Research Article

Antidiabetic Mechanism of Standardized Extract, Fraction and Subfraction of *Cinnamomum Iners* Leaves

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ABSTRACT

Diabetes mellitus is predicted to become one of the world's main disablers and killers within the next 25 years. One of therapeutic approach for treating diabetes is to reduce postprandial hyperglycemia by inhibition of carbohydrate hydrolyzing enzymes such as α-glucosidase and α-amylase. The objective of the present manuscript is to screen the *in vivo* and *in vitro* inhibitory effect of active methanol extract, chloroform fraction and sub-fraction 1 of *Cinnamomum iners* leaves together with cinnamic aldehyde, a well known antidiabetic compound presence in the samples against α-glucosidase and α-amylase enzymes. In addition, *ex vivo* study for inhibition of glucose absorption from the intestine by methanol extract, chloroform fraction and sub-fraction 1 of *C. iners* leaves was assessed. The results indicate that sub-fraction 1 of *C. iners* leaves showed appreciable inhibition activity against α-glucosidase, α-amylase enzymes and reduce glucose absorption by intestine. It could be speculated that the antidiabetic mechanism of *C. iners* is by decreasing postprandial hyperglycemia, thus it is useful in management of type 2 insulin independent diabetes mellitus. Cinnamic aldehyde was identified as the major bioactive constituent presence in the sub-fraction 1.

Keywords: α-glucosidase; α-amylase, *Cinnamomum iners*; cinnamic aldehyde, diabetes mellitus.

INTRODUCTION

World Health Organization (WHO) estimated that, there are 160,000 diabetics worldwide and these figures has double in the last few years and is expected to double once again in the year 2025.[1] The seriousness of this disease due to its high prevalence and potential deleterious effect on a patient cause this disease to remain as major health concern among community.[2] A medical approach for treating diabetes is the restriction of carbohydrate absorption by inhibiting carbohydrate hydrolyzing enzymes such as α-glucosidase and α-amylase.[3] Commercially available inhibitory drugs such as acarbose, miglitol and voglibose are related with serious side effect such as flatulence, abdominal discomfort and diarrhea.[4] Hence, the search for new α-glucosidase and α-amylase inhibitors from medicinal plants to be developed as an alternative drug with lesser adverse effects can lead to discovery of potent antidiabetic agent.[5] However, adequate research on these natural remedies to screen the pharmacological potential should be conducted with the aim to standardize and develop them into natural products which would effectively serves as an alternatives or supplement to the modern medicine.

In line with the foregoing objective, the present study using an ethnomedical-based drug discovery program, we evaluated the enzyme inhibitory activity of leaves of *Cinnamomum iners* which grows wild in lowland of Malaysia, India, Myanmar, Indonesia, Thailand, Singapore, Brunei and Philippines. This plant locally known as *kayu manis hutan*, *medang kemangi* and *teja*. The plant leaves has been heavily promoted for a wide range of pharmacological uses; from fever, headache, for digestive system problem, rheumatism, wound healing and diabetes.[6,7] An earlier hypoglycemic and anti-hyperglycemic screening of various extracts and fractions of *C. iners* revealed that methanol extract, chloroform fraction and sub-fraction 1 exhibited the most potent glucose lowering activity (unpublished data). Hence, the aim of the present study was to investigate the *in vitro* and *in vivo* α-glucosidase and α-amylase enzyme inhibitory effects of active methanol extract, chloroform fraction and sub-fraction 1 of *C. iners* leaves. In addition, the major compound presence in each active extract, fraction and subfraction were determined.

MATERIALS AND METHODS

Plant material

C. iners leaves were collected at USM (Universiti Sains Malaysia). The authentication work was carried out by a botanist from School of Biological Sciences, USM where the plant material was deposited. The voucher specimen number is 11014.

Extraction

Powdered dried leaves (500 g) of the plant were serially macerated in petroleum ether (60-80; 2500 mL), chloroform (2500 mL) and methanol (2500 mL) for 3 days each. The residue after methanolic extraction was macerated in water (2500 mL) for 24 hr to obtain water extract. All the leaves extract (pet.ether, chloroform, methanol and water) was filtered and concentrated under reduced pressure at 55 °C in a rotary evaporator. The concentrated extracts obtained were placed in the oven for 3 days to remove the remaining solvent. Then it

was placed in the freeze drier for 24 hours which yielded a sticky material.

Fractionations of the active extract (methanol extract)

Methanol extract (2 g) was suspended in distilled water (500 mL). Then the suspension obtained was placed into a 1L separatory funnel. Firstly, the solution was extracted with chloroform (3×250 mL). Next, the aqueous layer was extracted with ethyl acetate and *n*-butanol (3×250 mL) to obtain the respective fractions. All of the fractions obtained were concentrated using the rotary evaporator. Concentrated fractions were kept in freeze drier for 24 hours to remove the remaining solvents.

Subfractionation of the active fraction (chloroform fraction)

Chloroform fraction was further extracted in hexane-chloroform mixture (1:3). The supernatant formed was collected, filtered and concentrated using rotary evaporator and freeze dried to obtain sub-fraction 1 (SF 1). The residue was dried and then similarly washed with chloroform until no colour was formed. Again, this supernatant was filtered, concentrated using rotary evaporator and freeze dried to obtain sub-fraction 2 (SF 2).

Standardization of active extract, active fraction and active sub-fraction using cinnamic aldehyde

The chemical composition of the methanol extract, chloroform fraction and sub-fraction 1 was analysed using Agilent Gas Chromatography (GC 6890N, China), and Agilent Mass Spectrometer (MS 5973I, USA). HP-5MS column (30 m x 0.25 mm x 0.25 μm) was used. The inlet temperature was set at 220°C and MSD transferline heater at 225°C. GC was performed in splitless mode. The flow rate of carrier gas (helium) was maintained at 1.0 mL/min. The initial temperature of oven was 80 °C and then increased to 170 °C by 10 °C/min and maintained for 2 min. The standard cinnamic aldehyde was prepared at a concentration of (0.06-1.00 mg/mL) in methanol by serial dilution of stock solution. Samples were prepared at 1 mg/mL in methanol.

Animals

Healthy male Sprague Dawley (SD) rats between 2 to 3 months of age, and weighing 200-250 g were obtained from Animal Research and Service Centre (ARASC), USM, Penang. The animal were kept in clean and dry cages and maintained in a well-ventilated animal house with 12 h-light-12 h dark cycle. Rats were fed with standard diet (ARASC, USM) and water *ad libitium*. The study was approved by the Animal Ethic Committee of USM (Reference number: USM /Animal Ethics Approval / 2012 / (78) (393). For experimental purposes, animal were kept fasting overnight but had free access to water.

Induction of diabetes

Diabetes was induced by intraperitoneal injection (single dose) of STZ (55 mg/ kg body weight) in 0.9% NaCl to 16 hr fasted rats. Blood glucose level was measured after 72 hr of STZ injection. Rats with fasting blood glucose concentrations 12-22 mmol/L was considered as diabetic and used for the study.[8,9]

In vitro α-glucosidase inhibition study

In-vitro α-glucosidase inhibitory activity was evaluated as method previously described.[10] Fifty (50 μL) each of sample (extract/fraction/subfraction/ cinnamic aldehyde) and acarbose, positive control, were added to yeast α-glucosidase (0.1 U/mL) which was dissolved in 0.1 M phosphate buffer, (pH 7.0) in a 96-well microplate. This suspensions was pre-incubated at 25 °C for 10 min followed by addition of 50 μl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution (the enzyme substrate), in 0.1 M phosphate buffer (pH 7.0). As for the control (to allow 100% inhibition), equivalent amount (50 μl) of buffer solution was added instead of the extract. Then the reaction mixture was incubated for 5 min at 25 °C. Finally, the absorbance was measured at 405 nm on a microplate reader. The α-glucosidase inhibitory activity was calculated as follows:

% inhibition= (A-B/ A) × 100

A is the absorbance of control and B is the absorbance of samples containing extracts, acarbose or cinnamic aldehyde. The inhibitory concentration of the extract required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by regression analysis. The experiments were performed in triplicate. For blank incubation, enzyme solution was replaced by buffer solution. Pure cinnamic aldehyde was used as standard and acarbose as positive control.

In vitro α-amylase inhibition study

In vitro α-amylase inhibitory activity was evaluated as per standard procedure. [11] Each sample (0.5 mL) was added to 0.5 mL of 0.02 M sodium phosphate (pH 6.9 with 0.006M NaCl) containing porcine α-amylase solution (0.5 mg/mL) in the test tubes. This mixture was incubated at 25 °C for 10 min followed by addition of 500 μL of 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl). Next, all the test tubes were incubated at 25 °C for 10 min. Thereafter, the reaction was stopped with addition of 1mL dinitrosalicylic acid color reagent. Then, the reaction mixture were incubated again in boiling water for 5 min and cooled to room temperature. Lastly, all the test tubes were diluted with 10 mL of distilled water, and absorbance was measured at 540 nm. Control incubation represent 100% enzyme activity and were conducted in a similar way by replacing extracts with methanol. For blank incubation, enzyme solution was replaced by methanol. Acarbose was used as positive control and cinnamic aldehyde as pure standard. Percent inhibition was calculated as follows:

% Inhibition = (A-B/ A) × 100

A is the absorbance of control and B is the absorbance of samples containing extracts, acarbose or cinnamic

aldehyde. The concentration of the extract required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by regression analysis. Experiments were performed in triplicate.

Measurement of glucose absorption from the intestine

This test was carried out as per described. [12] SD rats weighing 200-250 g were sacrificed, and their abdominal walls were dissected. The jejunum (20 cm away from the pylorus) was removed and the isolated jejunum was everted. The everted part of jejunum was cut into 5 in length and place into oxygenated tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 1.0 mM MgCl2, 12.0 mM NaHCO3, 0.2 mM NaH2PO2 and 5.5 mM glucose). Each segment was filled with tyrode solution (1.0 mL) and tied at both ends to form a sac. Next, the sac was placed in test tubes filled with tyrode solution (14.4 mL) in the presence of test substances (0.6 mL) and gassed with 95% O2 and 5% CO2. The test substances were 1 mg/mL of *C. iners* methanol extract, chloroform fraction and sub-fraction 1. Acarbose (1.0 mg/mL), served as positive control while tyrode solution alone was used as negative control. Next, the tubes were incubated in water bath at 37 °C for 60 min. At the end of the incubation period, the sacs were removed from the test tubes. After that, 3 mL of peridochrome-glucose reagent was added into separate test tubes followed by addition of 30 µL of supernatant collected from the previously incubated tubes. This test tubes was placed in water bath at $37^{\circ}C$ for 20 min. Finally, glucose concentration in the mixture was determined by using a Stat Fax Analyzer (LabCommerce Inc, USA). The following calculation was used to calculate the amount of glucose transported into the intestine:

Amount of glucose transported= (GT – GS) /weight of intestine (g); GT = glucose concentration in tyrode solution; GS = glucose concentration outside the sac.

Confirmatory *in vivo* studies

Oral carbohydrate challenge tests were determined as per standard procedure. [13] The oral carbohydrate tolerance tests were carried out using starch, sucrose and glucose separately both in normal and STZ- induced diabetic groups of rats.

Starch tolerance test in normal and diabetic rats

In this test, all the 16h fasted rats (either normal or diabetic) were divided into groups of six each. All the rats were given orally methanol extract (1 g/kg), chloroform fraction (500 mg/kg), SF1 (250 g/kg) and acarbose (positive control, 10 mg/kg). Vehicle (20% Tween-20 in distilled water) serves as negative control. After 10 min, the rats were all administered with starch (3 g/kg) orally and blood collected via tail puncture for blood glucose estimation before (0 min) and at 30, 60 and 120 minutes after starch treatment. [11] Area under the curve (AUC) was calculated using the following formulae:

AUC (mmol/Lh)

$$= \frac{[(BG0 + BG30) \times 0.5 + (BG30 + BG60) \times 0.5 + (BG60 + BG120)]}{2}$$

BG, blood glucose

Sucrose tolerance test in normal and diabetic rats

This test was carried out with procedure identical as the starch tolerance test in normal and diabetic rats but using sucrose at dose, 4 g/kg body weight instead of starch.

Glucose tolerance test in normal and diabetic rats

The procedure for oral glucose tolerance test was similar to starch tolerance test. The test was carried out in normal and diabetic rats using glucose at dose, 2 g/kg body weight instead of starch.

Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM) for six animals per group. Statistical analysis was made using one-way analysis of variance (ANOVA) and post-hoc comparisons were done using Dunnett's t-test using SPSS computer software. *p*-values < 0.05 were considered to be statistically significance.

RESULTS

Main active constituent in *C. iners* leaves

The GCMS profiles of methanol extract, chloroform fraction and sub-fraction 1 of *C. iners* identified cinnamic aldehyde in all the samples. Calculation based on simple linear regression curve revealed that methanol extract, chloroform fraction and sub-fraction 1 of *C. iners* leaves contain 8.32%, 14.8 % and 33.2 % of cinnamic aldehyde respectively.

In vitro α-glucosidase and α-amylase inhibition studies

The *in vitro* α-glucosidase and α-amylase inhibition studies showed that the methanol extract, chloroform fraction and sub-fraction 1 of *C. iners* possess potent inhibitory activity against α-glucosidase *in vitro*. This activity of chloroform fraction and sub-fraction 1 was almost similar to cinnamic aldehyde, a standard antidiabetic compound. This relative inhibitory potential for α-glucosidase and α-amylase was clearly indicated by the IC_{50} values (Table 1).

Table 1: IC_{50} values of methanol extract, chloroform fraction, sub-fraction 1 and subfraction 2 of *Cinnamomum iners* leaves on *in vitro* α- glucosidase and α-amylase enzyme.

Analyte	Inhibitory Concentration IC_{50} (mg/mL)	
	α- glucosidase	α-amylase
Methanol extract	14.23	81.76
Chloroform fraction	8.36	60.43
Sub-fraction 1	6.00	45.84
Acarbose	1.94	2.56
Cinnamic aldehyde	3.05	39.80

Effect of treatment on sucrose tolerance tests in normal rats and diabetic rats

The effects of *C. iners* methanol extract, chloroform fraction, sub-fraction 1 and cinnamic aldehyde, acarbose on sucrose tolerance in normal and diabetic rats are demonstrated on Table 2. The test results in normal rats reveal that chloroform fraction and sub-fraction 1 demonstrated a potential inhibitory action against α-glucosidase *in vivo.* However, chloroform fraction failed to showed any significant reduction in blood glucose or AUC of diabetic animals. Only sub-fraction 1 had showed significant reduction of blood glucose (17.50 ± 1.95) and AUC (27.05 ± 2.86) after 120 min of sucrose loading in diabetic rats as depicted in Table 2.

Effect of treatment on starch tolerance tests in normal rats and diabetic rats

Methanol extract and chloroform fraction treated groups did not show any significant decrease in blood glucose and AUC (Table 3) compared to normal and diabetic control rats. Sub-fraction 1 demonstrates significant decreases in blood glucose with 17.80 and 18.10 percentage of reduction in normal and diabetic rats. Sub-fraction 1 also showed significant AUC (11.01 ± 0.18) in normal rats and diabetic rats (32.21 ± 1.86) respectively. Acarbose and sub-fraction 1 managed to reduce significantly ($p < 0.05$) blood glucose and AUC of normal and diabetic rats as shown in Table 3.

Effect of treatment on glucose tolerance tests in normal rats and diabetic rats

In normal and diabetic rats, none of the extract treated groups managed to reduce blood glucose and AUC significantly in comparison to control group. Both cinnamic aldehyde and acarbose also failed to significantly suppress the blood glucose at stipulated times after glucose loading.

Table 2: Effects of methanol extract, chloroform fraction and sub-fraction 1 of *C. iners* leaves on blood glucose (BG) (min 120) and area under the curve (AUC) after sucrose loading (4g/kg) in normal rats and STZ-diabetic rats.

Group	BG (mmol/L)	% reduction of BG	AUC	% reduction of AUC (mmol/L)
Normal rats				
Normal control	5.10 ± 0.10		10.98 ± 0.19	
Methanol extract	4.80 ± 0.32	5.80	9.88 ± 0.22	7.85%
Chloroform fraction	4.50 ± 0.26*	11.80	9.56 ± 0.15	7.96%
Sub-fraction 1	4.40 ± 0.18*	13.70	9.35 ± 0.31	14.45%
Acarbose	4.30 ± 0.24*	15.70	9.08 ± 0.24*	18.56%
Cinnamic aldehyde	4.40 ± 0.19*	13.90	9.26 ± 0.35*	18.06%
Diabetic rats				
Diabetic control	20.80 ± 1.85		40.60 ± 1.85	
Methanol extract	19.80 ± 1.06	4.80	39.65 ± 1.74	10.54
Chloroform fraction	19.60 ± 2.0	5.70	37.54 ± 2.45	11.06
Sub-fraction 1	17.50 ± 1.95*	15.80	27.05 ± 2.86*	33.04
Acarbose	15.70 ± 1.87*	24.50	23.25 ± 1.56*	38.05
Cinnamic aldehyde	16.70 ± 1.94*	19.70	25.40 ± 1.64*	35.18

Table 3: Effects of methanol extract, chloroform fraction and sub-fraction 1 of *C. iners* leaves on blood glucose (BG) (min 120) and area under the curve (AUC) after starch loading (3g/kg) in normal rats and STZ-diabetic rats

Group	BG (mmol/L)	% reduction of BG	AUC	% reduction of AUC (mmol/L)
Normal rats				
Normal control	5.50 ± 0.16		13.80 ± 0.16	
Methanol extract	5.20 ± 0.24	10.50	12.44 ± 0.30	10.85
Chloroform fraction	5.30 ± 0.31	12.10	12.05 ± 0.21	12.04
Sub-fraction 1	4.60 ± 0.16*	17.80	11.01 ± 0.18*	17.01
Acarbose	4.40 ± 0.18*	25.42	9.80 ± 0.32*	24.64
Cinnamic aldehyde	4.70 ± 0.22*	20.30	10.61 ± 0.46*	20.18
Diabetic rats				
Diabetic control	20.80 ± 1.65		37.45 ± 1.98	
Methanol extract	19.50 ± 1.52	6.20	34.19 ± 1.75	13.18
Chloroform fraction	19.60 ± 1.08	6.40	35.14 ± 1.95	11.16
Sub-fraction 1	18.10 ± 1.36*	18.10	32.21 ± 1.86*	15.10
Acarbose	15.80 ± 1.05*	24.0	23.35 ± 2.16*	34.26
Cinnamic aldehyde	17.20 ± 1.22*	11.5	26.51 ± 1.54*	33.10

Glucose absorption from the intestine

Figure 1 demonstrates the effect of acarbose and *C. iners* methanol extract, chloroform fraction and subfraction 1 on intestinal absorption of glucose in the everted sac segments. Only subfraction 1 produced a significant reduction in intestinal absorption of glucose. Acarbose and cinnamic aldehyde also showed significant inhibition in glucose uptake by intestine.
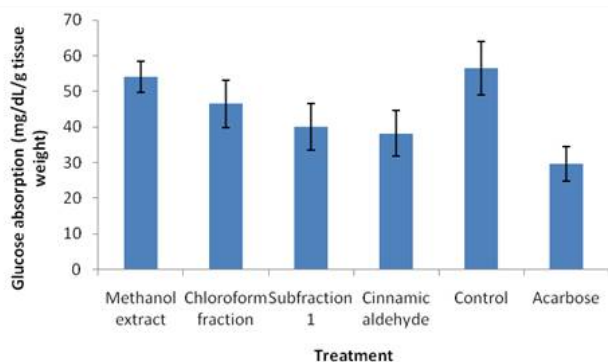

Figure 1: Effects of water extract, fraction and subfraction of *C. iners* and acarbose and cinnamic aldehyde on glucose absorption by everted sac technique. Each value represents the mean ± S.E.M. (n = 6); * indicates significant difference between treated groups compared with control group at *P* < 0.05.

DISCUSSION

Carbohydrate rich diet causes elevation in blood glucose level as the complex disaccharides in the food is rapidly absorbed in the intestine in the form of monosaccharides after breakdown aided by the a glucosidase and α–amylase enzyme. [14] Hence, inhibition of α–glucosidase and α–amylase enzymes play an important role in type 2 insulin independent diabetes mellitus by preventing increase of postprandial blood glucose level. [15] Therefore the *in vitro* and *in vivo* α-glucosidase and α-amylase inhibitory effect of *C. iners*, a plant with valuable therapeutic value, was investigated for the first time.

From the present investigation, it could thus be speculated that a dose dependent effect was observed on increasing the concentrations of the sample solution in *in vitro* α-glucosidase and α-amylase test. The results indicate that chloroform fraction and sub-fraction 1 of *C. iners* leaves showed appreciable inhibition activity, thus could be useful in management of postprandial hyperglycemia. However the *in vitro* inhibitory activity does not always correlate to the *in vivo* findings.[11] Hence preclinical animal studies were conducted as a proof of concept. As expected, our *in vivo* experiment demonstrated that sub-fraction 1 blunted postprandial hyperglycemic spike in normal rats loaded with sucrose and starch. Subsequently, the postprandial hyperglycemia amelioration of extract, fraction and subfraction was evaluated in the STZ induced diabetic rats. The result obtained showed that sub-fraction 1 exhibited potential α-glucosidase inhibitory activity in diabetic animal as well. Our findings reveal that one of the mechanisms by which

C. iners exhibiting their antidiabetic effect would be by inhibition of α-glucosidase and α-amylase activity leading to retardation of complex carbohydrate molecules such as starch and sucrose, eventually lowering postprandial blood glucose. The standard drug, acarbose similarly suppressed the postprandial glucose level. On the other hand, non treated animals showed an extremely high level of blood glucose that has been staying high even two hours after the sucrose and starch administration. Since the observed reduction in postprandial glucose could be due to the secretagogue activity and insulin sensitizing property of potential samples, we have evaluated the effect of extract, fraction and subfraction on glucose loading in the normal and diabetic rats. Sub-fraction 1 showed insignificant reduction on blood glucose of rats loaded with glucose (monosaccharide) but significant suppression on postprandial hyperglycemia after starch and sucrose (disaccharide) loading, which suggest that the major mechanism of action of postprandial glucose suppression is exhibited by inhibition of a-glucosidase.[16] Our GCMS evidence highlighted that the major compound presence in the sub-fraction 1 is cinnamic aldehyde. Cinnamic aldehyde is a well established compound of Cinnamomum species for antihyperglycemic properties.[17-20]

CONCLUSION

Sub-fraction 1 of *C. iners* serves as potential therapeutic approach to delay the quick digestion of starch and sucrose and lengthen the duration of carbohydrate absorption over time. *C. iners* might be useful for people on sulfonylurea or metformin medication, who need an additional medication to keep their blood glucose levels within a safe range. Our GCMS findings suggest that the enzyme inhibitory potential of sub-fraction 1 is due to the presence of cinnamic aldehyde, a well known antidiabetic compound.

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