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In vitro anti-amylase activity of some Indian dietary spices

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ABSTRACT

Pancreatic alpha amylase (PAA) inhibitory activity of cinnamon, cumin was evaluated in vitro to search new anti-diabetic agents as alternatives to synthetic medicines. Bark of cinnamon, seeds of cumin, fenugreek, nutmeg, fennel, and buds of clove were extracted with hot water, methanol, chloroform, benzene, and ethyl acetate and 30 extracts were tested for the presence of PAA inhibitory activity using qualitative and quantitative methods and their modes of inhibition were determined. Presence of alpha amylase inhibitors was identified in 18 extracts in quantitative assay. Benzene extracts of cinnamon, clove, fenugreek, and nutmeg and chloroform extract of cumin showed highest anti-amylase potential. The IC50 values of these potential extracts ranged between 2.09 ± 0.12 mg/ml and 2.89 ± 0.079 mg/ml with lowest IC50 value noted for benzene extract of cinnamon and highest IC50 value was noted for benzene extract of fenugreek. Based on the values of Vmax and Km compared to control, it was found that all these extracts display non-competitive mode of inhibition on amylase activity. In conclusion, active constituents of these five extracts possess anti-amylase properties and can be used in management of diabetes mediated complications.

1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by both postprandial and fasting hyperglycemia with disturbed carbohydrate fat and protein metabolism. According to the recent report of Indian Council for Medical Research, Institute for Health Metrics and Evaluation and Public Health Foundation, India, diabetes prevalence has increased by 64% across India over quarter century with a figure expected to reach 134 million by 2025. India currently represents 49% of the world's diabetes burden, which poses a serious public health challenge to a country [1]. The logical therapeutic approach used for management of diabetes is to decrease postprandial hyperglycemia. This can be achieved by delaying the absorption of glucose through the inhibition of carbohydrate hydrolyzing enzymes in the digestive tract.

Pancreatic alpha-amylase (PAA) is the principal carbohydrate hydrolyzing enzyme located in the brush border of small intestine. It catalyzes breakdown of the complex dietary carbohydrates (starch) into easily absorbable monosaccharides (glucose) or disaccharides (maltose). Inhibitors of α-amylase activity reduce the glucose levels slowing down the speed of starch to glucose or simple sugar conversion. Hence, α-amylase inhibitors are used as drug targets in the development of anti-diabetic drugs. Some inhibitors of α-amylase, currently in the clinical use are acarbose and miglitol. However, these synthetic hypoglycemic agents are non-specific, produce serious side effects, and fail to elevate diabetic complications. The associated side effects of these inhibitors are bloating abdominal discomfort, diarrhea, and flatulence [2].

In this regard, drug, diet, and recently included spice therapies are major approaches used for the treatment and management of DM [3]. A number of medicinal plants, traditionally used for over 1,000 years named rasayana are present in herbal preparations of Indian traditional health care systems. These alternative medicines are not only used by the rural masses for their primary health care in developing countries but are also used in developed countries where modern medicines dominate [4].

Spices are the integral part of Indian food which impart distinctive flavor and aroma to food. The chemical profile of spices showed presence of various phytochemicals such as glucosides, phenolics, saponins, flavonoids, alkaloids, coumarins, terpenoids, etc. These phytochemicals are expected to contribute for pharmacological properties of spices [5,6]. It is also known that dietary spices drive

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Table 1: Spices and their parts used for screening of anti-amylase activity.

Sr. no	Botanical name	Common name	Family	Parts used
1	*Cinnamomum zeylanicum Breyn.*	Cinnamon (Cn)	Lauraceae	Bark
2	*Cuminum cyminum L.*	Cumin (Cm)	Apiaceae	Seeds
3	*Syzygium aromaticum (L)*	Clove (Cl)	Myrtaceae	Buds
4	*Foeniculum vulgare*	Fennel (Fn)	Apiaceae	Seeds
5	*Trigonella foenum-graecum L.*	Fenugreek (Fg)	Fabaceae	Seeds
6	*Myristica fragrans*	Nutmeg (Nm)	Myristicaceae	Seeds

diverse metabolic and physiological actions in gastrointestinal, cardiovascular, reproductive and nervous systems, and exert beneficial health effects [3]. In the present study, we have screened benzene, methanol, ethyl acetate, chloroform, and aqueous extracts of six different spices routinely used in Indian cooking for the presence of α-amylase inhibitors. The details of selected spices along with their parts used are listed in Table 1. The lead extracts were used for determining their IC_{50} values, as well as mode of α-amylase inhibition.

2. MATERIALS AND METHODS

All spices that were used in this study (Table 1) were obtained from local market of Nanded (M.S), India. Porcine PAA and Acarbose were procured from Sigma Aldrich Co. (St. Louis, USA), while soluble starch and dinitro salicylic acid (DNSA) were obtained from HiMedia (Mumbai, India). Other chemicals and solvents were of analytical grade.

2.1. Preparation of Spice Extracts

Parts of different spices were washed with water to remove all impurities, dried under shade, and grounded to fine powder. The powdered spices were divided into six portions and each portion was individually extracted with hot water, methanol, chloroform, benzene, and ethyl acetate in 1:10 ratio. The resulting blends were collected, filtered, and evaporated in a rotary evaporator. Dried extracts were weighed and dissolved in dimethyl sulphoxide to yield stock solutions from which various concentrations of extracts were prepared.

2.2. Qualitative Assay of α-amylase Inhibition

Modified well plate method described by Fossum and Whitaker [7] was used to detect α-amylase inhibitory activity of different spice extracts. Briefly, wells (5 mm) were made on sterile starch agar plates with the help of sterilized cork borer. Two hundred microliters of pancreatic α-amylase (10 mg/ml) was pretreated individually with 200 µl of different solvent extracts (2.5 mg/ml) of selected spices for 10 minutes. Such pretreated enzyme was loaded in wells (100 µl) and the plates were incubated overnight at 37°C. After incubation, plates were flooded with iodine solution and observed for clear starch hydrolysis zones against dark purple background. Amylase solution without pretreatment was used as positive control. Resulting zones of starch hydrolysis were measured and the extracts showing zones lesser than positive control well were selected for further study.

2.3. Quantitative Assay of α-amylase Inhibition

Quantitative α-amylase inhibition assay was performed as per the method described earlier [8]. Briefly, 200 µl of each extract (2.5 mg/ml) was mixed with 200 µl of α-amylase (10 mg/ml) and 500 µl of 0.1 M phosphate buffer (pH 7.0). This mixture was pre-incubated at room temperature for 10 minutes. After incubation, 500 µl of starch (0.25%) solution was added and incubated at room temperature for further 10 minutes. This was followed by addition of 1 ml DNSA reagent to stop the reaction and incubation in boiling water bath for 10 minutes. The reaction mixture was removed from water bath, cooled at room temperature, and diluted to 10 ml with distilled water. Absorbance of the mixture was taken at 540 nm. Amylase activity in absence of spice extracts was used as positive control for comparison. Acarbose (100 µg/ml), a known α-amylase inhibitor, was used as a standard drug. Each experiment was performed in triplicate and the results are interpreted as mean. The percentage inhibition in amylase activity was calculated by using the following formula.

$$\%\text{inhibition} = \frac{\text{O.D of control} - \text{O.D of extract}}{\text{O.D of control}} \times 100$$

The extracts showing more than 50% inhibition were selected to study amylase inhibition at varying concentrations. The enzyme was pretreated with 200 µl of different concentrations (1–10 mg/ml) of spice extracts individually and the activity was determined as stated earlier. Concentrations of extracts resulting in 50% inhibition in amylase activity (IC_{50}) were determined by using regression analysis.

2.4. Mode of α-amylase Inhibition

The method for determining the mode of inhibition of α-amylase in presence of spice extracts involved pre-incubation of 200 µl of α-amylase solution with selected spice extracts at room temperature for 10 minutes. Control was prepared by pre-incubating α-amylase with 200 µl of phosphate buffer (pH 7.0). Reaction was started with addition of starch solution at increasing concentration (0.025%–0.25%). The reaction mixture was incubated for 10 minutes at room temperature and terminated by addition of 1 ml of DNSA. The contents were placed in boiling water bath for 10 minutes and diluted 10 ml with distilled water. The amount of reducing sugars released during reaction was determined spectrophotometrically at 540 nm. The concentration of reducing sugars was determined using standard curve of maltose. The concentrations of reducing sugar obtained such were converted to reaction velocities. The mode of α-amylase inhibition by the extract was determined by plotting a double reciprocal (Line weaver-Burk) plot between reaction velocity (1/V) and starch concentration (1/[S]) using Michaelis Menten kinetics. The changes in the values of V_{max} and K_m over control were considered to reveal inhibition mode of extract as explained earlier [9].

Table 2: Amylase inhibitory activity of selected spices on starch agar plate.

Extract	Spice/*Diameter of zone of hydrolysis (mm)					
	Cn	Fn	Cl	Cm	Fg	Nm
ME	16 ± 1.22	21 ± 0.65	16 ± 0.96	16 ± 0.037	20 ± 0.11	19 ± 0.065
BE	7.0 ± 0.43	21 ± 0.81	15 ± 1.01	20 ± 0.91	14 ± 0.245	10 ± 0.189
EAE	17 ± 0.87	20 ± 1.26	18 ± 1.92	14 ± 1.45	19 ± 0.16	20 ± 1.20
CE	14 ± 1.6	21 ± 0.34	18 ± 0.099	13 ± 0.01	20 ± 0.04	20 ± 0.43
AE	16 ± 1.18	19 ± 0.021	19 ± 0.14	19 ± 0.045	15 ± 0.23	15 ± 0.96

*Zone of starch hydrolysis in absence of extracts was 22 mm. The extract whose presence lowered the size of the zone was considered inhibitory for amylase activity. ME: Methanol extract, BE: Benzene extract, EAE: Ethyl acetate extract, CE: Chloroform extract, AE: Aqueous extract.

3. RESULTS AND DISCUSSION

Alpha amylase is the key digestive enzyme involved in the process of starch metabolism [10]. Inhibitors of such enzyme, therefore, can play an important role in the management of diabetes-related complications. Natural alpha-amylase inhibitors from food-grade herbal sources offer an attractive therapeutic approach to the treatment of postprandial hyperglycemia by decreasing glucose release from starch, which may be potentially useful in the treatment of diabetes mellitus and obesity [11]. In the present study, six dietary spices, namely, cinnamon, cumin, fennel, fenugreek, clove, and nutmeg were selected and α-amylase inhibitory activity in different parts (seeds, bark, and bud) of them was determined both qualitatively and quantitatively. Qualitative detection of anti-amylase activity was done by using starch agar plate assay and results were recorded in terms of diameters of starch hydrolysis zones in the presence and absence of extracts. Of the different spice extracts screened, benzene extracts of cinnamon (7.0 mm) and nutmeg (10 mm) showed the lowest starch hydrolysis zone on the starch agar plate, indicating highest inhibition of α-amylase activity. The other extracts showed moderate to least α-amylase inhibition (Table 2).

The anti-amylase potential of different solvent extracts of spices was also determined quantitatively. The details of spices screened, solvent extracts used, and percent anti-amylase activity in them are summarized in Table 3. At 2.5 mg/ml concentration, 18 out of 30 extracts showed amylase inhibition capacity. Remaining 12 extracts although showed a reduction in starch hydrolysis zones as determined qualitatively (Table 2), did not show inhibition effectively under assay conditions. The detected range of inhibition in presence of 18 extracts was between 16.20% and 69.92%.

Table 3: Inhibition of amylase activity by solvent extracts of different spices at 2.5 mg/ml.

Sr. no.	Name of the spices	Anti-amylase activity (%)				
		ME	BE	EAE	CE	AE
1	Cn	27.81	**69.92**	25.74	42.27	32.22
2	Cl	34.18	**53.21**	28.34	22.41	21.83
3	Cm	34.25	00	41.37	**52.29**	00
4	Fg	00	**51.89**	00	00	41.95
5	Nm	16.20	**57.80**	00	00	43.33
6	Fn	00	00	00	00	00

Bold values in the table indicates percent inhibition above 50%.

Benzene extract of cinnamon had the highest inhibition capacity, followed by benzene extracts of nutmeg (57.80%) whereas methanol extract of nutmeg showed least (16.20%) reduction in amylase activity among all extracts. Aqueous extracts of cinnamon have been reported to exhibit an appreciable inhibitory effect on pancreatic amylase by Sellami et al. [12] whereas, recently Bhutkar et al. [13] found 28.96% inhibition in amylase activity in presence of hot water extract of nutmeg seeds at 1,000 µg/ml concentration.

Nutmeg has shown to possess cytotoxic, hepatoprotective, antioxidant, anti-inflammatory, antithrombotic, hypolipidemic, anti-atherosclerotic, hypoglycemic, and antidiabetic activities [14–16]; however, its *in vitro* anti-amylase activity was less observed. 31.32% inhibition in amylase activity was observed in presence of 1 mg/ml ethanol extract of fenugreek leaves by Narkhede [17].

Clove is an aromatic flower bud, commonly used in Africa, Asia, and other parts of the world in preparation of various spicy rich dishes. It possesses antioxidant, anti-fungal, anti-viral, anti-microbial, anti-diabetic, anti-inflammatory, antithrombotic, anesthetic, pain relieving, and insect repellent properties [18]. Oboh et al. [19] investigated the effect of essential oil from clove bud on alpha-amylase and alpha-glucosidase activities and found a dose-dependent inhibition of amylase and glucosidase activities with EC_{50} values of 88.9 and 71.94 µl/l, respectively.

In the present study, we observed 52.29% inhibition in amylase activity in presence of chloroform extract of cumin seeds. The cumin seeds are also widely used as a spice due to their characteristic aroma and as a traditional medicine to treat diseases such as chronic diarrhea and dyspepsia, acute gastritis, diabetes, and cancer. The biological and biomedical activities of cumin are credited to its bioactive constituents as terpenes, phenols, and flavonoids [20]. Recently, Siow et al. [21] identified CSP4 and CSP6 as potent amylase inhibitory peptides from cumin seeds.

In all extracts of fennel, amylase inhibition was negligible or absent when studied quantitatively. Fennel is one of the widespread plants known for aromatic odor and high phenolic contents. Little information is available about its anti-diabetic properties. According to Abu-Zaiton et al. [22], extracted aerial parts of fennel with different solvents and tested *in vitro* potential of extracts to inhibit activities of angiotensin-converting enzyme, alpha amylase, and alpha glucosidase. Methanol: acetone: water (1:1:1) and methanol: acetone (1:1) were found suitable to extract active metabolites required for maximum inhibition of these

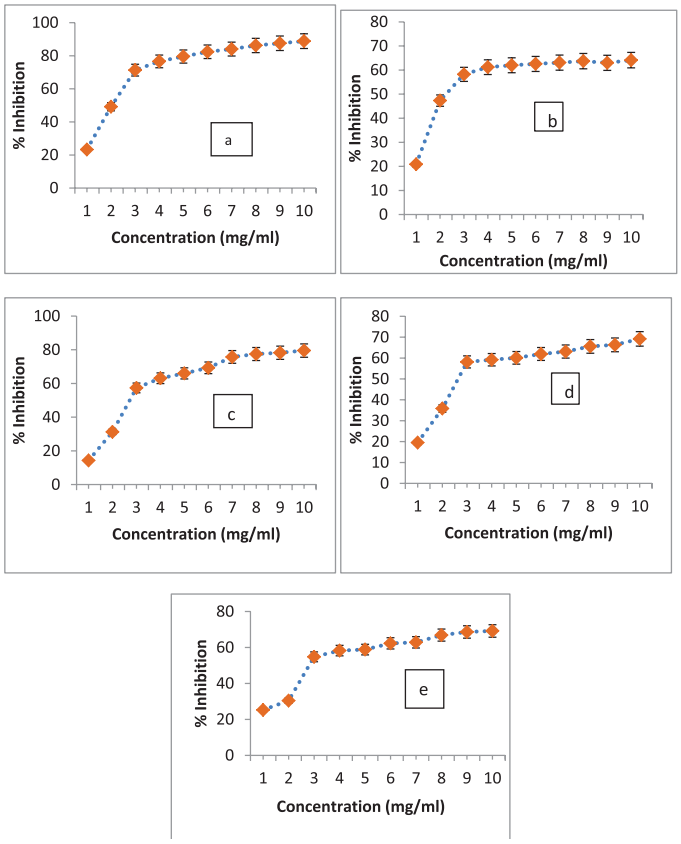

Figure 1: *Amylase inhibitory activity of benzene extract of cinnamon (a), nutmeg (b), and clove (c), chloroform extract of cumin (d), and benzene extract of fenugreek (e) at varying concentrations (1–10 mg/ml).*

enzymes. As we have not used methanol, acetone, or water in combination, it is possible that the active metabolites might not be extracted in required amounts needed for the inhibition of porcine pancreatic α-amylase (PPA) activity.

The five extracts, including benzene extract of cinnamon, clove, fenugreek, and nutmeg and chloroform extract of cumin showed inhibition potential above 50% and were tested for their activity at varying concentrations (1–10 mg/ml). Neither extract of fennel was able to inhibit amylase activity at selected concentration level. Acarbose at 100 µg/ml concentration showed 51.69% inhibitory effects on the α-amylase activity. All selected extracts showed a concentration-dependent increase in enzyme inhibition at the initial level with the curve approaching to minimal change at higher concentrations of extracts (Fig. 1a–e).

Table 4: Inhibitory concentration (IC_{50}) value of spice extract on alpha-amylase.

Sr. no.	Spices	Extract	IC_{50} (mg/ml)
1	Cm	CE	2.5 ± 0.89
2	Cl	BE	2.6 ± 0.043
3	Fg	BE	2.89 ± 0.079
4	Cn	BE	2.09 ± 0.12
5	Nm	BE	2.25 ± 0.28
6	Acarbose	ME	0.125 ± 0.39

Table 5: K_m and V_{max} values of extract of spices and their mode of inhibition.

Parameter	Control	Cm CE	Cl BE	Cn BE	Nm BE	Fg BE
K_m	0.0357	0.0355	0.0354	0.0356	0.0356	0.0358
V_{max} (µmoles/ minute)	0.00086	0.00041	0.00034	0.00012	0.00028	0.00054
Inhibition Mode*	-	NC	NC	NC	NC	NC

*NC: non-competitive; CE: chloroform extract; BE: benzene extract.

Analysis of the dose-response curves of selected extracts for determining their IC_{50} values showed that benzene extract of cinnamon contained the most potent alpha-amylase inhibitor with an IC_{50} value of 2.09 ± 0.12 mg/ml, followed by an effective inhibition determined in presence of other selected extracts with IC_{50} values ranged between 2.25 ± 0.28 mg/ml and 2.89 ± 0.079 mg/ml (Table 4). Sellami et al. [12] reported anti-amylase activity of both bark (IC_{50}: 214 ± 2 μg/ml–215 ± 10 μg/ml) and leaf (IC_{50}: 943 ± 28 μg/ml) of Ceylon cinnamon. Previous investigation on α-amylase inhibitory activity of bark of some economically important Cinnamomum species such as *C. zeylanicum, C. aromaticum*, and *C. loureiroi* showed their anti-amylase activity with IC_{50} values 1.23 ± 0.02 mg/ml, 1.77 ± 0.05 mg/ml, and >4.00 mg/ml, respectively [10]. The discrepancy observed between present study and previous investigations on anti-amylase activity may be due to the use of different solvents, extraction procedures, and source of α-amylase.

For determining the mode of inhibition of α-amylase by crude extracts of spices, α-amylase activity was determined both in the presence and absence of selected extracts at different substrate concentrations. The mode of inhibition of all these five extracts on alpha-amylase activity was determined using Line weaver-Burk plot (data not shown) which indicated that these extracts displayed non-competitive inhibition modes on the enzyme activity (Table 5). In presence of all tested extracts, the values of apparent K_m remain unchanged while reducing the V_{max}. This suggests that there is no competition between extract and substrate for binding with the active site of amylase and they reduce the activity of the enzyme by binding to free or substrate bound enzyme.

4. CONCLUSION

These findings suggest that although all selected extracts are commonly used in Indian food and their medicinal properties are known to some extent, but very few studies reported their PPA inhibitory potential. In the present study, we have not ascertained the chemical nature of the phytoconstituents responsible for PPA inhibitory activity and the active lead molecules need to be isolated and characterized through *in vitro* and *in vivo* studies. While some of the spice extracts used in this study showed negligible or no inhibition against PPA, five extracts exhibited good enzyme inhibitory activity. Hence, these extracts can be used to lower the postprandial high glucose levels by inhibiting PPA activity.

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