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Inhibitory potential of ginger extracts against enzymes linked to type 2 diabetes, inflammation and induced oxidative stress

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Abstract
Ginger (*Zingiber officinale* Roscoe) continues to be used as an important cooking spice and herbal medicine around the world. Gingerols, the major pungent components of ginger, are known to improve diabetes, including the effect of enhancement against insulin sensitivity. In the current study, ginger sequentially extracted with different solvents—namely, hexane, ethyl acetate, methanol, 70% methanol–water and water—were screened to determine the variations in phenolic-linked active constituents. The potential of these extracts to inhibit key enzymes relevant to type 2 diabetes and inflammation was studied. Phenolic compounds—namely, gingerols and shoagols—were quantified using high-performance liquid chromatography. Ethyl acetate extract showed higher activity compared with other extracts. These studies indicate that ginger has very good potential for α-glucosidase and α-amylase inhibition relevant for type 2 diabetes management and cyclooxygenase inhibition for inflammation.

Keywords: *α-Glucosidase, α-amylase, cyclooxygenase, reactive oxygen species, flow cytometer, C2C12*

Introduction

Ginger, the rhizome of *Zingiber officinale* Roscoe (Zingiberaceae), is a perennial herbaceous plant native to Southern Asia. It is the underground stem or rhizome of the plant and continues to be used as an important cooking spice and is valued for its pungency. It has been widely used in traditional system of medicines all over the world, for a wide array of unrelated ailments that include arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever and helminthiasis (Badreldin et al. 2008). Ginger represents a rich source of biologically active constituents. It is a strong antioxidant substance and may either mitigate or prevent generation of free radicals (Haksar et al. 2006, Kim et al. 2007). It is considered a safe herbal medicine with only few and insignificant adverse/side effects. Several reviews have appeared in the literature about this plant as a spice and a medicinal plant (Afzal et al. 2001, Chrubasik et al. 2005). The pungent constituents of ginger, gingerols, have been reported to possess strong anti-inflammatory activity, anti-fungal property, be a most potent cyclooxygenase (COX) inhibitor and are anti-platelet agents (Nurtjahja et al. 2003), and also lower blood pressure and reduce blood clotting (Thomson et al. 2002, Ghayur and Gilani 2005). Reactive oxygen species (ROS) are typically generated as the byproduct of cellular metabolic processes and are carefully controlled by cellular antioxidants or scavengers. Oxidative stress is considered the main cause for several chronic diseases, including diabetes (Wei et al. 2009). Oxidative stress occurs in the cell when the generation of ROS overwhelms the cells' natural antioxidant defense.

The present study involves the inhibitory effect of sequentially extracted Indian ginger on α-amylase, α-glucosidase and cyclooxygenase for anti-diabetic and anti-inflammatory properties and its reaction of ROS. High-performance liquid chromatography (HPLC) quantification of the active constituents present in the extracts was also carried out.

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Experimental

Plant material

Fresh ginger rhizomes (5 kg) procured from a local market at Thiruvananthapuram, Kerala, India were used. Voucher specimens have been kept in the Herbarium of the Institute. The rhizomes were chipped and dried at 50°C in a drier for 8 h to a 10% moisture level and were powdered for further studies.

Chemicals and reagents

α-Amylase from *Aspergillus oryzae*, α-glucosidase type 1 from baker's yeast, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), Hematin, Tris–HCl, dinitro-salicylic acid, *p*-nitrophenyl-α-D-glucopyranoside, arachidonic acid, 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA), indomethacin and *N*-vanillyl-nonanamide were purchased from Sigma-Aldrich (St Louis, MO, USA). The COX inhibitor assay screening kit was obtained from Cayman (Ann Arbor, MI, USA). Acarbose was obtained from Serva Electrophoresis GmbH (Mannheim, Germany). Sodium chloride, hydrogen peroxide, anhydrous sodium phosphate monobasic and anhydrous sodium phosphate dibasic were bought from Sisco Research Laboratories Pvt Ltd (Mumbai, India).

Cell culture and treatment

C2C12 cells purchased from the National Centre for Cell Science (Pune, India) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a 5% carbon dioxide incubator. When the cells were about to cover 80% of the flask area, they were disrupted and seeded on 24-well plates. After attaining ~70–80% confluency, the cells were rinsed twice with phosphate-buffered saline (PBS) and changed with medium containing extracts at different concentrations. After 24 h incubation, the cells were washed twice with PBS and 50 μM H_2O_2 was maintained in individual wells for 1 h at 37°C. These cells were detached by trypsin to assay by flow cytometry.

Sample preparation

Dried ginger powder (500 g having moisture content 10%) was successively extracted with 1 litre of each of the solvents at room temperature (27°C) with hexane (yield: 3.87%), ethyl acetate (yield: 1.93%), methanol (yield: 3.75%), 70% methanol–water (yield: 4.82%) and water (yield: 5.68%). The solvent was evaporated using a rotavapor under reduced pressure. Sample stock solution (5 mg/ml) was prepared for all the extracts in methanol and was used for diabetic studies. Different concentrations of the successive extracts (38, 95 and 190 μg/ml) were used for inflammatory studies.

In vitro anti-diabetic assays

α-Glucosidase inhibition assay. α-Glucosidase inhibition was assayed using different concentrations of sample stock solution (100–250 μg/ml), 100 μl of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/ml), and was incubated in 96-well plates at 25°C for 10 min. After pre-incubation, 50 μl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 min. Before and after incubation, absorbance readings were recorded at 405 nm by a Synergy 4 Biotek multiplate reader (Biotek Instruments Inc., Highland Park, PO Box 998, Winooski, Vermont-0504-0998, USA) and compared with a control that had an adequate amount of buffer solution in place of the extract (Apostolidis et al. 2007). Acarbose was used as the standard. The α-glucosidase inhibitory activity was expressed as the inhibition percentage and was calculated as follows:

$$\%\ \text{inhibition} = (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100 \quad (1)$$

where A_{control} is the absorbance of control without sample and A_{sample} is the absorbance of the sample. The concentration of the extract having 50% inhibition (IC_{50}) was calculated from the concentration-inhibition response curve.

α-Amylase inhibition assay. The α-amylase inhibition assay was carried out by the method of Apostolidis et al. (2007). Briefly, 500 μl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/ml) and different concentrations of the stock solution of extracts (500–1,250 μg/ml) were incubated at 25°C for 10 min. After pre-incubation, 500 μl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml dinitro-salicylic acid color reagent. The test tubes were then incubated in a boiling-water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm using a Synergy 4 Biotek multiplate reader. Acarbose was used as the positive control. The percentage inhibition was calculated using Equation (1). A graph was plotted with concentration along the *x* axis and percentage inhibition along the *y* axis to obtain the IC_{50} value.

Anti-inflammatory activity

In vitro evaluation of COX inhibitory activity. Enzymatic activity of COX was measured according to the method of Copeland et al. (1994) with slight modifications using

a chromogenic assay based on the oxidation of TMPD during the reduction of PGG_2 to PGH_2. Briefly, various concentrations of the sample solution contained (30–200 μg/ml) Tris–HCl buffer (100 mM, pH 8.0), Hematin (15 μM), ethylenediamine tetraacetic acid (3 μM), enzyme (100 μg COX). The mixture was pre-incubated at 25°C for 15 min and then the reaction was initiated by the addition of arachidonic acid and TMPD, in total volume of 1 ml. The enzymatic activity was determined by estimating the rate of TMPD oxidation for the first 25 sec of the reaction by following the increase in absorbance at 603 nm. Indomethacin was used as the standard. A low rate of non-enzymatic oxidation observed in the absence of COX was subtracted from the experimental value while calculating the percentage inhibition (see Equation (1)). The IC_{50} value was calculated from the concentration-inhibition response curve.

Evaluation of oxidative stress inhibition

Cytoprotective effect against the oxidative stress induced by H_2O_2 was measured by determining the intracellular content of ROS. Intracellular ROS levels were measured employing DCFH-DA. DCFH-DA is cleaved intracellularly by non-specific esterase and turn to high-fluorescent 2,7-dichlorofluorescein upon oxidation by ROS, which were analyzed with FACS Aria II (BD Bioscience, San Jose, CA, USA). C2C12 cells pretreated with ethyl acetate extract of ginger were incubated with DCFH-DA at 37°C for 1 h and then read in the FACS Aria II.

Quantification of gingerols and shoagols in ginger extracts

Sample preparation. A sample of 5 mg/ml stock solution of ginger extracts was prepared in methanol. *N*-Vanillylnonanamide dissolved in methanol was used as the standard at a concentration of 0.2 mg/ml.

Chromatographic conditions. The analytical HPLC was performed on a Waters liquid chromatograph equipped with a Rheodyne injector and a Waters 2487 (M/s. Waters Ges.m.b.H, Hietzinger Haupstasse 145, A 1130, Vienna, Austria, Europe) UV detector (wavelength 280 nm) using a C-18 column (150 × 4.6 mm, 5.0 μm). The mobile phase consisted of solvent A 1% acetic acid in water and solvent B acetonitrile (40:60). The flow rate was 1.5 ml/min.

Statistical analysis

The experimental results are expressed as the mean ± standard deviation of three parallel measurements. The results were subjected to one way analysis of variance and the significance of differences between sample means was calculated. $P \leq 0.05$ was considered significant.

Results and discussion

In the present study, potential anti-diabetic effect of ginger extracts obtained by sequential extraction of dried ginger powder with different solvents was investigated. The ability of ginger extracts to inhibit α-glucosidase and α-amylase was measured using four different dosages. α-Glucosidase inhibitory activity was measured using concentrations of 100, 150, 200 and 250 μg/ml. The ethyl acetate extract of ginger showed the highest activity (Figure 1) among the extracts and expressed in terms of IC_{50} value. A lower IC_{50} value indicates higher inhibition. The IC_{50} value of standard acarbose was 36 μg/ml and that of ethyl acetate extract was 180.13 μg/ml. The inhibitory potential of α-glucosidase on ginger extracts was related to its phenolic content, gingerol and shoagol. The results of HPLC quantification of different extracts presented in Table I indicate that ethyl acetate extract contains a higher amount of active compounds, gingerol and shoagol.

α-Amylase inhibitory activity was measured at various dosages (500, 750, 1,000 and 1,250 μg/ml). As in the case of α-glucosidase inhibitory activity, ethyl acetate extract ($IC_{50} = 980.21$ μg/ml) showed α-amylase inhibitory activity (Figure 2) compared with acarbose ($IC_{50} = 336.69$ μg/ml) as standard. Other extracts would not respond to the tests. Earlier reports show that phenolics play a role in mediating amylase inhibition and therefore have potential to contribute to the management of type 2 diabetes (McCue and Shetty 2004). Ginger extract gave mild α-amylase inhibitory activity compared with α-glucosidase. Previous reports had also indicated that excessive inhibition of α-amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon, and therefore mild α-amylase inhibition activity is useful (Horii et al. 1986).

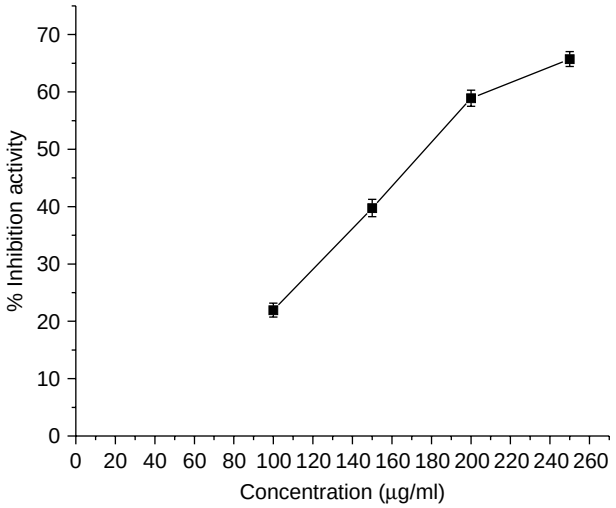

Figure 1. α-Glucosidase inhibition of ethyl acetate extract of ginger with varying concentrations.

Table I. HPLC quantification of gingerols and shoagols in successive extracts.

Sample number	Extract	Yield (%)	Total gingerols (%)	Total shoagols (%)	Total pungency (%)
1	Hexane	3.87	15.58	0.52	16.1
2	Ethyl acetate	1.93	17.22	0.72	17.94
3	Methanol	3.75	4.41	1.86	6.27
4	70% methanol–water	4.82	–	–	–
5	Water	5.68	–	–	–

Anti-inflammatory effects of the extracts were evaluated by percentage inhibition of three different dosages (38, 95 and 190 μg/ml) of extracts. Of these, ethyl acetate extract showed the highest activity, with an IC_{50} value of 145.04 μg/ml compared with standard indomethacin ($IC_{50} = 10.2$ μg/ml) against COX, as shown in Figure 3. COX was the key marker enzyme for the diseases with impaired arachidonic acid metabolism. Non-steroidal anti-inflammatory drugs bind to COX and inhibit the production of prostaglandins from arachidonic acid. COX catalyzes the conversion of arachidonic acid into prostaglandins, which play a significant role in health, and in disease in the gastrointestinal tract and in the renal, skeletal and ocular systems (Raju et al. 2010). The effectiveness of the ginger extract used in folk medicine to suppress inflammatory responses may thus be due to their capacity to reduce oxidative stress and to their inhibitory activity on COX enzymes.

The biological significance of increased formation of ROS may occur in diabetes for reasons possibly related to an increase in glucose level concentration in plasma and tissue. Interestingly, accumulation of intracellular ROS in H_2O_2-treated cells were decreased when cells were pretreated with ethyl acetate extract of ginger at 10, 20, 40, 80 and 100 μg/ml ($P < 0.05$) in a dose-dependent manner, as shown in Figure 4 (average mean fluorescence from triplicates is expressed per each group of cells). This could be explained as ginger inhibits intracellular oxidative stress and protects C2C12 cells from oxidative damage. No evidence of any increase or decrease in 2,7-dichlorofluorescein fluorescence was observed in cells incubated with extracts alone. Many experimental and clinical observations indicate oxidative stress to be an important

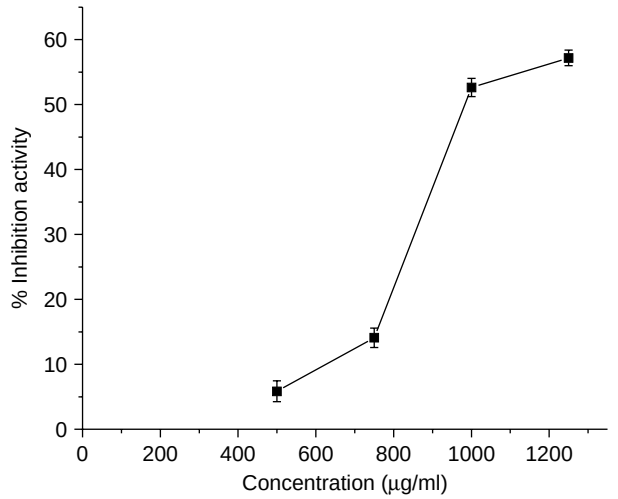

Figure 2. α-Amylase inhibition of ethyl acetate extract of ginger with varying concentrations.

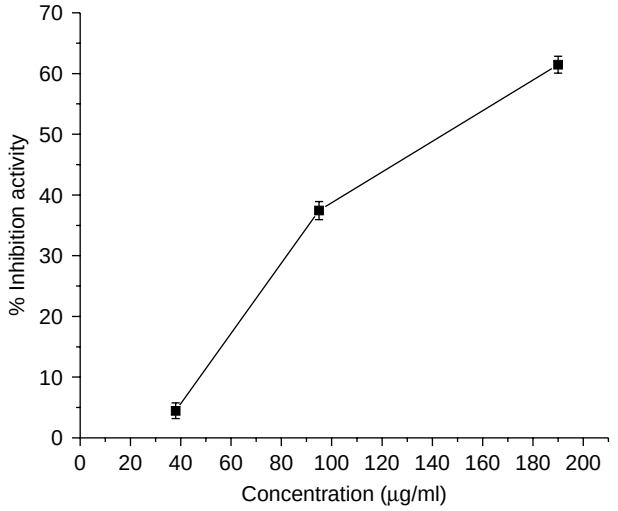

Figure 3. COX inhibition of ethyl acetate extract of ginger with varying concentrations.

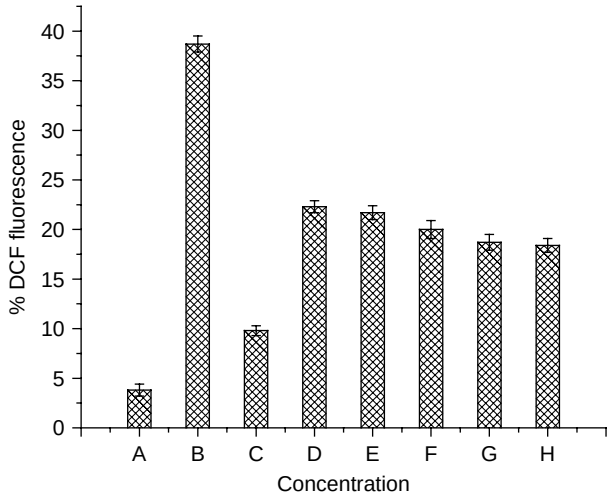

Figure 4. Evaluation of oxidative stress in C2C12 cell lines by flow cytometry. A, blank cells without any treatment; B, control cells treated with hydrogen peroxide; C, cells treated with hydrogen peroxide and ascorbic acid (25 μg/ml); D, cells treated with hydrogen peroxide + ethyl acetate extract of ginger (10 μg/ml); E, cells treated with hydrogen peroxide + ethyl acetate extract of ginger (20 μg/ml); F, cells treated with hydrogen peroxide + ethyl acetate extract of ginger (40 μg/ml); G, cells treated with hydrogen peroxide + ethyl acetate extract of ginger (80 μg/ml); H, cells treated with hydrogen peroxide + ethyl acetate extract of ginger (100 μg/ml).

mechanism in obesity-associated metabolic syndrome, in development of diabetes and its complications, heart disease and many satellite conditions.

The goal of the present study was to provide *in vitro* evidence for potential α-glucosidase, α-amylase and COX inhibitors to generate stronger biochemical rationale for further clinical studies. Among the five extracts, ethyl acetate extract showed the highest activity. The content of gingerol and shoagol, the pungent compounds in ginger, was determined by HPLC in the extracts (Table I). Owing to the insoluble nature of 70% methanol–water and water extracts, gingerol quantification was not done in these extracts. Even though the total pungency was almost comparable in ethyl acetate and hexane extracts, the reduced activity observed in hexane extract may be due to its dilution with other inactive or retarding constituents as is shown by its higher yield. Thus the anti-diabetic and anti-inflammatory activity of ginger revealed in the present study can be utilized to develop a suitable herbal formulation.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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