Original Article

Antidiabetic Activity of Ajwain Oil in Different In Vitro Models

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Abstract

Context: Ajwain oil is an essential oil with thymol as its major constituent which is known for many pharmacological activities. Aims: To evaluate the anti-hyperglycaemic potential of ajwain oil using different in vitro models. Methods and Material: In vitro α-amylase and α-glucosidase inhibitory effect of ajwain oil was carried out by the method of Bernfeld and Shibano et al. 1997 with minor modifications respectively. Cytotoxicity of the ajwain oil was assessed using MTT assay. Glucose uptake potential was assessed in differentiated L6 myotubes using fluorescent tagged 6-NBDG. Results: Ajwain oil showed very good α – amylase inhibitory activity. A maximum inhibition of 88.55 ±0.43 % was achieved at a concentration of 4µL/ml by ajwain oil which was comparable to that of standard acarbose, 90.96 ± 1.81%. The IC₅₀ of the extract was found to be 0.47µL/ml and for acarbose 0.69µL/ml. The maximum in vitro α – glucosidase inhibitory activity was found to be 89 ± 0.72 % and 91.67 ± 1.09% at 4µL/ml for ajwain oil and acarbose. The IC₅₀ of the extract and acarbose were found to be 0.37µL/ml and 0.41µL/ml respectively. Ajwain oil has enhanced glucose uptake in L6 myotubes in a dose dependent manner. Conclusion: The anti-hyperglycaemic activity of the ajwain oil strongly support its ability to decrease sugar level hence it may be further validated for its use as an antidiabetic agent.

Keywords: Ajwain oil, α-amylase, α-glucosidase, glucose uptake, inhibitory effect

Introduction

Diabetes mellitus is a noncommunicable disease, which is steadily increasing worldwide, and is a major cause of morbidity and mortality.[1] It is characterized by hyperglycemia, which may be due to absolute (or) relative deficiency of insulin, which not only leads to changes in the metabolism of carbohydrate, protein, and fat but also the water and electrolyte homeostasis.[2] There are many risk factors including lifestyle. However, early diagnosis can slow down or prevent the debilitating long-term complication of this chronic metabolic disorder. The long-term micro- and macrovascular complications of type 2 diabetes can be reduced by adequate glycemic control, initially with monotherapy followed by combination therapy in addition to diet and exercise. The most frequently prescribed oral hypoglycemic agent is metformin as per American diabetic association guidelines.[3] Combination therapy helps to maximize the glucose control and adherence.[4,5] Triple drug combination is also found to be a safe and excellent option for the management of type 2 diabetes.[6] However, the multidrug regimen increases the cost of patient care. Control of postprandial hyperglycemia in a natural way may be yet another option to have prolonged survival. Hence, phytotherapy has got much attention recently.

Many plant medications are explored for antidiabetic activity using different in vitro as well as in vivo models.[7,8] In this study, ajwain oil, an essential oil, was used to explore its antihyperglycemic effect. Essential oils contain monoterpenes as the most abundant components with small molecular weight coupled with high nonpolar nature. They are reported to have

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antioxidant, antimicrobial, and antidiabetic effects.[9] Essential oil of ajwain is composed of thymol, carvacrol, limonene, dillapiole, p-cymene, γ-terpinene, β-pinene, terpinene-4-ol, oleic acid, linoleic acid, palmitic acid, and xylene.[10] In this study, the effect of ajwain oil on α-amylase and α-glucosidase, its cytotoxicity, and glucose uptake potential was explored.

**Materials and Methods**

**Chemicals and extract**

The chemical used for the experiment such as α-Amylase enzyme, phosphate buffer, dinitrosaliclyc acid, 4-nitrophenyl α-d-glucopyranoside, α-glucosidase solution, sodium carbonate, p-nitrophenol methanol and hexane were of analytical grade. Ajwain oil was obtained as gratis from Synthite Industries, Kerala, India.

**Preparation of sample**

A total of 4 g of ajwain oil was extracted with 8 mL of hexane. Later, 8 mL of methanol/water (60:40, v/v) was added and then the mixture was vortexed vigorously for 2 min.

**In vitro α-amylase inhibitory assay**

The in vitro α-amylase inhibitory activity was carried out according to the method of Bernfeld.[11] In brief, 0.02–4 µL of the test extract from the solvent-extracted oil was allowed to react with 200 µL of α-amylase enzyme (HiMedia RM 638, Mumbai), and 100 µL of 2 mM of phosphate buffer (pH, 6.9). After 20-min incubation, 100 µL of 1% starch solution was added. The same was performed for the controls, where 200 µL of the enzyme was replaced by buffer. After incubation for 5 min, 500 µL of dinitrosaliclyc acid reagent was added to both control and test. They were kept in boiling water bath for 5 min. The absorbance was recorded at 540 nm using spectrophotometer, and the percentage inhibition of α-amylase enzyme was calculated using the following formula:

\[ \% \text{ Inhibition} = \left( \frac{[\text{Control} - \text{Test}]}{\text{Control}} \right) \times 100 \]

Suitable reagent blank and inhibitor controls were simultaneously carried out. All values were expressed as mean ± standard error of mean (SEM) (n = 3).

**α-Glucosidase inhibitory activity**

The enzyme inhibition activity for α-glucosidase was evaluated according to the method previously reported by Shibano et al. (1997) with minor modifications.[12] The reaction mixture consisted of 50 µL of 0.1-M phosphate buffer with pH of 7.0, 25 µL of 0.5 mM 4-nitrophenyl α-d-glucopyranoside (dissolved in 0.1-M phosphate buffer with pH of 7.0), 0.02–4 µL of the oil and 25 µL of α-glucosidase solution (a stock solution of 1 mg/mL in 0.01-M phosphate buffer with pH of 7.0 was diluted to 0.1 unit/mL with the same buffer with pH of 7.0 just before assay). This reaction mixture was then incubated at 37°C for 30 min. Then, the reaction was terminated by the addition of 100 µL of 0.2-M sodium carbonate solution. The enzymatic hydrolysis of substrate was monitored by the amount of p-nitrophenol released in the reaction mixture at 410 nm using microplate reader. Individual blanks were prepared for correcting the background absorbance, where the enzymes were replaced with buffer. Controls were conducted in an identical manner replacing the test extract with methanol. Acarbose was used as positive control. All values were expressed as mean ± SEM (n = 3).

**Cell culture studies**

**Preparation of cell culture**

L6, a monolayer myoblast culture (obtained from the National Center for Cell Science, Pune, Passage no. 27), was cultured in dulbecco’s modification of eagle medium (DMEM) with 10% fetal bovine serum (FBS) and supplemented with penicillin (120 units/mL), streptomycin (75 µg/mL), gentamycin (160 µg/mL), and amphotericin B (3 µg/mL) in a 5% CO₂ environment. For differentiation, the L6 cells were transferred to DMEM with 2% FBS for 4 days, after confluence. The extent of differentiation was established by observing the multinucleate cells.

**Cytotoxicity study—(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay**

Cytotoxicity of the ajwain oil was assessed by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[13] Cells were plated in 48-well plate at a concentration of 5 × 10⁴ cells/well. After 24 h of incubation, it was washed with 200 µl of 1× phosphate-buffered saline (PBS; pH, 7.4) and starved by incubation in serum-free medium for an hour at 37°C in CO₂ incubator. After starvation, cells were treated with different concentrations (0.03–4 µL/mL) of the oil for 24 h. At the end of the treatment, media from control and oil-treated cells were discarded and 50 µL of MTT-containing PBS (5 mg/mL) was added to each well. Cells were then incubated for 4 h at 37°C in CO₂ incubator. The purple formazan crystals formed were then dissolved by adding 150 µL of dimethyl sulfoxide and mixed effectively by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured using Multimode reader (Perkin Elmer, USA) at 570 nm. Optical density of each sample was compared with control optical density and graphs were plotted.
Antidiabetic activity of ajwain oil was assessed in differentiated L6 myotubes using fluorescent-tagged 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yi) amino)-2-deoxyglucose (6-NBDG). L6 myotubes (10,000 cells/well) were seeded in 96-well plates and allowed to confluence around 80%. Then, the cells were differentiated using 2% FBS and different concentrations of the oil (0.25–4 µL/mL) was added. At the end of treatment, 10 µM of insulin was added to stimulate glucose uptake and incubated for 15 min. A total of 20 µg/200 mL of 6-NBDG was added and incubated for 10 min at dark. Glucose uptake (in percentage) was measured using Multimode reader (PerkinElmer) with an excitation/emission filter of 466/540 nm.[14]

RESULTS
The results showed good α-amylase inhibitory activity of the ajwain oil compared with that of standard acarbose [Figure 1]. A maximum inhibition of 88.55% ± 0.43% was achieved at a concentration of 4 µL/mL by the oil, which was comparable to that of standard acarbose inhibition of approximately 90.96% ± 1.81%. The 50% Inhibitory concentration (IC₅₀) of the extract and acarbose was found to be 0.47 and 0.69 µL/mL, respectively [Figure 1].

The in vitro α-glucosidase inhibitory activity of the oil was investigated. The maximum inhibition of the extract was found to be 89% ± 0.72% at 4 µL/mL compared to that of standard acarbose, which showed its maximum inhibition of 91.67% ± 1.09% at 4 µL/mL. The IC₅₀ of the extract and acarbose was found to be 0.37 and 0.41 µL/mL, respectively [Table 1].

The cytotoxicity assay was carried out for the oil at different concentrations of 0.03–4 µL/mL at an interval of 24 h. From the results, it was observed that both the oil and standard metformin exhibited a dose-dependent decrease in the % cell proliferation, which was ≤50% even at a maximum dose of 4 µL/mL. The IC₅₀ of oil and metformin was found to be 0.56 and 1.87 µL/mL, respectively [Figure 2].

The glucose uptake potential of the oil was evaluated at different concentrations of 0.25–4 µL/mL. Insulin (10 µM) and standard metformin (10 µg/mL) were used as positive controls. It was shown from the results that the ajwain oil enhanced glucose uptake in L6 myotubes in a dose-dependent manner, which was compared with metformin. The maximum percentage of uptake was found to be 62.28% ± 0.40% for ajwain oil, whereas metformin at 10 µg/mL showed 76.99% ± 0.22% of glucose uptake and insulin showed 84.48% ± 0.05% [Figure 3]. The photo document showing the glucose uptake potential of the ajwain oil at different concentrations are presented in [Figure 4].

**Table 1: α-Glucosidase inhibitory activity of ajwain oil**

| Concentration (µL/mL) | % Inhibition of α-glucosidase |
|-----------------------|-------------------------------|
|                       | Ajwain oil                    | Acarbose                     |
| 0.02                  | 6.88 ± 1.14                   | 6.16 ± 0.36                  |
| 0.03                  | 16.30 ± 1.09                  | 15.58 ± 1.09                 |
| 0.06                  | 23.91 ± 0.35                  | 22.46 ± 1.45                 |
| 0.25                  | 38.04 ± 3.26                  | 37.32 ± 1.81                 |
| 0.50                  | 53.62 ± 1.16                  | 53.99 ± 1.81                 |
| 1.0                   | 63.04 ± 1.45                  | 63.04 ± 0.72                 |
| 2.0                   | 78.99 ± 1.27                  | 71.01 ± 0.71                 |
| 4.0                   | 89.86 ± 0.72                  | 91.67 ± 1.09                 |

**Figure 2:** Graphical representation of cytotoxicity (MTT) assay of ajwain oil using L6 myotubes
**DISCUSSION**

In different health-care systems, the direct costs of patients with diabetes have shown a threefold rise. Moreover, the chance for acute and chronic complications to uncontrolled disease is more with patients with diabetes.[15] Plant therapy has got enough popularity recently as it has less side effects being natural and it acts by different mechanisms. In this study, ajwain oil showed a dose-dependent inhibitory effect on \( \alpha \)-amylase and \( \alpha \)-glucosidase activity compared to standard acarbose without producing cytotoxic effect. \( \alpha \)-Amylase and \( \alpha \)-glucosidase are the key enzymes of carbohydrate metabolism. \( \alpha \)-Amylase hydrolyses the 1, 4-glycosidic linkages of polysaccharides to disaccharides. \( \alpha \)-Glucosidase in turn converts the disaccharides to monosaccharide, resulting in postprandial hyperglycemia. Hence, inhibitors of these enzymes are a good option for the control of postprandial hyperglycemia.[16]

Plants such as *Garcinia mangostana*, *Nephelium lappaceum* L., *Barringtonia racemosa*, and *Phyllanthus acidus* were shown to have \( \alpha \)-glucosidase inhibitory activity.[17] The plant *Cymbopogon martinii* (Roxb.) is reported to have \( \alpha \)-glucosidase inhibitory effect and thereby reducing the postprandial hyperglycemia in streptozotocin-induced diabetes in rats.[18] Studies show that the flavonoids, alkaloids, terpenoids, anthocyanins, glycosides, curcuminoids, and phenolic compounds present in the plant are responsible for the \( \alpha \)-glucosidase inhibitory activity.[19] Study conducted by Sales et al.[20] also proved amylase inhibitory activity of phenolic compounds and flavonoids. The rationale behind the activity of ajwain oil may be the presence of various monoterpenes as they are known for their antidiabetic activity.

Antioxidants have great therapeutic potential in diseases associated with oxidative stress, such as cancer, diabetes mellitus, and neurodegenerative disorders, as these agents can prevent cellular damage by neutralizing free radicals.[7] Therefore, the antioxidant property of the ajwain oil also can contribute for the antidiabetic activity.

Non-metabolizable fluorescent glucose analog, 6-NBDG, was used for the glucose uptake study. It is increasingly used to study cellular transport of glucose. Intracellular accumulation of exogenously applied 6-NBDG is assumed to reflect concurrent gradient-driven glucose uptake by glucose transporters (GLUTs). Ajwain oil showed a comparable glucose uptake potential in L6 myotubes.

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**Figure 3:** Graphical representation of 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose glucose uptake potential of ajwain oil in L6 myotubes

**Figure 4:** Photo document showing the glucose uptake potential of the ajwain oil at different concentrations
uptake with that of metformin showing its usefulness in diabetic condition. The usefulness of rhinacanthins-rich extract from *Rhinacanthus nasutus* leaf and its marker compounds in diabetes was reported because of its potent glucose uptake stimulatory activity in 3T3-L1 adipocytes and L6 myotubes.\[^{[23]}\] *Tinospora cordifolia* leaves extract showed 62.0\% ± 1.79\% glucose uptake compared with the standard insulin (1 IU/mL), which showed 90\% ± 2.5\% glucose uptake. The presence of alkaloids, flavonoids, glycosides, proteins, saponins, tannins, terpenoids, and anthraquinones were responsible for its pharmacological properties.\[^{[22]}\] All these studies support the ability of plants to control postprandial hyperglycemia. Ajwain oil showed maximum glucose uptake 62.28\% ± 0.40\% at 4 µL/mL, whereas metformin at 10 µg/mL showed 76.99\% ± 0.22\% of glucose uptake and insulin (10 µM) showed 84.48 ± 0.05. All these data show the antihyperglycemic effect of ajwain oil is comparable to other studies to recommend its usage for diabetic care after proper in vivo studies. Plants such as *Dioscorea villosa* and *Caralluma fimbriata* also show antidiabetic activity by inhibiting the enzymes of carbohydrate metabolism.\[^{[8,23]}\]

**Conclusion**

Ajwain oil is a good choice for the management of diabetes as it can effectively inhibit the key enzymes of carbohydrate metabolism such as α-amylase and α-glucosidase and thereby decreasing the postprandial hyperglycemia. Moreover, it has proved its ability to promote glucose uptake in the L6 myotubes.

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**Conflicts of interest**

There are no conflicts of interest.

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