 100
\]

where, \(AC\) - O.D of Control at 570 nm, \(AT\) - O.D of Test samples at 570 nm.

In Vitro glucose uptake activity

3T3-L1 cells monolayer was maintained at sub confluent conditions in growth media containing DMEM with 4.5 g/l glucose, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10 % fetal bovine serum. Cells were cultured on 6 well plates and incubated for 48 h at 37 °C in a CO₂ incubator. When semi confluent monolayer was formed, the culture was renewed with serum free DMEM containing 0.2 % BSA and incubated for 18 h at 37 °C in the CO₂ incubator. Insulin was used as a positive control and wells containing medium and cells served as the negative control. After 18 h, the media was discarded and cells were washed with PBS buffer once. The cells were treated with Insulin, Crude and Sub-Fraction of varying concentration (10, 50 and 100 μg/mL), then glucose (1M) was added and incubated for half an hour. The supernatant was collected for glucose estimation and glucose uptake was terminated by washing the cells thrice with 1 mL ice-cold DPBS buffer. Cells were subsequently lysed by freezing and thawing thrice. Cell lysate was collected for glucose estimation [29]. Glucose uptake was calculated as the difference between the initial and final glucose content in the incubated medium by GOD-POD method.

Glucose concentration in mg/dl = \(\frac{A_{\text{sample}}}{A_{\text{standard}}}\times 100\)

whereas, \(A_{\text{sample}}\) - Absorbance of test sample,

\(A_{\text{standard}}\) - Absorbance of standard drug.

Results

TLC analysis of ethyl acetate extract of *T. ammi*

The ethyl acetate extract yielded a dark greenish-brown substance which was subjected to TLC. Prepared TLC Plates were developed with solvent systems which separated components into a wide range of Rₚ values as shown in Table 1 and Figure 1. The bioactive compounds present in ethyl acetate crude extract of *T. ammi* were identified using TLC. The most suitable TLC solvent mixture was Toluene:Methanol:Chloroform (5:1:4) with the largest discriminating power under ultra violet light at 365 nm (Figure 1(a)). Different solvent systems and the Rₚ values obtained in each solvent systems is shown in Table 1. The components in TLC plates were visualized by placing under visible and U.V. light (312 and 365 nm). The best resolution of compounds was identified in UV Light of 365 nm as shown in Figures 1(a) - 1(d) shows the under UV light of 312 nm.
Table 1. Rf Values of Crude Ethyl Acetate Extract of T. ammi in Different Running Solvent Mixtures.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Solvent Mixtures</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Toluene:Methanol:Chloroform (5:1:4)</td>
<td>0.18, 0.22, 0.27, 0.62, 0.67, 0.98</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform:Methanol:Toluene:Acetone (2:1:2:0.5)</td>
<td>0.67, 0.80, 0.89, 0.98</td>
</tr>
<tr>
<td>3</td>
<td>Chloroform:Ethyl acetate:Hexane (70:15:15)</td>
<td>0.22, 0.33, 0.44, 0.89, 0.98</td>
</tr>
<tr>
<td>4</td>
<td>Ethyl Acetate:Toluene:Methanol (1:8:1)</td>
<td>0.16, 0.24, 0.29, 0.38, 0.42</td>
</tr>
</tbody>
</table>

Figures 1(a) - 1(d) Compound Separation by Thin Layer Chromatography in Different Running Solvent Mixtures. (a) TLC Plate in Toluene:Methanol:Chloroform (5:1:4) at UV-365 nm, (b) TLC Plate in Chloroform:Methanol:Toluene:Acetone (2:1:2:0.5) at UV-365 nm, (c) TLC Plate in Chloroform:Ethylacetate:Hexane (70:15:15) at UV-365 nm, and (d) TLC Plate in Ethyl Acetate:Toluene:Methanol (1:8:1) at UV-312 nm.

Column chromatography for separations of fractions

80 eluted column fractions were collected at regular volume of 5 mL each and run in TLC to separate the similar compounds with same Rf values. They were pooled together giving 9 number of major fractions.

Primary screening of fractions by starch-agar gel diffusion assay for α-amylase inhibition

The fractions were subjected to starch agar-gel diffusion assay to screen the fraction having high α-amylase inhibiting activity. All the fractions exhibited different percentage of α-amylase inhibitory activity as given in Table 2 and Figure 2. The activity observed in blank was highest which was considered as 100 % amylase activity. Acarbose inhibited 100 % amylase activity acting as standard.
Figure 2 Starch-Agar gel diffusion assay for α-Amylase inhibition by fractions of *T. ammi*, B: Blank (Alpha-amylase), S: Standard (Acarbose), 1: Fraction 1, 2: Fraction 2, 3: Fraction 3, 4: Fraction 4, 5: Fraction 5, 6: Fraction 6, 7: Fraction 7, 8: Fraction 8, 9: Fraction 9.

Table 2 Percent Inhibition of α-amylase Activity by the 9 Different Fractions of Ethyl Acetate Extract of *T. ammi*.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Fractions</th>
<th>Zone of inhibition in cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Blank (Alpha-amylase)</td>
<td>1.54 ± 0.04</td>
</tr>
<tr>
<td>S</td>
<td>Standard(Acarbose)</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Fraction 1</td>
<td>1 ± 0.14</td>
</tr>
<tr>
<td>2</td>
<td>Fraction 2</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>Fraction 3</td>
<td>0.53 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>Fraction 4</td>
<td>0.86 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>Fraction 5</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>Fraction 6</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>Fraction 7</td>
<td>1.25 ± 0.03</td>
</tr>
<tr>
<td>8</td>
<td>Fraction 8</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Fraction 9</td>
<td>0.48 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± S.D. of triplicates. NA: No inhibitory activity exhibited by fractions.

The fraction with high activity was found to be fraction-8 screened by starch-agar gel diffusion assay. This fraction was further subjected to column chromatography to separate it into subfractions. The TLC of fraction performed the solvent system was further used for obtaining subfractions. These subfractions were run in TLC and based on the same Rf values they were pooled together leading to main 4 subfractions namely subfraction-I, subfraction-II, subfraction-III and subfraction-IV. All the 4 subfractions were subjected to further determining of hypoglycemic activity by *in vitro* glucose uptake assay by yeast cells.

**Secondary screening of sub-fractions by glucose uptake assay by yeast cells**

Amount of transportation of glucose across the cell membrane was studied in an *in vitro* system comprising of yeast cells suspended in 20 mm glucose concentration. The rate of glucose transport across cell membrane in yeast cells was directly proportional to the increase in the concentration of subfractions of ethyl acetate extract of *T. ammi*. Subfraction-I showed maximum activity than other subfractions. The high uptake of the glucose was seen in 20 mm glucose concentration. The results were depicted in Figure 3. The amount of glucose remaining in the medium after a specific time interval serves as an indicator of glucose uptake by the yeast cells. The Subfraction-I exhibited significantly higher glucose uptake activity than the other subfractions. Therefore, the active subfraction-I along with crude ethyl acetate extract of *T. ammi* was further subjected to *in vitro* testing in 3T3-L1 Cell line.
Figure 3 Effect of Subfractions of Ethyl Acetate Extract of T. ammi on the uptake of Glucose (20 mm) by Yeast Cells.

*In vitro* toxicity testing by MTT assay
MTT assay measures the viability and proliferation of cells. This assay works with the principle of reduction of yellow color (MTT) to purple formazan by the enzyme reductase which is present in living cells. The cytotoxicity of crude extracts and subfraction-I on 3T3-L1 cell line at various concentrations was evaluated in the present study (Table 3). The control (untreated cells) showed 100 % cell viability. The cells treated with crude extracts and subfraction-I showed no toxicity between 10 - 500 µg/mL concentration (Figure 4).

Table 3 MTT assay for crude and fraction-8 of ethyl acetate extract of T. ammi treated in 3T3-L1 cell line.

<table>
<thead>
<tr>
<th>Concentration of samples in µg/mL</th>
<th>Percentage of viability (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude</td>
<td>Fractions</td>
</tr>
<tr>
<td>10</td>
<td>92.66 ± 2.52</td>
<td>93.33 ± 6.03</td>
</tr>
<tr>
<td>50</td>
<td>80 ± 2.64</td>
<td>91.33 ± 1.53</td>
</tr>
<tr>
<td>100</td>
<td>68.33 ± 3.05</td>
<td>78.33 ± 7.64</td>
</tr>
<tr>
<td>250</td>
<td>45.33 ± 3.51</td>
<td>62.33 ± 3.05</td>
</tr>
<tr>
<td>500</td>
<td>20.66 ± 1.15</td>
<td>50.67 ± 1.15</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.D; n = 3
Figure 4 Cytotoxic activity of crude and Sub-Fraction-I of ethyl acetate extract of *T. ammi* in 3T3-L1 cell line.

**Glucose uptake activity (insulin mimetic activity) in 3T3-L1 cell line**

In 3T3-L1 cell lines the glucose uptake assays, a significant level of insulin mimetic activities were observed in the case of crude extract and subfraction-I of *T. ammi*. All observed values of glucose uptake activity are compared using the control (untreated cells). These values are normalized with MTT cell viability assay values (10, 50, 100, 250 and 500 μg/mL) for the crude and subfraction-I. The insulin mimicking activity was found to be the best at 100 μg/mL concentration of subfraction-I (Figure 5).

Figure 5 Glucose uptake activity in 3T3-L1 cells. Cells were incubated with crude, sub-fraction and Insulin along with 1M glucose.

**Discussion**

Traditional medication from plant materials for treatment of Diabetes Mellitus is advised as good source for novel drug or leading to preparation of novel drug. Prescribed by the traditional healers, different plant extract preparations conceivably been accustomed by users for Diabetes Mellitus. Ethno botanical information reports almost 800 plants possess anti-diabetic potential [30,31]. Glucose Control in blood is important to help lower the diabetic complications. Acarbose and miglitol are presently used synthetic inhibitory drugs which have negative effects such as diarrhea, vomiting, abdominal pain, nausea, abnormal weight gain, low blood glucose or allergic reactions [32,33]. Plant natural products are useful in discovering lead candidates with potential antidiabetic activity which plays an important role in
prospective drug development [34]. Acarbose and miglitol, competitive inhibitors of α-amylase and α-glucosidase reduces the digestion of starch and polysaccharides, disaccharides, thereby resulting in lower absorption of glucose through the inhibition of carbohydrates hydrolyzing enzymes in the digestive tract delaying carbohydrate digestion time [35,36]. In the present study, acarbose was used as a standard which showed inhibitory activity on α-amylase during starch assay.

The inhibitory activities of the extracts may be as a result of phytochemicals like alkaloids, steroids and fatty acids which are majorly present in them. Previous studies on medicinal herbs recommend that a number of capable inhibitors belong to alkaloids and steroids class that has features to inhibit α-amylase and α-glucosidase [37]. Phytomolecules such as flavonoids, phenols, alkaloids, glycosides, saponins, glycolipids, dietary fibres, polysaccharides, peptidoglycans, carbohydrates, amino acids and others obtained from various plant sources have been reported with hypoglycemic and antihyperglycemic agents [38,39]. Plant based α-amylase and α-glucosidase inhibitors were proved to contain potential therapeutic strategy to control hyperglycemia [40,41]. In this study, fraction-8 of T. ammi exhibited high inhibitory activity suggesting it to be a good source of α-amylase inhibitors.

Acarbose-like drugs, which inhibit α-glucosidase present in the brush border of epithelial lining, were demonstrated in decreasing post-prandial hyperglycemia [42] and improved impaired glucose metabolism in NIDDM patients [43]. These drugs are useful for patients taking sulfonylurea medication or metformin, to keep their blood glucose level within a safe range. Hence, delayed absorption of carbohydrates with a plant based α-amylase and α-glucosidase inhibitors offers a prospective therapeutic approach for the management of type 2 Diabetes Mellitus and borderline patients [44]. With this regard the ethyl acetate extract of Trachyspermum ammi seems to be a promising alternative for the treatment of Diabetes Mellitus by reducing postprandial hyperglycemia.

TLC technique was used for the separation of compounds present in the crude EA extract and to select the solvent system capable of showing better resolution. TLC fingerprints of plant extracts shows complex mixtures of non-polar to polar compounds. TLC helps in monitoring composition of the extracts to ensure that no components are lost during processing. Toluene:Methanol:Chloroform solvent system was a suitable solvent mixture for fractionation of ethyl acetate extract of T. ammi, since this solvent system was giving good resolution and showing 5 - 6 spots on exposure to iodine vapor by forming the intense brown color at the spotted area. So the same solvent systems have been selected for the further column chromatographic study [45].

With the help of column chromatography, ethyl acetate crude extract of T. ammi yielded 9 major fractions which were screened by α-amylase inhibition assay by starch-agar gel diffusion method. The highest activity was observed in blank containing only α-amylase solution which was considered as 100 % amylase activity having 1.54 ± 0.04 as zone of measurement. Acarbose was used as a positive control which inhibits α-amylase enzyme and does not produce zone of measurement, considered as 100 % inhibitory activity on anylase enzymes. The percentage of inhibition shown by fraction 8 was highest (100 %) since no zone of measurement was obtained. Fraction 8 inhibited α-amylase enzymes which was comparable to standard acarbose. The present study reports were in agreement with Bale and Gawade, [46] (2014) in which aqueous stem bark extracts of 34 plants species were screened for their α-amylase inhibitory activity by using Starch-agar gel diffusion assay out of which bark extracts of 5 plant species showed 100 % inhibition equivalent to acarbose. Similarly, Chandrashekar et al. [47] (2012) investigated antidiabetic activity of aqueous extracts of stem bark of Bauhinia purpurea, which showed significant α-amylase inhibitory activity as compared to acarbose standard drug.

For a second time the fraction 8 was run in the column chromatography yielding 4 subfractions. These subfractions were subjected to glucose uptake assay by yeast cells. It was studied that the glucose uptake rate increased with increasing concentration of the extract and decreased with increasing extracellular glucose. The subfraction-I exhibited up to 96 % increase in glucose uptake by yeast cells. Transport of glucose across yeast cell membrane occurs by facilitated diffusion down the concentration gradient. Hence glucose transport occurs only if the intracellular glucose is effectively utilized [48]. The reports obtained clearly suggests that the subfraction-I is capable of effectively enhancing glucose uptake which in turn suggests that it is capable of enhancing effective glucose utilization thereby controlling blood glucose level.

Scharff, [49] (1961) explained the mechanism of glucose transport across the yeast cell membrane has being receiving attention as in vitro screening method for hypoglycemic effect of various compounds/medicinal plants. It has been reported that glucose transport in yeast cells (Saccharomyces cerevisiae) is extremely complex and it is generally agreed that glucose is transported in yeast cells was by facilitated diffusion process. Facilitated carriers are specific carriers that transport solutes down the concentration gradient. This means that effective transport is only attained if there was removal of
intracellular glucose [50]. Heera et al. (2014) testified the in vitro antidiabetic activity of methanolic and ethyl acetate extracts of Alpinia galanga. The plant extracts were studied for their effect on increased glucose uptake percentage rate into yeast cells which was found to be linear in different glucose concentrations used. Maximum increase was seen in 20 mm glucose concentration exhibited by methanolic extract of Alpinia galanga [51]. Hence, 20 mm glucose concentration was used here for the glucose uptake assay by yeast cells.

From the present investigation, Crude and subfraction-I of ethyl acetate extract of T. ammi was screened using MTT for its cytotoxicity against 3T3-L1 cell line at different concentrations which was found to be dose dependent. Skeletal muscle is a major tissue involved in insulin induced stimulation of glucose uptake. Functional transport of glucose molecules increases insulin uptake in skeletal muscles in the plasma membrane. Contractile activity also stimulates transport of glucose in skeletal muscles. Defects in insulin mediated glucose uptake in skeletal muscle are common pathological condition of NIDDM. Medicinal plants enhances glucose uptake by glucose transport molecules (GLUT4) translocation and were proven by in vitro glucose model. 3T3-L1 cell lines are finest studied cellular model for glucose uptake study [52]. Hence, in this study 3T3-L1 cell lines are used to determine the glucose uptake activity of ethyl acetate extract of T. ammi. Insulin and Metformin were standard antidiabetic drugs.

Several plant bioactive compounds (alkaloids, polyphenols) have been reported for antidiabetic activity in 3T3-L1 cell lines. Berberine alkaloid from Coriatis Rhizoma increased glucose-stimulated insulin secretion in Min6 cells via an enhanced insulin/insulin-like growth factor-1 signaling cascade [53]. Akuammicine, an indole alkaloid, isolated from chloroform extract of seeds of Picralima nitida (Apocynaceae) stimulated glucose uptake activity in differentiated 3T3-L1 adipocytes cell line [54]. Kumar et al. (2013) treated the 3T3-L1 cells with mangiferin and mahanimbine which increased the glucose utilization in a dose-dependent manner [55]. Based on these reports, it can be concluded that the alkaloid content of T. ammi may be responsible for the antidiabetic activity in 3T3-L1 cell lines.

The results in 3T3-L1 cell line assay on glucose uptake demonstrated that crude and subfraction-I of ethyl acetate extract of T. ammi could increase the glucose consumption. Subfraction-I of ethyl acetate extract of T. ammi showed better glucose uptake effect in 3T3-L1 cell line than the crude extract. Insulin and metformin had highest glucose uptake effect in 3T3-L1 cell lines. It is suggested that the T. ammi had antidiabetic activity by facilitating the glucose utilization in adipose tissues and skeletal muscles.

Conclusions

The present work of α-amylase inhibition activity of fraction-8 from ethyl acetate extract of T. ammi confirmed its antidiabetic nature. Glucose uptake assay in yeast model showed the hypoglycemic nature of subfractions of ethyl acetate extract of T. ammi. Further the in vitro antidiabetic activity by cell line assay revealed their hypoglycemic nature of crude and subfraction-I of ethyl acetate extract of T. ammi. Hence the hypoglycemic activity of ethyl acetate extract of fruit of T. ammi has been proven to be significant in few in vitro experimental models. Since in vitro activity does not always correlate to the corresponding in vivo activity, proof of concept have to be demonstrated in animal studies to extend its scientific use.

References


