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In Vitro Antidiabetic and Anti-oxidant Activities of Methanol Extract of *Tinospora Sinensis*

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ABSTRACT

In the present study the methanol extracts of *Tinospora sinensis* Lour (Merr.) was studied for alpha (α)-amylase and alpha (α)-glucosidase inhibition using an *in vitro* model. The plant extracts were also examined for its antioxidant activities by using free radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging method, ABTS radical scavenging ability, reducing power capacity, estimation of total phenolic content, flavonoid content and flavonol content. The study revealed that the different concentrations of the extract possessed a very good amount of total phenolics, flavonoid and flavonol and exhibit potent radical scavenging activity using DPPH and ABTS as substrate. The methanol extract exhibited significant α-amylase and α-glucosidase inhibitory activities with an IC_{50} value 0.75µg and 0.80 µg dry extract respectively and well compared with standard acarbose drug. Thus, it could be concluded that due the presence of antioxidant components the plant extract have well prospective for the management of hyperglycemia, diabetes and the related condition of oxidative stress. This knowledge will be useful in finding more potent antidiabetic principle from the natural resources for the clinical development of antidiabetic therapeutics.

1. INTRODUCTION

Diabetes mellitus is an important chronic metabolic disorder that affects the metabolism of carbohydrate, fat and protein. It includes a group of metabolic diseases characterized by hyperglycemia, in which blood sugar levels are elevated either because the pancreas do not produce enough insulin or cells of the body do not respond properly to the insulin produced. The effects of diabetes mellitus include long-term complications include heart disease, stroke, dysfunction and failure of various organs [1]. There are three forms of diabetes. The three main types of diabetes are type 1, type 2, and gestational diabetes. Both women and men can develop diabetes at any age. The only therapy of type 1 diabetes is the substitution of insulin. Many and diverse therapeutic strategies for the treatment of type 2 diabetes are known. The conventional treatments for diabetes include the reduction of the demand for insulin, stimulation of endogenous insulin secretion, enhancement of the action of insulin at the target tissues and the inhibition of degradation of oligo- and disaccharides [2, 3]. One group of drugs introduced in the management of type 2 diabetes is represented by the inhibitors of α-glucosidase. The enzymes summarized as α-glucosidase are responsible for the breakdown of oligo- and/or disaccharides to monosaccharides. The inhibitory action of these enzymes leads to a decrease of blood glucose level, because the monosaccharides are the form of carbohydrates which is absorbed through the mucosal border in the small intestine. Another effective method to control diabetes is to inhibit the activity of α-amylase enzyme which is responsible for the collapse of starch to more simple sugars (dextrin, maltotriose, maltose and glucose) [4]. This is contributed by α-amylase inhibitors, which delays the glucose absorption rate thereby maintaining the serum blood glucose in hyperglycemic individuals [5]. Some inhibitors currently in clinical use are acarbose and miglitol which inhibit glycosidases such as α-glucosidase and α-amylase while others such as and voglibose inhibit α-glucosidase. However, many of these synthetic hypoglycemic agents have their limitations, are non-specific, produce serious side effects and fail to elevate diabetic complications. The main side effects of these inhibitors are gastrointestinal *viz*., bloating, abdominal discomfort, diarrhea and flatulence [6].

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Recently herbal medicines are getting more importance in the treatment of diabetes as they are free from side effects and less expensive when compared to synthetic hypoglycemic agents [7-8]. Phytochemical constituents like saponin, phenols, flavonoids etc studied in various plants such as *Proteus vulgaris, Euphorbia hirta, Cassia glauca* showed potential α-amylase inhibitors [9]. The role of medicinal plants in disease prevention is attributed to its antioxidant properties due to the presence of bioactive constituents [10]. *Tinospora sinensis* (Lour.) Merr belongs to the family Menispermaceae, and the stem of the plant is used as medicine. The plant grows wild in most parts of India, both in forests and plains. The plant is reported to be used for fumigation in piles and ulcerated wounds, and for the preparation of medicated baths for liver-complaints. The boiled roots are given in fever. Fresh leaves and stems are used in chronic rheumatism and also a muscle relaxant [11]. Thus, in this study, the antioxidant and antidiabetic activities of the methanol extract from the stem of *T. sinensis* were carried out and a relationship between antioxidant and antidiabetic activities was established. To determine the potential of *T. sinensis* stem extract as antidiabetic agents, we investigated the effect of extracts on the α-glucosidase and α-amylase inhibitory activities.

2. MATERIALS AND METHODS

2.1 Plant materials

The stem of *Tinospora sinensis* was collected from Hooghly district West Bengal, India and authenticated from Botanical Survey of India, Howrah. The voucher specimens were preserved in our department under registry no PHYSIOL/ BM/AB 001. The plant parts were shed-dried, pulverized and stored in an airtight container for further extraction.

2.2 Chemicals

Alpha (α)-Glucosidase, porcine pancreas alpha (α)-amylase, *p*-nitrophenyl-α-D-glucopyranose (*p*-NPG), 3,5-dinitrosalicylic acid (DNS), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxytoluene (BHT), gallic acid, rutin, quercetin, ascorbic acid and acarbose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soluble starch, sodium potassium tartarate, sodium dihydrogen phosphate (NaH_2PO_4), Di-sodium hydrogen phosphate (Na_2HPO_4) sodium chloride, sodium hydroxide, butylated hydroxytoluene (BHT), potassium persulfate, sodium carbonate, Folin-Ciocalteu (FC) reagent, potassium ferricyanide, potassium per sulphate, aluminum chloride, ferric chloride ($FeCl_3$) were from Merck Chemical Supplies (Damstadt, Germany). All the chemicals used including the solvents, were of analytical grade.

2.3 Extraction of plant material

The air-dried and coarse powdered plant sample of *T. sinensis* (10 g) was extracted with100 ml methanol by maceration on an orbital shaker with agitation for five days at room temperature. The extracts was filtered and residue was again extracted with same solvent for another five days and filtered. The filtered extracts were combined and concentrated using a rotary evaporator, under reduced pressure at approximately 40°C and lyophilized to obtain the powdered extract. The powdered extract were analysed for their α-glucosidase and α-amylase inhibition assays. The total phenolic, flavonoid and flavonol content, reducing power and their free radical scavenging capacity of the methanol extract of the plant were also investigated.

2.4 Estimation of total phenolic content

The amount of total phenolic content of crude extracts was determined according to Folin-Ciocalteu procedure [12]. The tested extracts (100 µl) were introduced into test tubes. One ml of Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured (UV-visible spectrophotometer Shimadzu UV 1800).

2.5 Estimation of total flavonoids

Total flavonoids were estimated using the method of Ordonez et al., 2006 [13]. To 0.5 ml of extracts, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After one hour, at room temperature, a yellow color developed, indicated the presence of flavonoids and the absorbance was measured at 420 nm (UV-visible spectrophotometer Shimadzu UV 1800).

2.6 Estimation of total flavonols

Total flavonols in the plant extracts was estimated using the method of Kumaran and Karunakaran, 2006 [14]. To 1.0 ml of extracts 1.0 ml of 2% $AlCl_3$ ethanol and 3.0 ml (50 g/L) sodium acetate solutions were added. The absorption at 440 nm (UV-visible spectrophotometer Shimadzu UV 1800) was read after 2.5 h at 20°C for the estimation of total flavonol content in the plant extract.

2.7 Measurement of reducing power

The reducing power of the extracts was determined according to the method of Oyaizu, 1986 [15**]**. The plant extracts (100 µl) was mixed with phosphate buffer (2.5 ml, 0.2 M, *p*H 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of 10% trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min.

The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm and reducing power is determined.

2.8 Determination of DPPH free radical scavenging activity

The free radical scavenging activity of the plant extract and butylated hydroxyl toluene (BHT) as positive control was determined using the stable radical DPPH (1,1-diphenyl-2-

picrylhydrazyl) [16]. 100µl of the tested extracts were placed in test tubes and 3.9 ml of freshly prepared DPPH solution (25 mg L^{-1}) in methanol was added in each test tube and mixed. 30 min later, the absorbance was measured at 517 nm (UV-visible spectrophotometer Shimadzu UV 1800). The capability to scavenge the DPPH radical was calculated, using the following equation:

DPPH scavenged (%) = {(Ac – At)/Ac} x 100

Where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration in mg of dry material per ml (mg / ml) that inhibits the formation of DPPH radicals by 50%. Each value was determined from regression equation.

2.9 Scavenging activity of ABTS radical cation

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\cdot+}$)-scavenging activity was measured according to the method described by Re *et al***.** [17]. ABTS was dissolved in water to a 7 mM concentration. The ABTS radicals were produced by adding 2.45 mM potassium persulphate (final concentration).The completion of radical generation was obtained in the dark at room temperature for 12–16 h. This solution was then diluted with ethanol to adjust its absorbance at 734 nm to 0.70 ± 0.02. To determine the scavenging activity, 1 ml of diluted $ABTS^{\cdot+}$ solution was added to 20 ,40 & 80 µl of plant extracts, and the absorbance at 734 nm was measured 6 min after the initial mixing, using ethanol as the blank. The percentage of inhibition was calculated by the equation:

ABTS scavenged (%) = $(A_{cont} - A_{test}) / A_{cont} \times 100$

where A_c and A_s are the absorbencies of the control and of the test sample, respectively. From a plot of concentration against % inhibition, a linear regression analysis was performed to determine the IC_{50} value of the sample.

2.10 *In Vitro* α-amylase inhibitory assay

A starch solution (1% w/v) was prepared by stirring 1g starch in 100 ml of 20 mM of phosphate buffer (pH 6.9) containing 6.7mM of sodium chloride. The enzyme solution was prepared by mixing 27.5 mg of porcine pancreatic amylase α-amylase (PPA) in 100 ml of 20 mM of phosphate buffer (PBS, pH 6.9) containing 6.7mM of sodium chloride. To 100 µl of (2,4,8,10,15 µg/ml) plant extracts, 200 µl porcine pancreatic amylase was added and the mixture was incubated at 37 ºc for 20 min. To the reaction mixture 100 µl (1%) starch solution was added and incubated at 37 ºC for 10 min. The reaction was stopped by adding 200 µl DNSA (1g of 3,5 di nitro salicylic acid, 30g of sodium potassium tartarate and 20 ml of 2N sodium hydroxide was added and made up to a final volume of 100 ml with distilled water) and kept it in a boiling water bath for 5 minutes. The reaction mixture diluted with 2.2 ml of water and absorbance was read at 540 nm. For each concentration, blank tubes were prepared by replacing the enzyme solution with 200 µL in distilled water. Control, representing 100% enzyme activity was prepared in a similar manner, without extract. The experiments were repeated thrice using the same protocol [18].

2.11 *In Vitro* α -Glucosidase Inhibition Assay

The inhibition of α-glucosidase activity was determined using the modified published method [19]. One mg of α-glucosidase was dissolved in 100 ml of phosphate buffer (pH 6.8). To 100 µl of (2, 4, 8, 10, 15 µg/ml) plant extracts, 200 µl α -glucosidase were added and the mixture was incubated at 37ºC for 20 min. To the reaction mixture 100 µl 3mM *p* -nitrophenyl α -D-glucopyranoside (*p*-NPG) was added and incubated at 37 ºC for 10 min. The reaction was terminated by the addition of 2ml Na_2CO_3 0.1M and the α-glucosidase activity was determined spectrophotometrically at 405 nm on spectrophotometer UV-VIS (Shimadzu UV-1800) by measuring the quantity of -nitrophenol released from *p*-NPG. Acarbose was used as positive control of α-amylase and α-glucosidase inhibitor. The concentration of the extract required to inhibit 50% of α-amylase and α -glucosidase activity under the assay conditions was defined as the IC_{50} value.

2.12 Method for calculation of α-amylase and α -glucosidase inhibitory concentration (IC50)

The concentration of the plant extracts required to scavenge 50% of the radicals (IC50) was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated by

I % = (Ac-As)/Ac X 100,

where Ac is the absorbance of the control and As is the absorbance of the sample [20].

Values are presented as mean ± standard error mean of three replicates. The total phenolic content, flavonoid content, flavonol content, reducing power and IC_{50} value of each plant material was calculated by using Linear Regression analysis.

3.RESULTS AND DISCUSSION

3.1 Total phenol, flavonoid and flavonol content of the extracts

Polyphenols have been said to be important phytochemicals with significant antioxidant capacities and other important medicinal characteristics. Total phenolic content in the plant extract was determined by the FC method and the calibration curve developed using Gallic acid. A regression equation was obtained from the standard curve and the amount of Gallic acid in the methanol extract of *T. sinensis* was calculated from the regression equation: y = 0.0013x + 0.0498, R^2 = 0.999 where y was the absorbance and x was the Gallic acid equivalent (mg/g).

The investigation showed that the plant under study contain a very good amount of total phenolics (21.34 ±0.25 mg GAE/mg plant extracts) which is well compared with the methanol extract of *Terminalia arjuna* (20.862 mg GAE/g plant extracts) [21]. Total flavonoid contents in the plant extracts were calculated as rutin equivalent (mg/g) using the equation based on the calibration curve: y = 0.0182x - 0.0222, R^2 = 0.9962, where y was

the absorbance and x was the rutin equivalent (mg/g) . The flavonol content in the plant was estimated as quercetin (mg/g) equivalent using the equation based on the calibration curve: $y = 0.0049x + 0.0047$, $R^2 = 0.9935$, where y was the absorbance and x was the quercetin equivalent (mg/g). The experimental result showed the presence of appreciable amount of flavonoid 0.35±0.01 mg/gm) and flavonol (0.31±0.01 mg/gm) in the methanol extract of *T. sinensis*. The result of investigation also showed a very good reducing power (0.52±0.01 mg/gm dry extract) with the methanol extract of the plant. The reducing power of the extract was evaluated in ascorbic acid equivalent (AAE) in milligram per gram (mg/g) of dry extract using the following equation based on the calibration curve: $y = 0.0023x - 0.0063$, $R^2 = 0.9955$ where y was the absorbance and x was the ascorbic acid equivalent (mg/g). It has been established that phenolic, flavonoids and flavonols compounds are regarded as one of the most widespread groups of natural constituents found in the plants which can adsorb and neutralize the free radicals and showed antioxidant activity through scavenging or chelating process [22-23]. Thus after determining the total phenol content of the plants, the antioxidant activities of the plants were estimated for their free radical scavenging activity.

3.2 DPPH free radical scavenging activity

The evaluation of anti-radical radical scavenging properties of the methanol extract of *T. sinensis* was executed by DPPH radical scavenging assay. The 50% inhibition of DPPH radical (IC_{50}) by the plant extract was determined (Table 1), a lower value would reflect greater antioxidant activity of the sample. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts [24]. The antioxidant effect is proportional to the disappearance of the purple colour of DPPH in test samples. Thus antioxidant molecules can quench DPPH free radicals by providing hydrogen atom or by electron donation and a colourless stable molecule 2,2- diphenyl-1-hydrazine is formed and as a result of which the absorbance (at 517 nm) of the solution is decreased. Hence the more potent antioxidant, more decrease in absorbance is seen and consequently the IC_{50} value will be minimum. In the present study the potent radical scavenging activity (IC_{50} = 0.50± 0.01 mg/gm dry ext) was shown by the extract of *T. sinensis*.

3.3 Scavenging activity of ABTS radical cation

The antioxidant activity of the methanol extract of *T. sinensis* using ABTS assay was also carried out. The antioxidant effect is proportional to the disappearance of the colour of ABTS in test samples. Concentration of sample that could scavenge 50 % free radical (IC_{50}) was used to determine antioxidant capacity of sample compared to standard. Sample that had IC_{50} < 50 ppm, it was very strong antioxidant, 50-100 ppm strong antioxidant, 101-150 ppm medium antioxidant, while weak antioxidant with IC_{50} > 150 ppm. A strong inhibition was observed (IC_{50} =0.12 ±0.01 mg/gm dry ext.) with the methanol extract of the plant under investigation.

3.4 *In Vitro* α -amylase inhibitory assay

In this study the *in vitro* α –amylase inhibitory activities of the methanol extract of *T. sinensis* was investigated. The result of experiment showed that, there was a dose-dependent increase in percentage inhibitory activity against α-amylase enzyme. The methanol extract (2-15µg/ml) of the plant exhibited potent α -amylase inhibitory activity in a dose dependent manner. The extract showed inhibitory activity from 3.28±0.04 to 20±0.02% with an IC_{50} value of 0.75 µg dry extract (Table 2). Acarbose is a standard drug for α -amylase inhibitor. Acarbose at a concentration of (2-15 µg/ml) showed α -amylase inhibitory activity from 6.99±0.03 to 56.17±0.05% with an IC_{50} value 0.32 µg dry extract. A comparison of α–amylase inhibitory activity between the standard drug and plant extracts has been depicted in fig. 1. So the plant extract might be used as starch blockers since it prevents or slows the absorption of starch in to the body mainly by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose, maltriose and other simple sugars.In our study, the methanol extract of the plant showed maximum α- amylase inhibitory activity (IC_{50} = 0.75 µg dry extract) which could be attributed to the presence of polyphenols (21.34±0.25 mg/g) and flavonoids (0.35±0.31) because polyphenols are not only capable of reducing oxidative stress but also of inhibiting carbohydrate hydrolyzing enzymes because of their ability to bind with proteins [1].

Our results are in accordance with the previous study wherein, there is a positive relationship between the total polyphenol and flavonoid content and the ability to inhibit intestinal α-glucosidase and pancreatic α-amylase [27-28] [25-26].

Table 1: Anti-oxidant activities of the methanol extract of *T. sinensis*

Name of the plant	Total phenolic content	Total flavonoid content	Total flavonol content	Reducing power	DPPH Free radical scavenging ability	ABTS Free radical scavenging ability
	(GAE mg / g dry extract)	(Rutin equivalent mg / g dry extract)	(Quercetin equivalent mg / g dry extract)	(Ascorbic acid equivalent mg / g dry extract)	(IC_{50} mg / g dry extract)	(IC_{50} mg / g dry extract)
T. sinensis	21.34±0.25	0.35±0.01	0.31±0.01	0.52±0.01	0.50± 0.01	0.12 ±0.01

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

Table 2: *In vitro* antidiabetic activity of the methanol extract of *T. sinensis* using alpha amylase method and comparison with standard drug acarbose.

Sl No	Plant extract/ standard drug	Concentration µg/ml	% of Inhibition	IC $_{50}$ µg dry extract
1	Methanol extract of *T. sinensis*	2	3.28±0.04	0.75
		4	5.14±0.06	
		8	10.71±0.03	
		10	14.32±0.05	
		15	20.00±0.02	
2	Acarbose	2	6.99±0.03	0.32
		4	11.58±0.05	
		8	26.45±0.02	
		10	41.64±0.06	
		15	56.17±0.05	

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM.

Table 3: *In vitro* antidiabetic activity of the methanol extract of *T. sinensis* using alpha glucosidase method and comparison with standard drug acarbose.

Sl No	Plant extract/ standard drug	Concentration µg/ml	% of Inhibition	IC $_{50}$ µg dry extract
1	Methanol extract of *T. sinensis*	2	5.74±0.09	0.80
		4	8.13±0.07	
		8	13.88±0.12	
		10	17.70±0.09	
		15	19.14±0.11	
2	Acarbose	2	29.57± 0.14	0.46
		4	31.58±0.17	
		8	34.93±0.12	
		10	38.28±0.09	
		15	41.82±0.08	

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM.

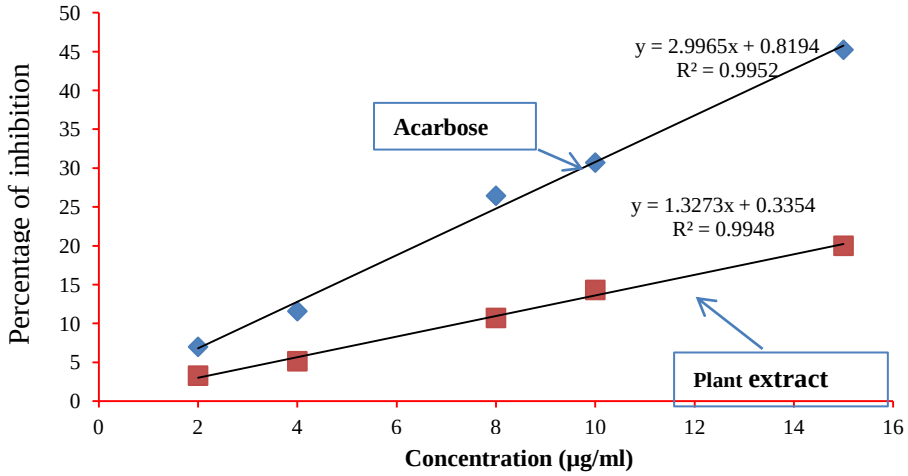

Fig. 1. *α* -Amylase inhibitory activity of Acarbose vs methanol extract of *T. sinensis.*

3.5 *In Vitro α* -glucosidase inhibitory assay

The results of antidiabetic activity using *α*- glucosidase inhibitory assay of the methanol extracts of *T. sinensis* stem are shown in Table 3. The extract revealed a significant inhibitory action of *α* -glucosidase enzyme. The percentage inhibition at 2-15 µg/ ml concentrations of *T. sinensis* extract showed a dose dependent increase in percentage inhibition.

The percentage inhibition varied from 19.62% - 5.74% for highest concentration to the lowest concentration. Thus the inhibition of the activity of *α*-glucosidase by *T. sinensis* would delay the degradation of carbohydrate, which would in turn cause a decrease in the absorption of glucose, as a result the reduction of postprandial blood glucose level elevation [30].

A comparison of *α*–glucosidase inhibitory activity between the standard drug and plant extracts has been depicted in fig. 2.

In this study acarbose was also used as a standard drug for α-glucosidase inhibitor. Acarbose at a concentration of (2-15 µg/ml) showed α-glucosidase inhibitory activity from 29.57 ±0.14 to 41.82±0.08 % with an IC_{50} value 0.46 µg dry extract. This indicates that the methanolic extract of *T. sinensis* is very potent α-amylase and α-glucosidase inhibitor in comparison with acarbose. This could be justified that the nature of some extract constituents (phenols, flavonoids saponins, steroids, alkaloids, terpenoids) present in the extract could be responsible as being effective inhibitors of α-amylase and α-glucosidase.

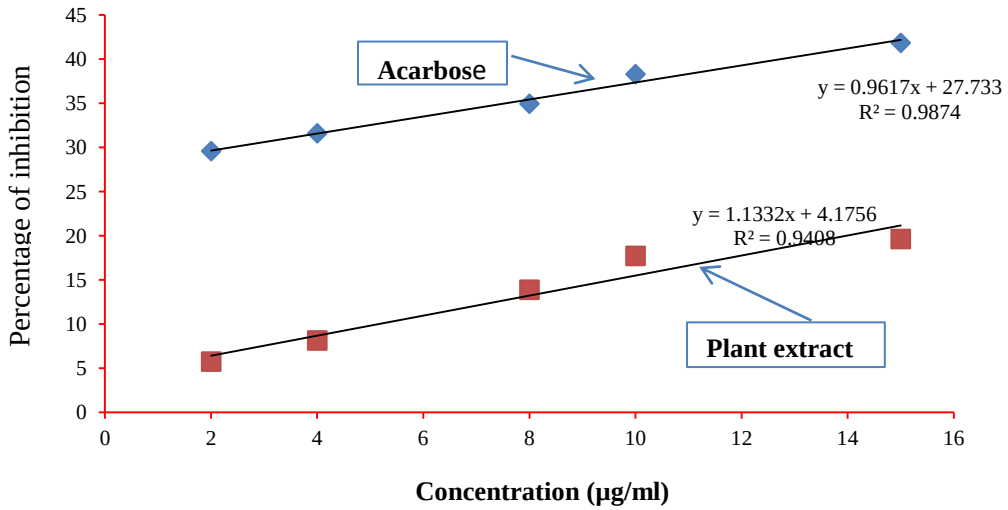

Fig. 2. *α* -Glucosidase inhibitory activity of Acarbose vs methanol extract of *T. sinensis.*

4. CONCLUSION

To investigate the biological activities of *T. sinensis* tuberous stem, the antioxidant and antidiabetic activities of the methanol extract of the plant has been analysed. As a result, we found that the extract of *T. sinensis* have free radical scavenging activity and inhibitory activity against α-amylase and α-glucosidase and this therapeutic potentiality could be exploited in the management of post prandial hyperglycemia in the treatment of type 2 diabetes mellitus. Although the effects of *T. sinensis* extract have been established *in vitro*, these results indicate that *T. sinensis* has potential as a crude drug and a dietary health supplement. The plant showed significant enzyme inhibitory activity, so the compound isolation, purification and characterization which are responsible for inhibiting activity, have to be done for the usage of antidiabetic agent. Further studies are also required to elucidate whether the plant have antidiabetic potential by *in vivo* for corroborating the traditional claim of the plant.

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6. Conflict of Interests: There are no conflicts of interest.

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