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INHIBITORY EFFECT OF A POLYHERBAL FORMULATION ON α-GLUCOSIDASE, AND PORCINE PANCREATIC AMYLASE IN TYPE-II-DIABETES.

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ABSTRACT

Diabetes has become a common global health problem that affects >170million people worldwide. It is one of the leading causes of death and disability. It is estimated that by 2030, the number will rise to 366 million (www.who.int). The majority of diabetes (~90%) is type 2 diabetes (T2D) caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver. Traditional Medicines obtained from medicinal plants are used by about 40-60% of the world's population. Though there are many approaches to control diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost. In the present study focused on screening of *cinnamomum cassia* extract for antidiabetic activity with respect to two different enzymatic assay as such α -glucosidase and α – amylase inhibition assay. It was reported by many researchers that diabetic patients are under oxidative stress due to an imbalance between the free radical generation and radical scavenging capacities. Therefore, use of antioxidants can be beneficial for diabetic patients. Further, the screening of *cinnamomum cassia* had been done using two antioxidant assay-ABTS & DPPH radical scavenging assay in *in vitro*. Extracts of *cinnamomum cassia* exerted *in vitro* inhibitory potential against α -amylase with IC50 ranging from 100.53 to170.70 µg/ml but shows no inhibitory effect against α -glucosidase. The antioxidant activities of the extract, measured in terms of IC50 values for ABTS and DPPH radical scavenging assay are 60.90-88.54 µg/ml and above 200 µg/ml, respectively. The above results support that the

cinnamomum cassia has the ability to inhibit the enzyme alpha amylase and also acts as an antioxidants.

KEY WORDS; *Cinnamomum cassia* DPPH ABTS Type 2 Diabetes.

INTRODUCTION

In humans, the digestion of starch involves several stages. Initially, partial digestion by the salivary amylase results in the degradation of polymeric substrate into shorter oligomers. Later on in the gut these are further hydrolyzed by pancreatic α-amylase into maltose, maltotriose and small malto-oligosaccharides. The digestive enzyme (α-amylase) is responsible for hydrolyzing dietary starch to maltose, which breaks down to glucose, prior to absorption. Inhibition of the α-amylase should reduce the unfavorable high postprandial blood glucose peak in diabetes. α-amylase inhibition is also a useful target in obesity[9,10] Pancreatic α-amylase hydrolyses the 2-chloro-4- nitrophenol α-D - maltotrioside (CNP-G3) to release 2-chloro-4-nitrophenol and form 2-chloro-4- nitrophenol α-D - maltoside (CNPG2), maltotriose and glucose. The rate of formation of the 2-chloro-4-nitrophenol can be measured at 405nm.α- glucosidase which is a membrane bound enzyme located at the epithelium of the small intestine, catalyses the cleavage of glucose from disaccharides. Thus, the retardation of the action of α-glucosidase may be one of the most effective approaches to control diabetes.[12] Since carbohydrate intake influences obesity, α-glucosidase inhibition may be useful in obesity.[13] α-glucosidase activity can be measured *in vitro* by determination of the reducing sugar (Glucose) arising from hydrolysis of maltose by α-glucosidase enzyme, isolated from small intestine of rat.

MATERIALS AND METHODS

α-amylase (EC 3.2.1.1) (Type VI-B: From porcine pancreas, 500,000 units [19.6 units/mg solid at pH 6.9]). CNP-G3 reagent [2-chloro-4-nitrophenol α-D-maltotrioside] Acarbose.Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$). Di-sodium hydrogen phosphate dihydrate ($Na_2\ HPO_4.\ 2H_2O$). Microwell plate: Costar 384 well polystyrene, non-treated.

The assay is performed as per Gella *et al*[11] with modifications. In brief, a pre-incubation mixture of 22.5µl contains 14.5µl 40mM phosphate buffer pH 6.9 / vehicle buffer/ positive control / test sample of various concentrations and 8µl of enzyme (0.4998units/ml). Mix and

pre-incubate at 37°C for 10 minutes. Following pre-incubation, add 31µl substrate (2.3mM CNP-G3) and incubate at 37°C for 8 to 14 minutes. Measure the absorbance at 405nm in kinetics mode (Pherastar). A control reaction is carried out without the test sample.

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

i. IC_{50} is calculated using log-probit analysis.
ii. Appropriate solvent and colour corrections should be done (in case of coloured samples & non- aqueous solutions).
iii. Methanol can be used up to 1% of the pre-incubation volume.
iv. DMSO can be used up to 5 % of the pre-incubation volume.

Phosphate buffer (40mM, pH 6.9 at 25ºC)

Solution A: 6.24g of sodium dihydrogen orthophosphate dihydrate is dissolved and made up to 1000ml with de-ionized water.

Solution B: 7.12g of di-sodium hydrogen phosphate dihydrate is dissolved and made up to 1000ml with de-ionized water.

Mix 45ml of solution A & 55ml of solution B & make up to 200ml with de- ionized water.

Enzyme (0.48412units/ml): 2.65mg of α-amylase is made up to 100 ml with 40 mM phosphate buffer pH 6.9. (Enzyme units are lot specific).

Substrate (2.3mM): CNP-G3 reagent ready to use.

(a)Positive control

Stock 1: (1mg/ml): 50mg of Acarbose dissolved in 50ml of 40mM Phosphate buffer, pH 6.9

Stock 2: Dilute to a concentration of 2.5µg/ml with 40mM Phosphate buffer, pH 6.9.

Working stock: Dilute to a concentration of 0.25µg/ml with 40mM Phosphate buffer, pH 6.9.

(b)Sample preparation

A sample stock of 310µg/ml was prepared for the sample by dissolving 3.1mg of the sample in 10ml with 40mM sodium phosphate buffer, pH 6.9. Further dilutions were made as required with 40mM sodium phosphate buffer, pH 6. 9

Enzyme: α-glucosidase isolated from rat intestine (store at -20°C).

Substrate: D (+) Maltose monohydrate (store at RT).

Positive control: Acarbose (store at RT).

Total protein estimation kit (Biuret method) store at 2-8°C.

Other reagents

- Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$).
- Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$).
- Glucose reagent (store at 2-8°C).
- Microwell plate - 96well flat, clear plate.

Phosphate buffer (80mM, pH 7.0 at 25ºC)

Solution A: 1.248g of sodium dihydrogen orthophosphate dihydrate is dissolved and made up to 100ml with de-ionized water.

Solution B: 1.242g of di-sodium hydrogen phosphate dihydrate is dissolved and made up to 100ml with de-ionized water.

Mix 39ml of solution A & 61ml of solution B and make up to 200ml with de- ionized water.

Enzyme: Rats are sacrificed, intestine is removed and chilled with ice cold 80mM phosphate buffers (pH 7.0). The intestine is then cut open, the mucosa is scraped off with a piece of glass rod and homogenized in homogenizer with four parts (v/v) of cold 80mM buffer (pH 7.0). The tube is chilled with crushed ice during homogenization. Nuclei and large cell debris are removed by centrifugation at 2000–4000 rpm for 10minutes and supernatant is aliquoted into 1.5ml vials and stored at –20ºC. [Protein content = approximately 0.5g/dl., by Biuret method].

Substrate (37mM): 0.632g of sucrose is made up to 25ml with 80mM phosphate buffer pH 7.0.

(a) Positive control

Stock 1 (1mg/ml): 50mg of Acarbose dissolved in 50ml of 80mM Phosphate buffer, pH 7.0.

Working Stock: Dilute to a concentration of 5□g/ml with 80mM Phosphate buffer pH 7.0.

(b) Sample preparation: A sample stock of 600µg/ml was prepared sample by dissolving 1mg of sample dissolved in 1.66ml with 80mM phosphate buffer (pH 7.0).

The assay is performed as per of *Vogel & Vogel*[14] with modifications. In brief, a pre-incubation mixture of 300µl contains 250µl of 80mM phosphate buffer pH 7.0 / positive control / test sample of various concentrations, add 50µl of enzyme. Mix and pre-incubate at 37°C for 30minutes. Following pre-incubation, add 500µl substrate (37mM sucrose) and incubate at 37°C for 20minutes. Arrest the reaction by keeping in boiling water bath for 2minutes, then cool, Add 250µl of glucose reagent to 5µl of reaction mixture and mix. Incubate at 25°C. for 10mins, Measure the absorbance at 510nm spectrophotometrically (Molecular devices Versamax microplate reader). A control reaction is carried out without the test sample.

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

i. IC_{50} is calculated using log-probit analysis.
ii. Appropriate solvent and color corrections should be done (in case of colored samples & non- aqueous solutions.
iii. DMSO can be used up to 2 % of the pre-incubation volume.

RESULTS AND DISCUSSION

1.*α-Amylase inhibition assay*

Sample	Conc. (µg/ml)	% inh.	IC_{50} (µg/ml)	Solubility
Ref. Inh.(Acarbose)	0.1	24.25	0.23 (0.18-0.28)	
	0.25	58.43		
	0.5	69.19		
	1	83.23		
Sample *Cinnamomum cassia*	10	5.68	126.49 (100.53-170.70)	approx. 80%
	25	18.46		
	50	27.41		
	100	44.34		
	200	60.99		

The IC_{50} of the *Cinnamomum cassia* was found to be 126.49µg/ml.

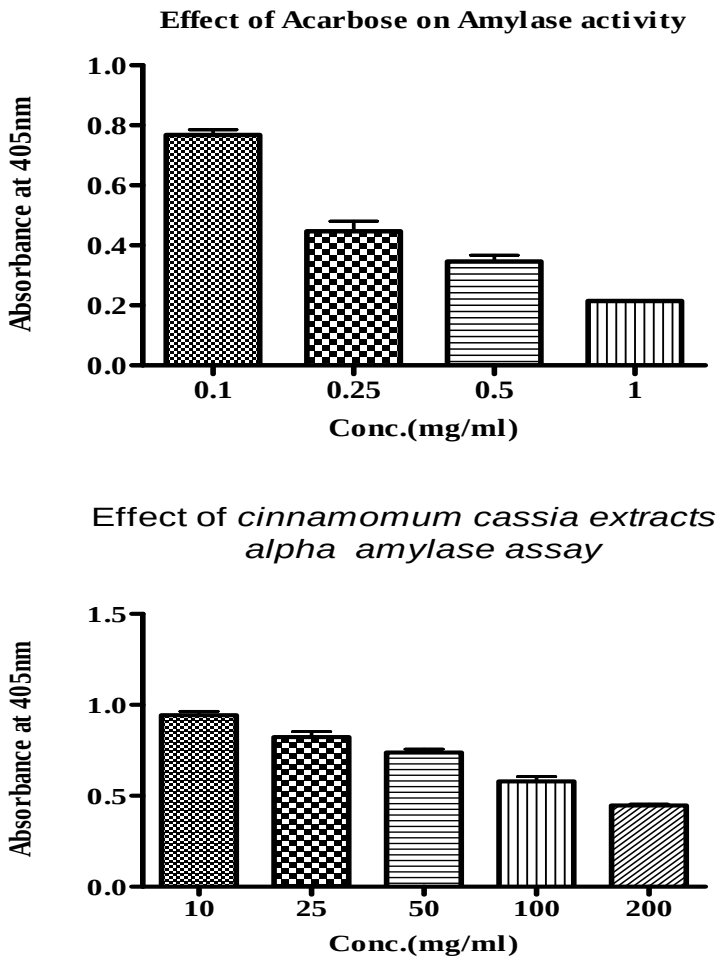

Fig.01 Graph for standard (Acarbose) and *Cinnammomum Cassia* Extract

2.*α-Glucosidase inhibition assay*

Sample	Conc. (µg/ml)	% inh.	IC_{50} (µg/ml)	Solubility
Positive Control (Acarbose)	0.5	36.26	1.09 (1.41-0.79)	
	1	44.86		
	2	64.77		
	4	72.99		
Sample *Cinnamomum cassia*	10	-10.84	NIL	100% in 2% DMSO
	25	-11.96		
	50	-22.71		
	100	-19.16		
	200	-17.10		

The sample of *cinnammomum cassia* is inactive showing negative inhibition at given concentration.

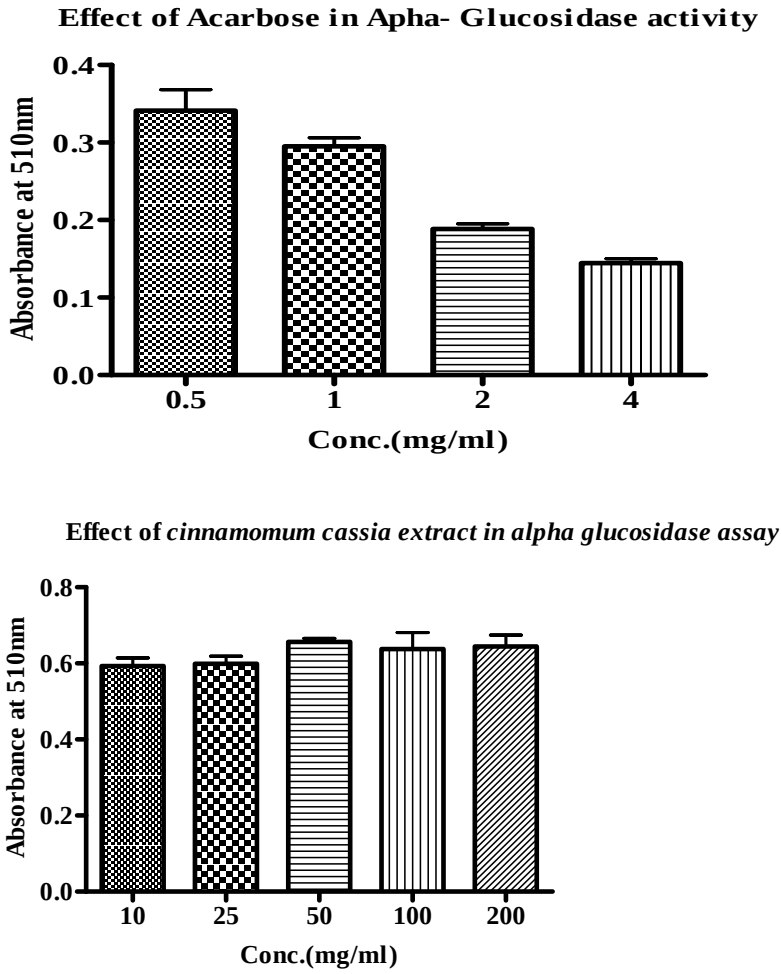

Fig.02 Graph for standard (Acarbose) and *Cinnammomum Cassia* Extract

Diabetes is one of the major complications, the world faces today. Many reports suggest that *Cinnamomum cassia* (Lauraceae)) has potent antidiabetic activity. The plant was selected based on their uses in traditional medicine. By using micro plate reader the % inhibition of the extract was carried out by using suitable enzyme, substrate and control followed by The IC_{50} was evaluated. The results of the present study demonstrated that *Cinnamomum cassia* extracts shows the inhibitory activity for the enzyme alpha amylase but show no inhibitory effect for alpha glucosidase enzyme. It has been proved that through scientific researches, the oxidative stress is responsible for causing diabetes. So, the antioxidants assays DPPH and ABTS radical scavanging assays were been done using *Cinnamomum cassia* extracts. In this case, *Cinnamomum cassia* extract shows good antioxidant activity in ABTS assay in comparision with DPPH assay. We conclude that further bioassay-guided fractionation approaches will be required on these species to identify the compounds responsible for their promising *in vitro* anti-diabetic activity.Besides this finding, Richa soni *et.al.,2009* confirms

that *cinnamomum cassia* is beneficial in reducing glycemic load and improves the blood sugar response both (fasting and post prandial) in diabetes (2003) on 60 type 2 diabetics randomly assigned to three groups receiving 1,3,6 g of cinnamon/ day for 40 days. Cinnamon supplementation significantly reduced the fasting and postprandial blood glucose. Anuradha and Devi (2004) also reported significant reduction in both fasting and post prandial blood glucose after 4 g cinnamon supplementation for 90 days.In recent studies by A.senghal,2011 shows that 1 gm of daily adjunct cinnamon, in addition to usual care lowers HbA1c by 0.83%, compared with usual care alone, which lowered HbA1c by 0.37%. Thus, cinnamon can be useful for lowering HbA1c in type 2 diabetics which helps to control diabetes.

CONCLUSSION

Diabetes is one of the major complications, the world faces today. Many reports suggest that *Cinnamomum cassia* (Lauraceae)) has potent antidiabetic activity. The plant was selected based on their uses in traditional medicine.By using micro plate reader the % inhibition of the extract was carried out by using suitable enzyme, substrate and control followed by The IC_{50} was evaluated. The results of the present study demonstrated that *Cinnamomum cassia* extracts shows the inhibitory activity for the enzyme alpha amylase but show no inhibitory effect for alpha glucosidase enzyme. It has been proved that through scientific researches, the oxidative stress is responsible for causing diabetes. So, the antioxidants assays DPPH and ABTS radical scavanging assays were been done using *Cinnamomum cassia* extracts. In this case, *Cinnamomum cassia* extract shows good antioxidant activity in ABTS assay in comparision with DPPH assay. We conclude that further bioassay-guided fractionation approaches will be required on these species to identify the compounds responsible for their promising *in vitro* anti-diabetic activity.Besides this finding, Richa soni *et.al.,2009* confirms that *cinnamomum cassia* is beneficial in reducing glycemic load and improves the blood sugar response both (fasting and post prandial) in diabetes (2003) on 60 type 2 diabetics randomly assigned to three groups receiving 1,3,6 g of cinnamon/ day for 40 days. Cinnamon supplementation significantly reduced the fasting and postprandial blood glucose. Anuradha and Devi (2004) also reported significant reduction in both fasting and post prandial blood glucose after 4 g cinnamon supplementation for 90 days.In the studies by A.senghal,2011 shows that 1 gm of daily adjunct cinnamon, in addition to usual care lowers HbA1c by 0.83%, compared with usual care alone, which lowered HbA1c by 0.37%. Thus, cinnamon can be useful for lowering HbA1c in type 2 diabetics which helps to control diabetes.

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