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***Cinnamomum iners* Leaves as an Alternative Therapy for Diabetes**

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ABSTRACT

This study was designed to investigate the antidiabetic property of *Cinnamomum iners* leaves which were used traditionally to combat diabetes. Firstly, the antidiabetic activity of petroleum ether, chloroform, methanol and water extract of *C. iners* leaves was evaluated. Next, the potential extract which was the methanol extract was fractionated to obtain ethyl acetate, n-butanol, chloroform and aqueous fraction. The active chloroform fraction was subjected to subfractionation process to obtain subfraction 1 (SF 1) and subfraction 2 (SF 2). *Cinnamomum iners* leaves active portion (methanol extract, chloroform fraction and subfraction 1) was standardized using cinnamic aldehyde. The antidiabetic activity of standardized *C. iners* leaves was assessed by using Intra-Peritoneal Glucose Tolerance Test (IPGTT). The antidiabetic mechanism of active portion of *C. iners* leaves were also determined by measuring glucose absorption from the intestine. This study showed significant glucose tolerance effect of active portion of *C. iners* leaf after repeated oral administration in STZ-induced diabetic rats. The antidiabetic mechanism of subfraction 1 and chloroform fraction of *C. iners* leaf is by inhibiting glucose absorption from intestine.

Key words: Antidiabetic, intra-peritoneal glucose tolerance test, standardization

INTRODUCTION

Cinnamomum iners plant appears as dense, bushy and dull green in colour. It grows wildly along the roadside (Chooi, 2004). There is numerous report on pharmacological activities of this plant such as antioxidant, antimicrobial, anticancer and analgesic (Mustaffa *et al.*, 2010a, b; Pang *et al.*, 2009; Mustaffa *et al.*, 2011). However, there is no scientific research yet to establish the antidiabetic properties although this plant had been used traditionally to treat diabetes.

Based on the report by World Health Organization (WHO., 1991), diabetes is regarded as one of the chronic disease that serves as the major cause of death around the world (WHO., 1991). Diabetes mellitus encounter as a major global health problem, affecting nearly 15 million of world populations due to a combination of lifestyle changes, genetics, nutritional and environmental factors (Burke *et al.*, 2003). As for Malaysia, Mafauzy (2005) reported that 73% of patients from 29 hospitals in Malaysia are diabetics. From this value, 55% had hypertension, 24% had diabetic nephropathy, 37% had diabetic retinopathy, 61% had diabetic neuropathy and 12% with coronary artery disease (Mafauzy, 2005). In recent trend, the treatment of hyperglycemia involves the use of synthetic drug such as biguanides, thiazolidinediones, sulphonylurea and alpha-glucosidase inhibitor (Vijayakumar *et al.*, 2005). However, some of these antidiabetic agents have its own range of side effects which may even worsen the condition of disease in some cases. For example sulphonylureas can lead to hypoglycemic coma and hepatorenal disturbances that may be

responsible for the safety issue of human health (Suba *et al.*, 2004). Hence, there is a massive need for the exploration of herbals which is economically wise in managing this disease. In line with the national and global need to search for alternative treatment in controlling diabetes, this research had been conducted.

MATERIALS AND METHODS

Plant material: Cinnamomum iners leaf were collected at Universiti Sains Malaysia (USM). 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.

Animals: Male Sprague Dawley (SD) rats between 2-3 months of age and weighing 200-250 g were obtained from Animal Research and Service Centre (ARASC), USM. The animals were kept in clean and dry cages and maintained in a well-ventilated animal house with 12 h light and 12 h dark cycle. The study was approved by the Animal Ethic Committee of Universiti Sains Malaysia [Reference number: USM/Animal Ethics Approval/2012/(780)393]. For experimental purpose, animal were kept fasting overnight but had free access to water.

Preparation of herb extracts: Herb extracts preparation took around 1 month (April, 2014). Firstly, the plant leaf were washed with water to remove dirt prior to the drying process. The leaf were then crushed into fine powder. Powdered dried leaf (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 for 24 h to obtain water extract. Then, the leaf extract was filtered and concentrated under reduced pressure at 55°C in a rotary evaporator. The concentrated extract obtained was placed in the oven at 60°C for 3 days to remove the remaining solvent. The aqueous extract was placed in freeze drier instead of oven.

Fractionations of the active extract (methanol extract): Fractionations procedures were carried out for almost 2 months (May-June 2014). Methanol extract (2 g) was suspended in distilled water (500 mL). Then, the suspension obtained was placed into a 1 L 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 three respective fractions. All fractions obtained were concentrated using the rotary evaporator. Chloroform fraction, ethyl acetate fraction and n-butanol fraction were kept in oven at 60°C to remove the remaining solvents. The aqueous fraction was placed in freeze drier instead of oven.

Subfractionation of the active fraction (chloroform fraction): Subfractionation procedure were carried out for 1 month (June, 2014). Chloroform fraction was further extracted in hexane-chloroform mixture (1:3). The supernatant formed was collected, filtered and concentrated using rotary evaporator and dried in oven at 60°C to obtain subfraction 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 subfraction 2 (SF 2).

Standardization of active extract, active fraction and active subfraction using cinnamic aldehyde: Standardization procedure was carried out continuously with determination of limit of detection and quantification (July-August, 2014). Sample solution of the methanol extract,

chloroform fraction and subfraction 1 (1 mg mL⁻¹) was prepared by dissolving in ethanol. Sample (1 µL) was injected and the profile was acquired using the GC-MS. The peak identification and quantification was carried out using the retention time and mass spectrum provided by the MS library.

Limit of quantification and detection: Limit of quantification and detection were determined in July until August 2014. The ICH Harmonised Tripartite Guideline procedure was employed for the determination of Limit of Quantification (LOQ) and Limit of Detection (LOD). Signal-to-noise (S/N) method was used for the determination of LOD and LOQ of cinnamic aldehyde. The LOD is the minimum concentration of the analyte that can be differentiated from assay background. The LOD is calculated in S/N ratio of 3:1. The LOQ is the minimum concentration of the analyte that can be quantified and determined by peak-to-peak noise in ratio of 10:1 (ICH Harmonised Tripartite Guideline, 1996).

Precision: Precision of standardization procedure was determined in July 2014. The ICH Harmonised Tripartite Guideline procedure was used for the inter-day and intra-day precision determination. Inter-day and intra-day precision were determined by calculating the percentage of relative standard deviation (RSD%) (ICH Harmonised Tripartite Guideline, 1996). Percentage of RSD of calculated using the following formula:

$$\text{RSD (\%)} = (\text{SD/M}) \times 100$$

Where:

M = Experimentally determined concentration

SD = Standard deviation of M

Intra-Peritoneal Glucose Tolerance Test (IPGTT) in normal and diabetic rats: This study was carried out for 2 months (August-September, 2014). Firstly, 36 male Sprague Dawley rats (200-250 g) either normal or diabetic were equally divided into six groups (n = 6). The first group received cosolvent 10 mL kg⁻¹ as control. The second group was treated with metformin 500 mg kg⁻¹ b.wt. The third, fourth, fifth and sixth group received extract 1 g kg⁻¹ b.wt., (petroleum ether, methanol, chloroform and water) or 1 g kg⁻¹ b.wt., active extract (methanol), 500 mg kg⁻¹ b.wt., active fraction (chloroform), 250 mg kg⁻¹ b.wt., active subfraction (subfraction 1) of *C. iners* leaf and cinnamic aldehyde (20 mg kg⁻¹ b.wt.) for IPGTT test of active portion of *C. iners* leaf extract. After 1 h, the rats were administrated with glucose (1 g kg⁻¹ b.wt.) intraperitoneally and blood samples were withdrawn via tail vein at times 0 (before treatment), 15, 30, 45, 60, 90 and 120 min after the glucose loading for determination of blood glucose level using a glucose meter (Hassan *et al.*, 2010). As for the IPGTT after multiple administration of active portion of *C. iners* leaf, samples were administered twice daily for 12 days and test was conducted on day 13 using the above mentioned procedure.

Measurement of glucose absorption from the intestine: This test was carried out as per described by Hassan *et al.* (2010) (2 weeks, October 2014). The 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 cm in length and place into oxygenated tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM

hCaCl₂ • 2H₂O, 1.0 mM MgCl₂, 12.0 mM NaHCO₃, 0.2 mM NaH₂PO₄ 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⁻¹ of *C. iners* methanol extract, chloroform fraction and subfraction 1. Acarbose (1.0 mg mL⁻¹) 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 reagent was added into separate test tubes followed by addition of 30 µL of supernatant collected from the previously incubated tubes. Peridochrome will bind to the glucose and increase the intensity of colour with increase of glucose. These test tubes were placed in water bath at 37°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:

$$\text{Amount of glucose transported} = \frac{\text{GT-GS}}{\text{weight of intestine (g)}}$$

Where:

GT = Glucose concentration in tyrode solution (mg)

GS = Glucose concentration outside the sac (mg)

RESULTS

Standardization of active extract, fraction and subfraction: Active portion of *C. iners* leaf [from anti-hyperglycemic test (published data)] which was methanol extract, chloroform fraction and subfraction 1 was standardized using cinnamic aldehyde as the marker compound. The GC-MS profiles of methanol extract, chloroform fraction and subfraction 1 of *C. iners* leaf were compared with standard cinnamic aldehyde. The analyses show the presence of cinnamic aldehyde in all samples but in varying proportions. Mass spectroscopy of cinnamic aldehyde identified in the samples showed major characteristic fragmentations pattern ($m/z = 45$, $m/z = 59$, $m/z = 69$, $m/z = 77$, $m/z = 89$, $m/z = 103$, $m/z = 111$, $m/z = 119$, $m/z = 131$, $m/z = 152$, $m/z = 161$, $m/z = 179$ $M^+ = 204$) exactly identical to the standard cinnamic aldehyde mass fragmentations pattern. The retention time of pure cinnamic aldehyde was 10.12 min whereas the retention times of the samples were 10.2±0.2 min. The regression curve of cinnamic aldehyde is linear with y-intercept of 814 051 and value of r^2 is 0.9921. The amount of cinnamic aldehyde in methanol extract, chloroform fraction and subfraction 1 is presented in Table 1.

LOD and LOQ: The LOD and LOQ of cinnamic aldehyde were 15.6 and 70.5 ppm, respectively.

Precision: The intra-day and inter-day precision for determination of cinnamic aldehyde was presented in Table 2. The percentage of RSD value for intra-day were 0.0008, 0.0028, 0.0018 and 0.00097, respectively. The percentage of RSD for inter-day precision were 0.006, 0.003, 0.002 and 0.00078, respectively.

Table 1: Amount of cinnamic aldehyde in methanol extract, chloroform fraction and subfraction 1 of *C. iners* leaf

Materials	Amount of cinnamic aldehyde (ppm)	Amount of cinnamic aldehyde (%)
Methanol extract	83.2	8.32
Chloroform fraction	148.0	14.8
Subfraction 1	332.0	33.2

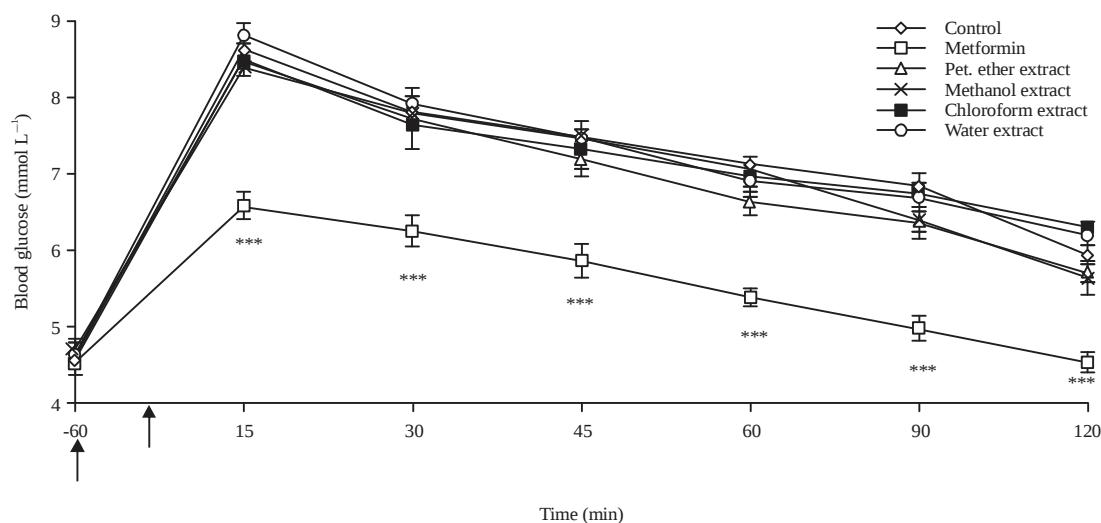


Fig. 1: Effect of oral administration of extracts ($1 \text{ g kg}^{-1} \text{ b.wt.}$) on blood glucose level of fasting normal rats loaded intraperitoneally with glucose ($1 \text{ g kg}^{-1} \text{ b.wt.}$) after 1 h. Results represented as Mean $\pm$ SEM (n = 6). ***p<0.001 indicates statistically significant differences compared to the control rats (untreated) analyzed by using one way ANOVA followed by *post hoc* Dunnett's test

Table 2: Precision and recovery for determination of cinnamic aldehyde

Concentration of cinnamic aldehyde (ppm)	Mean $\pm$ SD	Intra-day		Mean $\pm$ SD	Inter-day	
		Recovery (%)	Precision (RSD%)		Recovery (%)	Precision (RSD%)
62.5	61.0 $\pm$ 0.05	97.60	0.0008	60.8 $\pm$ 0.38	97.28	0.006
125	124.0 $\pm$ 0.35	99.20	0.0028	123.0 $\pm$ 0.46	98.4	0.003
250	248.5 $\pm$ 0.45	99.40	0.0018	249.1 $\pm$ 0.51	99.64	0.002
1000	998.4 $\pm$ 0.97	99.84	0.00097	996.1 $\pm$ 0.78	99.61	0.00078

IPGTT in normal rats: Figure 1 indicated the effect of *C. iners* leaf various extracts on blood glucose level following a glucose challenge compared to metformin. Glucose loading ($1 \text{ g kg}^{-1} \text{ b.wt.}$) results in the rise of blood glucose level of rats. None of the extracts inhibited the rise of blood glucose after glucose loading. On the other hand, metformin, an anti-hyperglycemic agent significantly inhibited the rise of blood glucose on 15th min and sustained lower glucose levels compared to the control throughout the duration of the experiment (p<0.001).

IPGTT in diabetic rats: Glucose loading ($1 \text{ g kg}^{-1} \text{ b.wt.}$) results in the rise of blood glucose level of diabetic rats. The intra-peritoneal glucose tolerance test in diabetic rats after single administration (Fig. 2) did not show significant difference as compared to control diabetic rats. Only metformin indicated a significant inhibition of the rise of blood glucose at p<0.05 on 45 min till the end of the study. As for the glucose tolerance test after repeated oral administration of treatment twice daily for 12 days (Fig. 3), subfraction 1 and cinnamic aldehyde treated groups significantly inhibited the rise of blood glucose after glucose loading compared to diabetic control beginning on first hour of experiment and the activity persist till the end of the experiment. Methanol extract and chloroform fraction also significantly inhibited the rise of blood glucose of glucose loaded diabetic rats during the second hour of the experiment (120 min).

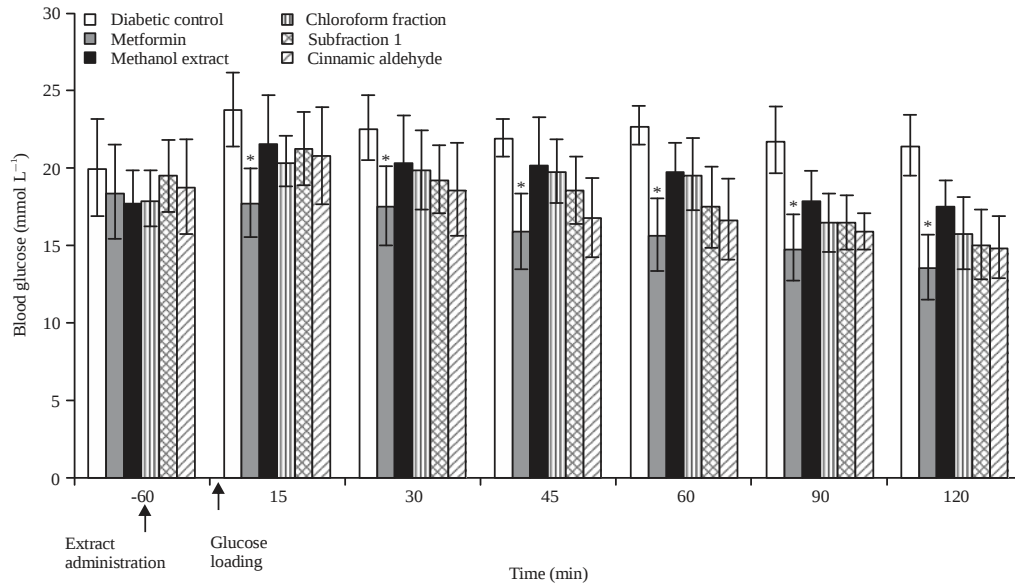


Fig. 2: Effect of single oral administration of methanol extract ($1 \text{ g}^{-1} \text{ b.wt.}$), chloroform fraction ($500 \text{ mg kg}^{-1} \text{ b.wt.}$), subfraction 1 ($250 \text{ mg kg}^{-1} \text{ b.wt.}$) of *C. iners* and intra-peritoneal 1 g kg^{-1} glucose loading at 0 min on blood glucose level of diabetic rats. Results represented as Mean $\pm$ SEM (n = 6). *p<0.05 indicates statistically significant decrease compared to the diabetic control (untreated) rats. Data was analyzed using one way ANOVA followed by *post hoc* Dunnett's test

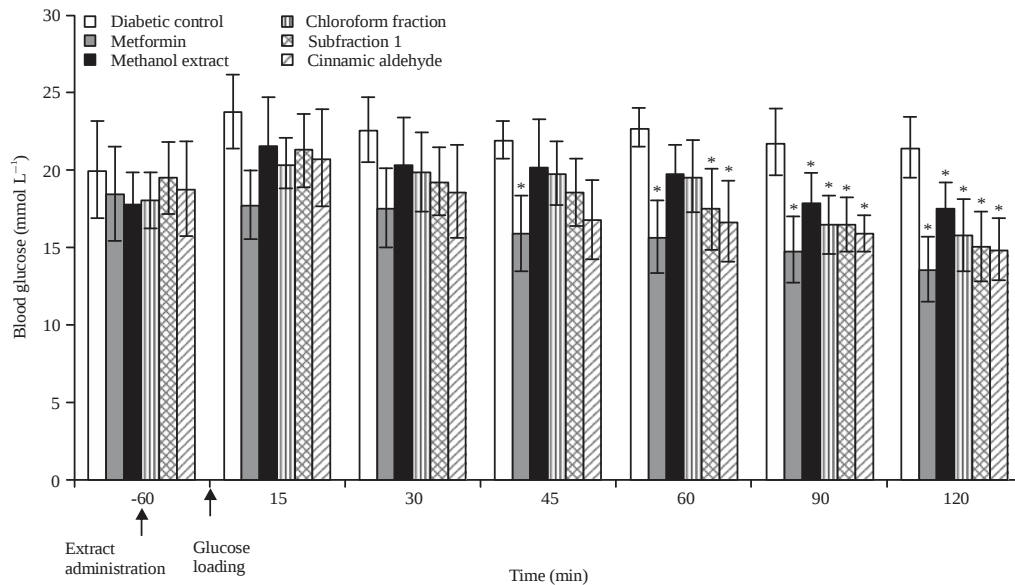


Fig. 3: Effect of repeated oral administration twice daily for 12 days of methanol extract ($1 \text{ g}^{-1} \text{ b.wt.}$), chloroform fraction ($500 \text{ mg kg}^{-1} \text{ b.wt.}$) and subfraction 1 ($250 \text{ mg kg}^{-1} \text{ b.wt.}$) of *C. iners* leaf on blood glucose level of glucose ($1 \text{ g kg}^{-1} \text{ b.wt.}$) loaded in diabetic rats. Results represented as Mean $\pm$ SEM (n = 6). *p<0.05 indicates statistically significant decrease compared to the diabetic control (untreated) rats. Data was analyzed using Dunnett's test

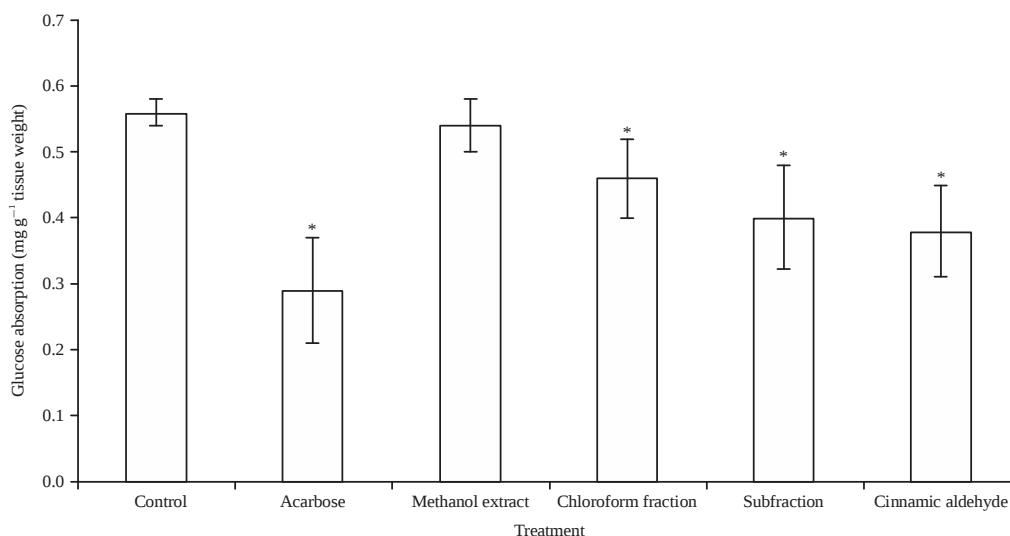


Fig. 4: Effects of methanol extract (1 g kg⁻¹ b.wt.), chloroform fraction (500 mg kg⁻¹ b.wt.) and subfraction 1 (250 mg kg⁻¹ b.wt.) of *C. iners* leaf and acarbose on glucose absorption by intestine. Each value represents the Means±SEM (n = 6). *p<0.05 indicated statistically significant difference compared with control (untreated)

Measurement of glucose absorption from the intestine: Figure 4 demonstrates the effect of acarbose, cinnamic aldehyde and *C. iners* methanol extract, chloroform fraction and subfraction 1 on intestinal absorption of glucose in the everted sac segments. As for the extract, only subfraction 1 produced a significant reduction in intestinal absorption of glucose. The rate of glucose absorption of intestine for the subfraction 1 (0.4±0.08 mg g⁻¹ tissue weight) treated group is almost similar to cinnamic aldehyde (0.38±0.07 mg g⁻¹ tissue weight).

DISCUSSION

Herbal medicine is a complete system of healing developed shortly after the beginning of human culture. However, owing to the fact that there is lack of both qualitative and quantitative standards of herbal medications, standardization serves as a technique for identification and authentication of herbs and their products which will then assure the quality control of these materials (WHO., 1991). Hence, *C. iners* leaf active portion had been standardized using cinnamic aldehyde, which acts as its bioactive compound.

All extracts (methanol, chloroform, pet. ether, water) caused no significant decrease in glucose level of normal rats in IPGTT. All the active portion of *C. iners* leaf (methanol extract, chloroform fraction and subfraction 1) did not produce any improvement in glucose tolerance of STZ-induced diabetic rats after single oral administration (Fig. 2). However, the present study indicates the significant glucose tolerance effect of methanol extract, chloroform fraction and subfraction 1 of *C. iners* leaf after repeated oral administration in STZ-induced diabetic rats (Fig. 3). This results is comparable with other plant with potential antidiabetic property such as *Tinospora bakis*, *Nauclea latifolia* and *Randia nilotica* which were used in Sudanese folk medicine to cure diabetes (Alamin *et al.*, 2015; Stanely *et al.*, 2000; Adoum *et al.*, 2012). This showed that hypoglycemic effect of *C. iners* leaf extract works only in diabetic conditions and not in normal conditions. This suggests that the active compound in *C. iners* leaf extract showed their action only in insulin-deprived situations e.g., in streptozotocin induced diabetic rats. These results indicate that possibly in

normal rats, the extracts of *C. iners* leaf does not alter the release of insulin but in a diseased state (hyperglycemia), it increases the insulin sensitivity for effective glucose disposal (Gokhale *et al.*, 1998).

The measurement of glucose absorption study by intestine showed that subfraction 1 and chloroform fraction could inhibit glucose absorption by the intestine. The inhibition of glucose absorption by intestine with the presence of *C. iners* leaves was found to be better than inhibitory effect of glucose intake by *Gynura procumbens* leaves which was used traditionally by Malaysian for diabetes (Hassan *et al.*, 2010). This indicate that subfraction 1 and chloroform fraction of *C. iners* leaf exerts extra-pancreatic effects similar to alpha-glucosidase inhibitor. Example of plants which exerts alpha-glucosidase inhibitory effects similar to *C. iners* leaves were *Cinnamomum zeylanicum*, *Artocarpus altilis*, *Piper betel* and *Artocarpus heterophyllus* plants (Nair *et al.*, 2013). Similarly some plants of Korean medicinal plants namely *Euonymus sachalinensis*, *Rhododendron schlippenbachii*, *Astilbe chinensis*, *Juglans regia*, *Meliosma oldhamii* and *Symplocos chinensis* demonstrated extra-pancreatic effect to combat diabetes by inhibiting glucose absorption from intestine (Sancheti *et al.*, 2011). Alpha-glucosidase inhibitors are very useful for people taking sulfonylurea medication or metformin, who need an additional medication to maintain the blood sugar level within a safe range. It could thus be speculated that subfraction 1 of *C. iners* leaf have an effect on glucose absorption from gut and may prolong absorption process and thus was suppressing the rise in blood glucose level. The results obtained suggest that the antihyperglycemic effect of subfraction 1 and chloroform fraction might be due to the presence of cinnamic aldehyde in which mimic the antidiabetic mechanism of cinnamic aldehyde by inhibiting glucose absorption from intestine (Hansotia and Drucker, 2005). The overall study showed that *C. iners* leaves active portion could improve postprandial glucose level of STZ-induced diabetic rat which is poorly controlled due to impaired insulin production. The antidiabetic effect of methanol extract might be brought by other blood glucose lowering mechanisms such as glucose uptake by muscle or increasing insulin secretion.

CONCLUSION

This study showed that *C. iners* leaves which is economically beneficial can serve as natural alternative therapy for the treatment of diabetes.

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